INVESTIGATING THE DEVELOPMENTAL ROLES FOR THE FUNCTIONAL AMYLOID SYSTEM OF *STREPTOMYCES VENEZUELAE*

INVESTIGATING THE DEVELOPMENTAL ROLES FOR THE FUNCTIONAL AMYLOID SYSTEM OF *STREPTOMYCES VENEZUELAE*

BY

DAVID A. CRISANTE, B.SC.

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AUTHOR: David Allen Crisante, B.Sc. (McMaster University)

SUPERVISOR: Dr. Marie Elliot

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Abstract

Amyloid proteins are found in all domains of life, and have a number of defining characteristics, including considerable β-sheet secondary structure and the ability to self-assemble into large, insoluble fibers. These insoluble aggregates are often deleterious to the cell, evidenced by amyloid proteins being involved in Alzheimer's, Parkinson's, and Huntington's disease. Remarkably however, some organisms have found ways to circumvent the toxicity of amyloid proteins, and instead co-opt them as beneficial aspects of their development and survival. An example of this can be seen in the bacteria genus *Streptomyces*. *Streptomyces* bacteria have a complex, multicellular life cycle that involves progressing through a number of distinct developmental stages. The reproductive phase of the life cycle requires the activity of amyloid-forming proteins known as chaplins - hydrophobic proteins that polymerize on the cell surface, ultimately promoting reproductive development.

Due to limitations in other model *Streptomyces*, key questions regarding the function of chaplins have not yet been addressed. The emerging model species *Streptomyces venezuelae* is unique given its rapid growth, its ability to develop in liquid, and its potential to adopt two distinct life cycle programs. This work sought to characterize how chaplins influence these processes. We created a number of chaplin mutants, and determined that chaplins contribute to these process in mostly redundant ways, but when deleted in bulk cause significant phenotypic changes. We have also shown that the requirement of chaplins in development goes beyond what was previously understood - as their loss affects development in all classical life cycle stages, and further impacts alternative life cycle programs. To understanding how chaplins are regulated in *S. venezuelae*, mutagenesis screens were conducted to identify mutants with altered chaplin regulation. These yielded promising candidates for further investigation. Collectively, this work has advanced our understanding of chaplin proteins, specifically how they are regulated, and how they affect various modes of *Streptomyces* growth and development.

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5-FC	5-fluorocytosine
5-FU	5-fluorouracil
A	adenine, alanine
bld	*bald* (gene denotation)
bp	base pairs
C	cytosine; cysteine
Cas	CRISPR associated
CDS	coding sequence
chp	chaplin (gene denotation)
cm	centimeter
codAS	cytosine deaminase (gene denotation)
CRISPR	clustered regularly interspaced short palindromic repeats
D	aspartic acid
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid; Difco Nutrient Agar
dNTPs	deoxyribonucleotides
E	glutamic acid
EMS	ethyl methanesulfonate
Fe	iron
fwd	forward
G	guanine; glycine
g	gravity
H	histidine
h	hour(s)
K	lysine
kb	kilo base pairs
L	leucine
LE	left editing template fragment
LB	lysogeny broth
M	methionine
Mb	mega base pairs
min	minutes
mL	milliliter
mM	millimolar
mm	millimeter(s)
MS	mannitol-soy flour
MYM	maltose-yeast extract-malt extract
N	asparagine
OD	optical density
P	proline
PCR	polymerase chain reaction
pH	potential hydrogen
PNPG	p-nitrophenyl β–D-glucuronide
Q	glutamine

R	arginine
rdl	rodlin (gene denotation)
RE	right editing template fragment
rev	reverse
RNA	ribonucleic acid
rpm	revolutions per minute
S	serine
seq	sequencing
SOB	Super Optimal Broth
T	thymine; threonine
U	uracil
V	valine
whi	white (gene denotation)
X-gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium
YP	yeast extract-peptone
µg	microgram(s)
µL	microliter(s)
µM	micromolar

Chapter One:
Introduction

1.1 Amyloid proteins

1.1.1 Characteristics of amyloid proteins

The term "amyloid" was first coined to describe a substance found in various diseased tissue that had a similar staining profile to that of starch (Virchow, 1971). It was not until much later that this substance was identified as proteinaceous, and later still that the world would come to realize the precarious nature of these proteins and the profound implications that amyloids have on health and disease (reviewed in Kyle, 2001). The amyloid distinction is reserved for proteins that meet a number of defined criteria; notably, amyloid proteins are rich in β-sheet secondary structure, a feature that enables them to self-polymerize into long fibrils (Greenwald & Riek, 2010). Interestingly, many proteins – even those whose native state contains few β-sheet structures – have the capacity to form amyloid fibrils upon encountering denaturing conditions (Chiti *et al*., 1999). Fibrils are formed when the β-sheets of the proteins stack linearly and perpendicularly to the fibril axis (hence the often used "cross-β sheet" term) and subsequently combine together in a helical nature to generate insoluble mature amyloid fibers (Figure 1.1; Colvin *et al*., 2016; Greenwald & Riek, 2010; Harper *et al*., 1997). These fibers are easily distinguished due to their non-branching appearance, their strong resistance to proteinases and other denaturing conditions, and their ability to bind specific dyes such as Congo Red or Thioflavin-T (Nilsson, 2004). The prevalence of amyloid proteins is vast; they are found in all domains of life with many species encoding a number of proteins with amyloid-forming potential (Greenwald & Riek, 2010).

1.1.2 Amyloid fibers and factors that affect fiber formation

Amyloid fiber formation begins when individual proteins aggregate together to commence the nucleation stage; this typically requires three to four proteins, and

is a relatively slow and rate-limiting process (Knowles *et al.*, 2009). This nucleus "seeds" the polymerization of additional monomers, and a rapid elongation phase follows. These polymers of amyloid proteins are termed "protofilaments", and will helically associate length-wise to produce fibrils, with as many as six protofilaments per fibril (Jimenez *et al.*, 2002; Walsh *et al.*, 1999). Rapid polymerization depends on a number of factors, with well-documented examples including the presence of oligomeric species, a high concentration of free monomers, and agitation of the amyloid protein-containing solution – all of which hasten the polymerization process. In addition to these factors, however, a number of other conditions are important for determining whether rapid polymerization will occur, including temperature, the presence of denaturants, the oxidative state of the solution, and the pH of the solution (Chiti *et al.*, 2001; Chiti & Dobson, 2006; Harper *et al.*, 1997).

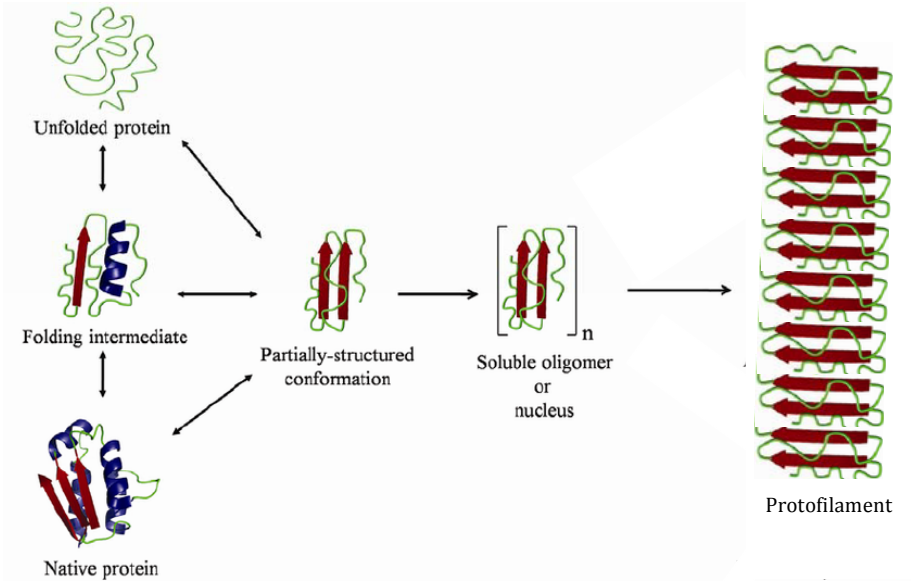


Figure 1.1 General pathway of amyloid formation. Amyloid proteins form when proteolytic cleavage, denaturing conditions, a change in solvent composition, or other factors cause β-sheet conformations to be favoured. When these structures stack together, soluble oligomers form after a prolonged nucleation phase, which is followed by a rapid elongation phase (modified from Kumar & Udgaonkar, 2010).

1.1.3 Harmful amyloids

Given the propensity of amyloid proteins to form large, insoluble fibers, it is perhaps no surprise that the medical and scientific communities viewed them as strictly deleterious and disease-causing after their initial discovery (Virchow, 1971). Indeed, in humans alone, 36 different ailments are caused or exacerbated by the presence of amyloid proteins, including Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis (ALS), and Type II diabetes, amongst many others (Chiti & Dobson, 2006; Sipe *et al*., 2016). Beyond impacting human health, amyloid-related toxicity has also been a major issue in the agricultural industry, with scrapie and bovine spongiform encephalopathy prions – both of which adopt amyloid characteristics during infection – causing mass fatalities to sheep and bovine livestock respectively (Aguzzi & Calella, 2018; Prusiner *et al*., 1983). Interestingly, although the process of amyloid fiber formation is generally similar among amyloidogenic proteins, the biological toxicity of different amyloid diseases varies widely. In some instances, the deposition of large quantities of amyloid fibers leads to impairment of cellular function. An example of this is seen in the disease 'amyloid light-chain amyloidosis', where the accumulation of amyloid fibers in the kidney is so great as to displace the extracellular matrix and lead to renal failure (Herrera *et al*., 1999). However, in other instances it is the soluble oligomeric species formed early in the amyloid pathway that are responsible for disease symptoms (Chiti & Dobson, 2006). This is seen in the case of Alzheimer's disease, where the oligomeric intermediates of amyloid-β protein are the specific causative agents of inflammation and downstream cellular dysfunction (Sengupta *et al*., 2016).

1.1.4 Functional amyloids

In recent years it has become apparent that a strict classification of amyloids as harmful entities is incorrect, with there being many instances where organisms rely on amyloids for fitness and survival. Amyloids that serve vital roles are now known as "functional amyloids". The ubiquity of harmful amyloids is mirrored in that of functional amyloids, with these systems serving a variety of roles – with unique modes of action – in species across all domains of life (reviewed in

Iconomidou & Hamodrakas, 2008). In humans for example, amyloid fibers are used as scaffolds in the biosynthesis of melanin, and in mice, as fertilization-promoting factors that assist with sperm adherence to oocytes. In both of these examples, the potentially harmful effects of the amyloid fibers is circumvented by housing them within structures with tightly regulated acidic pH values – outside of these structures and the neutral pH leads to rapid depolymerization, eliminating potential harm (Guyonnet *et al*., 2014; Mcglinchey & Lee, 2018).

1.1.4.1 The curli proteins – functional amyloids used in biofilms

Perhaps the best-studied cases of functional amyloids are not those found in humans, but those seen in microbes. In *Escherichia coli*, for example, the curli system is an intricate network of amyloid-forming proteins, regulatory proteins, and secretion proteins that collectively function to assemble extracellular amyloid fibers (curli fibers). These fibers contribute to the extracellular matrix during biofilm formation, enabling *E. coli* cells to establish firm attachments to cells and other surfaces (Gerven *et al*., 2015). Without an intact curli system, the structure and integrity of *E. coli* biofilms is compromised, and in the case of infection, these cells are more susceptible to immune clearance. Clearly, these functional amyloids are of crucial importance for the development and survival of *E. coli* cells (Biesecker *et al*., 2018; Hung *et al*., 2013). In order to maintain this system in a functional and not harmful state, an amyloid inhibitor is encoded within the curli operon. The gene *csgC* encodes an amyloid capping protein that lowers the polymerization kinetics of the amyloid subunit, CsgA, within the periplasm. In concert with this, the chaperone CsgE also contributes to reducing inappropriate amyloid polymerization while directing proteins to the curli secretion apparatus (Nenninger *et al*., 2012). In this way, rapid polymerization does not occur until CsgA monomers are secreted, providing an effective way of directing potentially fatal reactions outside of the cell, where they will not be harmful (Evans *et al*., 2015; Sleutel *et al*., 2017). In fact, in all instances where bacteria employ functional amyloids, they are associated with spatial segregation – a mechanism effectively preventing the potentially adverse

effects associated with uncontrolled formation of amyloid fibers (Taglialegna *et al*., 2016).

1.1.4.2 Hydrophobins – functional amyloids used for development and virulence

In the case of fungi, amyloid proteins are employed as developmental determinants (or in rare cases, virulence factors), rather than biofilm constituents. At least 20 species of fungi encode developmental proteins known as hydrophobins, many of which have amyloidogenic capabilities (Mackay *et al*., 2001; Wessels, 1996). When hydrophobins are secreted, they polymerize into fibers and associate at air-water or water-surface interfaces – with the latter example requiring a hydrophobic surface such as a plant leaf (Wosten *et al*., 1994a; Wosten *et al*., 1994b). In this way, the amphipathic hydrophobins allow fungal hyphae to breach water surfaces and grow into the air. This is important because the newly emerging aerial hyphae will give rise to fungal spores (reviewed in Kershaw & Talbot, 1998). Similar to *E. coli* biofilms aiding with immune evasion (mentioned above), hydrophobin activity can sometimes promote fungal pathogenicity. In the case of Dutch Elm Disease, the secreted hydrophobin (cerato-ulmin) by the fungi *Ceratocystis ulmi* is a virulence factor that damages the leaves of elm trees and increases fungal pathogenicity (Takai, 1974; Temple *et al*., 1997).

1.1.4.3 Chaplins – developmental amyloids from *Streptomyces*

The *Streptomyces* bacteria produce an interesting class of functional amyloids known as the chaplin proteins. Similar to fungal hydrophobins, the chaplins are secreted amyloids that function as developmental determinants enabling *Streptomyces* to progress through their life cycle. The chaplins also exert their effects by reducing surface tension at air-water interfaces (Claessen *et al*., 2003; Elliot *et al*., 2003).

1.2 The Streptomycetes

The Streptomycetes are a family of Gram-positive, soil dwelling bacteria that are scientifically intriguing due to their complex and multicellular development and their unparalleled ability to produce antibiotics and other clinically useful metabolites. As their name suggests (with *Streptomyces* translating to "twisted fungus") these bacteria were first mistakenly identified as fungi after observations of their hyphal structures and multicellular life cycle (Hopwood *et al*., 1970). Historically, many biological questions have been addressed in the model species *Streptomyces coelicolor* and *Streptomyces griseus*; however, *Streptomyces venezuelae* is an emerging model system and is the focus of my work here.

1.2.1 *Streptomyces venezuelae* as a model organism

Many questions in *Streptomyce*s biology have been addressed using the model organism *S. coelicolor*. Among the fascinating features that have been studied in this species, perhaps the most intensive work has been in trying to understand its complex growth and developmental cycles. The utility of a species that has served as an excellent model organism for over 70 years notwithstanding, *S. coelicolor* has some features that present challenges when addressing developmental questions including its relatively slow growth and its inability to fully develop in liquid medium (Hopwood *et al.*, 1970). In contrast, *S. venezuelae* has garnered attention in recent years due to its rapid growth (completing its life cycle in less than 48 h, compared to the approximately 96 h required by *S. coelicolor*), its ability to complete its life cycle in liquid medium, and its newly discovered ability to assume two distinct life cycles – each with vastly different morphologies – in response to varying medium conditions (Glazebrook *et al*., 1990; Jones *et al*., 2017). Therefore, *S. venezuelae* was the organism chosen to complete the following work.

1.2.2 The two life cycles of *S. venezuelae*

The *Streptomyces* have a complex multicellular life cycle, which involves the progression through distinct morphological stages. Beginning life as a spore, a germ tube emerges and grows into the surrounding substrate, eventually forming an extensive mass of branching hyphae known as a vegetative mycelium. From the

vegetative mass, and in response to lack of nutrients or other stressors, non-branching aerial hyphae are erected. These hyphae are sporogenic, and will undergo a synchronous round of cell division and septation resulting in chains of dormant spores. The spores are dispersed throughout the environment, and upon encountering favourable growth conditions, will begin the life cycle anew (Figure 1.2; Hopwood, *et al*., 1970) Recently, however, it has been determined that a number of streptomycetes, including *S. venezuelae,* are capable of progressing through an alternative developmental program. The above-described life cycle (what will hereafter be referred to as the "classical" life cycle) represents a relatively stagnant and slow mode of growth, but Jones *et al*. have found that in response to certain signals, such as the presence of other soil microbes, low glucose, or high pH, select streptomycetes are capable of adopting an "exploration" mode of growth (Figure 1.2). While exploring, a colony will rapidly spread across biotic and abiotic surfaces as non-branching vegetative hyphae – a morphology not seen in the classical life cycle. Exploration is not yet fully understood, and there are a likely a multitude of factors and signals that may influence *Streptomyces*' ability to explore (Jones *et al*., 2017).

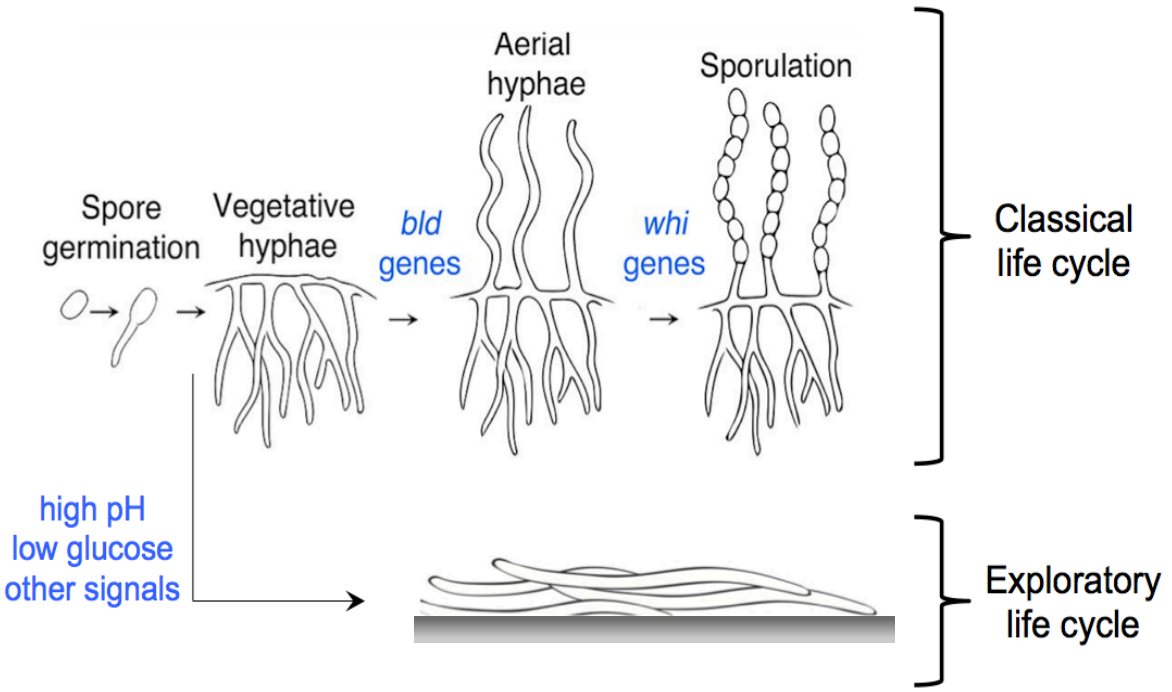


Figure 1.2 The classical and exploratory life cycles of *Streptomyces*. In the classical life cycle, a dormant *Streptomyces* spore resumes active growth after detecting favourable growth conditions. Growing by hyphal tip extension, a vegetative mycelial mass is soon established. Aerial structures grow upwards out of this vegetative mass in response to environmental stressors, and these aerial structures differentiate into spores that can be dispersed to new niches. In the exploratory life cycle, non-branching vegetative hyphae rapidly spread outwards from the colony centre, and traverse many different surfaces in their path. Shown in blue font are important genes or factors necessary for the progression to that stage of the life cycle. Modified from Jones *et al*., 2017.

1.2.3 Classical *Streptomyces* growth and development

As a *Streptomyces* colony grows and develops, it assumes a number of discrete cell types and morphologies. Accordingly, the genetic regulation governing these changes is extensive and highly intricate, and has been broadly divided into two classes of regulatory genes and proteins: the bald family and the white family. The bald (*bld*) genes are those whose products are important in the transition from the vegetative state to the aerial state. The white (*whi*) genes are those whose products are important for allowing aerial hyphae to differentiate into chains of mature spores (Figure 1.2). Collectively, the *bld* and *whi* genes are indispensable for development, with mutations in either family abolishing proper development (Flärdh & Buttner, 2009).

1.2.3.1 Bald genes in the developmental cascade

The *bld* genes were first identified from a series of mutants that were incapable of erecting aerial structures, with the ensuing colonies retaining a shiny – or bald – appearance (Merrick, 1976). A number of *bld* genes have been identified to date (*bldA*, *B*, *C*, *D*, *F*, *G*, *H*, *I*, *J*, *K*, *L, M, N O*) and their effects on *Streptomyces* growth are summarized in the table below (Table 1.1).

Table 1.1 The *bld* genes of *Streptomyces*. A list of the identified *bld* genes, together with a brief description of their function.

***bld* gene**	**Brief description of function**
bldA	Encodes a tRNA that translates the rare UUA codon. This is

significant because this codon is found in the mRNA of *bldH* (also known as *adpA*) and *ramR* (required for SapB production, where SapB is another important developmental determinant). *bldA* is therefore required for the translation of the corresponding proteins (Kelemen & Buttnert, 1998; Takano *et al*., 2003).

bldB Encodes a homodimeric DNA binding protein that indirectly affects its own expression and influences antibiotic production. Strains mutated in *bldB* are impaired in their ability to catabolize carbon. (Eccleston *et al*., 2002; Pope *et al*., 1998).

bldC Encodes a small, dimeric DNA binding protein that binds to the promoters of a number of developmental genes, including many *whi* genes (Hunt *et al*., 2005; Schumacher *et al*., 2018).

bldD Encodes a DNA binding "master regulator" protein that controls numerous genes (usually via repression) involved in aerial development and sporulation. Many developmental processes are directly or indirectly affected by BldD. Mutants in *bldD* appear bald because repression relief of such a large number of development genes causes highly accelerated development, and colonies readily form spores while bypassing aerial development (Elliot *et al*., 2001; den Hengst *et al*., 2010; Tschowri *et al*., 2014).

bldF Poorly characterized (Schauer *et al*., 1991).

bldG Encodes an anti-anti-sigma factor that liberates sigma factors involved in development (with σ^H, being the best characterized example; Champness, 1988; Sevcikova *et al*., 2010).

bldH Also known as *adpA*, it encodes a DNA binding protein that binds hundreds of promoters, many of which belong to developmental genes. In addition to activating expression of *bldA*, the *bldH* transcript contains a UUA codon and relies on BldA for efficient translation (Higo & Horinouchi, 2011; Takano *et al*., 2003; Yao *et al*., 2013)

bldI Poorly characterized, but appears to be involved in the expression of *bldA*, either directly or indirectly (Leskiw & Mah, 1995).

bldJ	Mutations in *bldJ* result in cells that have impaired siderophore export and import ability, likely affecting development through an iron starvation process. Specific role of encoded protein unclear (Lambert *et al*., 2014).
bldK	The *bldK* locus encompasses a number of genes (*bldK A-E*) that collectively form an ATP-binding cassette (ABC) oligopeptide transporter channel (Akanuma *et al*., 2011). BldK likely functions to transport siderophores (Lambert *et al*., 2014).
bldL	Poorly characterized (Nodwell *et al*., 1999)
bldM	Encodes an orphaned response regulator (not genomically associated with a sensor kinase) whose expression is activated by σ^{BldN} (Bibb *et al*., 2000; Molle & Buttner, 2000).
bldN	Encodes an extracytoplasmic function sigma factor (σ^{BldN}) that binds directly to the chaplin, rodlin, and *bldM* promoters and likely activates their expression (Bibb *et al*., 2012; Elliot *et al*., 2003).
bldO	Encodes a DNA binding protein that directly regulates the expression of a sporulation specific gene (*whiB*), repressing its expression until the onset of sporulation. Despite *whiB* being the only target of BldO, *bldO* mutants are classified as bald because they sporulate prematurely and bypass aerial development (Bush *et al*., 2017).

Although the *bld* gene products control the expression of a number of downstream genes, a prominent role shared by some of these products is to regulate (directly or indirectly) the expression of the *chp* genes. Due to the amphipathic characteristics of chaplin amyloids, these proteins allow hyphae to breach the surface of their hydrophilic medium and grow aerially (Claessen *et al*., 2003; Elliot *et al*., 2003). As such, these proteins are major developmental determinants, and will be described in detail below.

1.2.3.2 White genes in the developmental cascade

Once aerial growth has been achieved, the *Streptomyces* life cycle enters its final stage: sporulation. During this time, the multi-genomic, actively growing filamentous hyphae will metamorphose into chains of dormant exospores that can be dispersed to the surrounding environment. The products of the *whi* genes govern many of the processes needed to achieve this feat, including cytoskeleton rearrangement, chromosome segregation, and cell wall modification. The *whi* genes were initially identified due to the distinctive white colouration of their corresponding mutant colonies. The final stages of spore maturation involve the deposition of a pigmented polyketide metabolite into the cell wall of wild-type spores; failure to form mature spores by the *whi* mutants leave colonies appearing fuzzy and white (reviewed in Bush *et al*., 2015).

1.3 Introducing the chaplins

1.3.1 Identification and characteristics of chaplin proteins

The chaplin proteins were first identified when global transcription data from an *S. coelicolor bldN* mutant were compared to that of a wild type counterpart and it was noted that eight homologous genes were significantly down regulated in this mutant (Elliot *et al*., 2003). Concurrently, extracts from spore walls that could impart hydrophobicity were analyzed and found to contain peptides specific to four particular proteins. Again, the coding sequence of these peptides was determined to result from the same family of eight homologous genes (Claessen *et al*., 2003). These genes were dubbed the *chp* genes (for coelicolor hydrophobic protein – although these genes are found throughout the streptomycetes and are not unique to *S. coelicolor*). *S. coelicolor* chaplins are split into two types: long and short. The long chaplin proteins (encoded by *chpA, B,* and *C*) are organized such that they have an N-terminal Sec secretion signal, followed by two 'chaplin domains' having strongly hydrophobic characteristics and predicted β-sheet structure. At the C-terminus of these long chaplins is a sorting signal that targets these proteins for covalent attachment to the cell wall (Figure 1.3). After the Sec secretion has been cleaved, the mature peptide length is ~200 amino acids. In *S. venezuelae*, homologues of only

chpB and *chpC* have been identified; *S. venezuelae* does not possess *chpA*. The short chaplins (encoded by *chpD, E, F, G, H*), also have an N-terminal Sec secretion signal, but instead of two chaplin domains, they have a single chaplin domain and no sorting signal (Figure 1.3). The length of mature short chaplins is ~50 amino acids. Each chaplin domain is associated with two conserved cysteine residues, with the only exceptions being that of *chpE* and the second chaplin domain of *chpB* (Claessen *et al*., 2003; Elliot *et al*., 2003). The Sec secretion signal found at the beginning of each chaplin peptide ensures that these nascent peptides are directed towards the membrane-spanning 'Sec' channel and are promptly exported in their primary conformation *(*Keyzer *et al*., 2003). The sorting signal at the C-terminus of the long chaplins comprises an LAXTG motif, followed by a string of hydrophobic residues and then a number of positively charged residues. This motif is recognized and cleaved by sortase enzymes, which then covalently attach the C-terminal end of the peptide to the peptide chains of nascent *N*-acetylmuramic acid in the peptidoglycan (Duong *et al*., 2012; Marraffini *et al*., 2006).

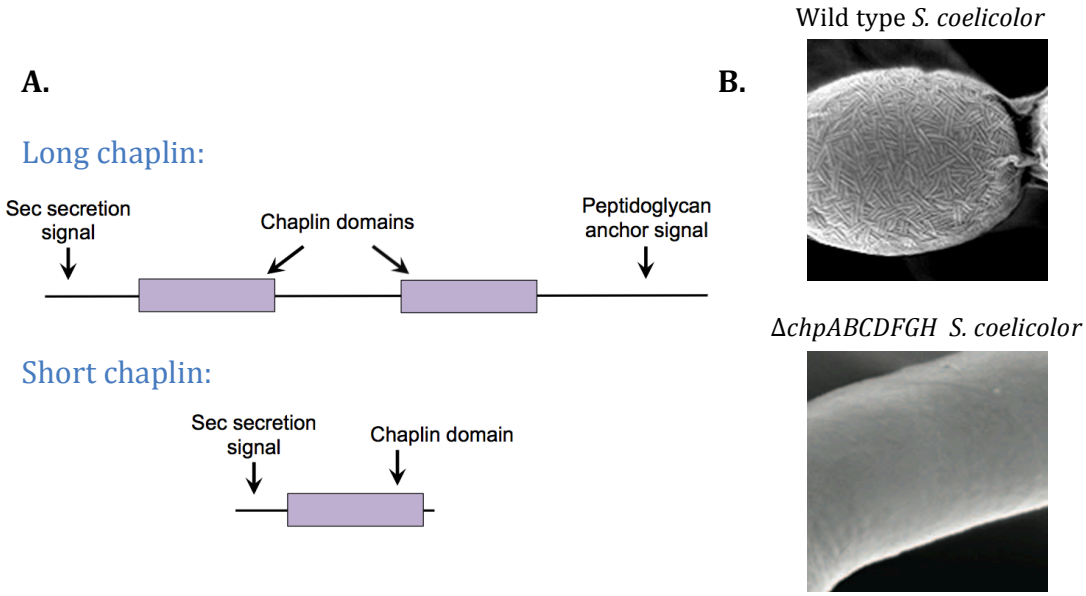


Figure 1.3 The long and short chaplins and their effects in *S. coelicolor*. (A) The typical domain architecture of long chaplins and short chaplins (in *S. venezuelae,* ChpB and ChpC are long and ChpD, E, F, G, H are short). **(B)** In *S. coelicolor*, the phenotypic consequences on the cell wall of a wild type strain are compared to that of a Δ*chpABCDFGH* strain (Di Berardo *et al*., 2008).

1.3.2 Chaplin E – a conditionally essential chaplin

In work conducted with *S. coelicolor*, there was an intriguing discovery made while attempting to create chaplin mutants: *chpE* could not be deleted in a wild type background. Indeed, only when a second, non-chromosomal copy was added, or second site-suppressors were present, could the *chpE* gene be readily deleted. This discovery, coupled with the fact that ChpE does not contain the same conserved cysteine residues of the other chaplins, nor the same hydrophobicity profile (Di Berardo *et al*., 2008), suggests that it may play a distinct and essential role in wild type *Streptomyces*. Interestingly, deletions of *chpE* were permitted in strains that were lacking the other *chp* genes, or in strains harbouring mutated rodlin or Tat-secretion pathway-encoding genes (where the Tat-secretion system functions to export proteins in their tertiary or quaternary states; Di Berardo *et al*., 2008; Natale *et al*., 2008).

1.3.3 The role of chaplins in classical development

Biochemical investigations have revealed a possible mechanism for the how chaplins exert their effects. The chaplin domain is amylodogenic, and chaplins self-assemble into amyloid fibers that coat the outside of the hyphae (Figure 1.3). These fibers reduce the surface tension of the hydrophilic growth medium and facilitate aerial hyphae up-growth (Claessen *et al*., 2003). In addition to their role in promoting aerial development, the chaplins are key structural features of *Streptomyces'* fimbriae (pili-like extensions used for surface adhesion; de Jong *et al*., 2009),

The eight *chp* genes of *S. coelicolor* are redundant to a degree, although they are collectively indispensable for the transition to aerial hyphae. This was determined by studying a *chp*-null strain in *S. coelicolor* which was unable to grow aerially or progress through its life cycle (Capstick *et al*., 2007). Importantly, a mutant containing only *chpC*, *chpE*, and *chpH* (known as the minimal *chp* strain) could raise aerial hyphae albeit with some developmental delays (Di Berardo *et al*., 2008). This is consistent with transcriptional expression data showing that *chpE* and *chpH* are expressed earlier and at much greater levels than the other six

chaplins; as well as the fact that *chpC*, *chpE*, and *chpH* are strictly conserved among all *Streptomyces* (Claessen *et al*., 2003; Elliot *et al*., 2003).

1.3.4 Other secreted proteins involved in development

When appreciating the roles that chaplins serve, it is important to consider other extracellular proteins that function similarly to the chaplin proteins. First, the rodlins are a family of secreted proteins that are proposed to interact with the chaplins. The current model involves the rodlins organizing the chaplins into the precise ultrastructure seen on the surface of aerial hyphae and spores (*e.g*., Figure 1.3; Claessen *et al*., 2004). Secondly, the SapB protein (also secreted) shares similar functionality with the chaplins in reducing surface tension and permitting aerial hyphal up-growth. Notably, SapB is only expressed during growth on rich medium with high osmolarity; under these conditions, aerial growth is promoted by both SapB and the chaplins, whereas during growth on more minimal-type medium, the chaplins are sufficient for aerial hyphae formation (Capstick *et al*., 2007; de Jong *et al*., 2012).

1.3.5 Regulation of *chp* gene expression

Expression of *chp* genes relies on the products of specific *bld* genes. To date, the two major *bld* gene products affecting chaplin expression are BldD and BldN (Bibb *et al*., 2000; Hengst *et al*., 2010a). More recently, the two-component response regulator of a two-component system (MtrA) has been revealed to bind the promoters of the *chp* genes (Som *et al*., 2017) and activate their expression as well (Zhang *et al*., 2017).

BldD is a master regulator of *Streptomyces* development, functioning as a global repressor of numerous genes that impact both aerial growth and sporulation (Elliot *et al*. , 2001; Elliot & Leskiw, 1999; Hengst *et al*., 2010). Increasing intracellular levels of the secondary messenger cyclic di-GMP promotes the dimerization of BldD, allowing it to bind to the promoters of approximately 170 genes – including its own promoter, those of several *bld* genes and those of several *whi* genes, (den Hengst *et al*., 2010; Tschowri *et al*., 2014). BldD affects chaplin

levels, indirectly, as some of its target gene products directly regulate *chp* expression themselves (*e.g*., *bldN* and *rsbN* – see below; Bibb *et al*., 2012).

σ^{BldN} is an extracytoplasmic sigma factor that directs RNA polymerase to a number of developmental promoters including those for the *chp* genes, and another *bld* gene, *bldM* (Bibb *et al*., 2000; Bibb *et al*., 2012). σ^{BldN}-mediated regulation of *bldM* is important for *Streptomyces* development because BldM, like BldD, plays a central role in the control of developmental genes. In addition to being transcriptionally repressed by BldD, σ^{BldN} is further regulated by an anti-sigma factor, RsbN (regulator of sigma BldN). RsbN is anchored to the membrane, and its cytosolic domain sequesters σ^{BldN} until an unknown extracellular signal causes its release (Bibb *et al*., 2012).

MtrA is a two-component system response regulator that is conserved throughout the actinobacteria. Mutations in *mtrA* result in colonies with a bald phenotype, reduced expression of numerous developmental genes [*chp* genes, rodlin genes, certain *bld* genes (*e.g*., *bldK*), certain *whi* genes (*i.e*., *whiH* and *whiI*)], and impaired antibiotic production. Not all MtrA target genes are activated by MtrA however, with *bldD* being dramatically upregulated in an *mtrA* mutant (Zhang *et al*., 2017). In *Streptomyces* it is not yet known what signal the sensor kinase, MtrB, is activated by, but in other actinobacteria, the activity of this kinase is modulated by its hydration status, allowing MtrB to indirectly respond to external solute concentrations (Moker *et al*., 2007).

1.4 Aims of this work

My thesis work has focused on understanding the function of the *chp* genes and chaplin proteins from *S. venezuelae*. It is well established that the *chp* genes are important for the development of *S. coelicolor*, but these genes/products have not been investigated in *S. venezuelae*. This is important because *S. venezuelae* provides an excellent platform to address questions in chaplin function that are not possible in other *Streptomyces* species such as rapid growth, liquid development, and the ability to undergo exploration. These latter two situations are interesting, as they include aqueous growth and vegetative growth respectively, and therefore should

not rely on the only defined function of chaplin proteins: promoting aerial development. Therefore, my first research aim is to address the question: are the chaplin proteins, which are crucial developmental determinants of the classical life cycle equally as important in less-studied developmental conditions? If so, what role may chaplins be providing in these unique environments? Given that there is much still unknown about the regulation of *chp* genes, my second research aim is to identify the regulators of these genes. It is known that σ^{BldN} requires additional transcription factors in order to initiate transcription, but it remains to be determined whether MtrA is solely that transcription factor. Because *chp* expression still occurs in an *mtrA* mutant, this suggests that there may be other unidentified factors involved in their expression and regulation. I have conducted random chemical mutagenesis as a means of identifying novel genes that may contribute to *chp* regulation.

Chapter Two:
Materials and Methods

2.1 Bacterial strains and culturing

2.1.1 Plasmids and Strains

All bacterial strains created in this work are listed in Table 2.1. All plasmids created in this work are listed in Table 2.2.

Table 2.1 Bacterial strains used in this work

Strain	Genotype, characteristic, use	Reference or source
E. coli strains		
DH5α	Routine cloning	Invitrogen
ET12567/pUZ8002	Methylation deficient, contains helper plasmid pUZ8002, used for conjugations with *Streptomyces*; kan^R, $chlor^R$.	MacNeil *et al*., 1992; Paget *et al*., 1999
BW25113/pIJ790	Recombinogenic, used for ReDirect-based deletions; $chlor^R$.	Gust *et al*., 2003
Streptomyces strains		
S. venezuelae ATCC 10712	Wild type	Bibb *et al*., 2012

Table 2.2 Plasmids created in this work

Plasmid	Genotype, characteristic, use	Reference or source
pCRISPomyces-2-based	Temperature-sensitive origin of replication, used for CRISPR-based deletions, $apra^R$	Cobb, Wang, Zhao, 2014
pMC311	+ *chpB* gRNA and editing template	This work
pMC312	+ *chpC* gRNA and editing template	This work
pMC313	+ *chpD* gRNA and editing template	This work
pMC314	+ *chpE* gRNA and editing template	This work
pMC315	+ *chpF* gRNA and editing template	This work
pMC316	+ *chpG* gRNA and editing template	This work
pMC317	+ *chpH* gRNA and editing template	This work
pMC318	+ *mtrA* gRNA and editing template	This work
pGUS-based	Transcriptional reporter *gusA*, *int* ϕC31, $apra^R$	Myronovski *et al*., 2011
	+ *chpH* promoter driving *gusA*	From Stephanie Jones
	+ *chpE* promoter driving *gusA*	From Stephanie Jones
pMS82-based	*int* φBT1, hyg^R	Gregory *et al.*, 2003
	+ P*chpH*–theophylline riboswitch–*codAS*	This work
	+ P*chpE*–theophylline riboswitch–*codAS*	This work
pSET*hyg-aac(3)IV*-	*int* ϕC31, hyg^R, $apra^R$ ($spec^R$/$strep^R$)	Sherwood & Bibb, 2013

based		
	+ *Sven_7287*	This work
	+ *Sven_3213*	This work
	+ *Sven_1160*	This work
pIJ82-based	*int* ϕC31, hyg^R	Gift from H. Kieser
	+ *chpH*, for complementation	This work
	+ *chpF*, for complementation	This work
	+ *chpB,* for complementation	This work

2.1.2 Conditions for growth

All *E. coli* strains were grown on solid or in liquid lysogeny broth (LB) medium (Miller, 1972) and incubated at 37°C unless stated otherwise. When selecting with the salt-sensitive antibiotic hygromycin B, Super Optimal Broth (SOB) liquid medium or Difco Nutrient Agar (DNA) solid medium was used instead (Hanahan, 1983). *S. venezuelae* and its derivative strains were grown in liquid or on solid maltose - yeast extract - malt extract (MYM) medium and incubated at 30°C unless stated otherwise (Yang *et al*., 2001). When conjugations were performed, *S. venezuelae* was grown on mannitol-soy flour (MS) solid medium and incubated at 30°C (Hobbs *et al*., 1989). Exploration analyses were conducted by growing *S. venezuelae* strains on yeast extract-peptone (YP) solid medium and incubating at 30°C. Where appropriate, antibiotics were added to growth medium at the following concentrations: apramycin (50 μg/mL), kanamycin (50 μg/mL), chloramphenicol (25 μg/mL), hygromycin B (50 μg/mL), streptomycin (100 μg/mL), spectinomycin (100 μg/mL), ampicillin (100 μg/mL), and nalidixic acid (25 μg/mL). Kanamycin was used at 50 μg/mL for *E. coli* and 25 μg/mL for *Streptomyces*. Liquid cultures were incubated while shaking at 200 rpm.

2.1.3 Long-term storage of *E. coli* strains

E. coli strains to be stored for future use were grown overnight in 5-10 mL of LB or SOB liquid medium with appropriate antibiotics. The overnight culture was mixed with 40% glycerol in a 1:1 ratio (500 μL of each) to bring the final concentration of glycerol to 20%. These *E. coli* glycerol stocks were then stored at -80°C.

2.1.4 Long-term storage of *Streptomyces* strains

S. venezuelae strains to be stored for future use were spread onto MYM solid medium and incubated at 30°C until sporulation was achieved (approximately 2-3 days). At this stage, the biomass was scraped off the medium into 10 mL of sterile water. The resulting biomass suspension was sonicated in a water bath for approximately 2 min in order to separate mature spores from mycelium. This suspension was then passed through a cotton syringe filter and the flowthrough was centrifuged at 3930×*g* for 5 min to pellet spores. Spore pellets were resuspended in 40% glycerol and stored at -20°C.

2.1.5 Monitoring *Streptomyces* growth in liquid culture

To monitor the growth profiles of various *chp* mutants and *chp* regulator mutants, overnight cultures of these strains were used to inoculate 10 mL of liquid MYM medium to a final optical density at 600 nm (OD_{600}) of 0.05. Samples were taken at intervals over a 30 h – 56 h time course, where 250 µL of the culture aliquot would be mixed with 750 µL of fresh liquid MYM medium and the OD_{600} value for this diluted sample was recorded. Concurrently, a 10 µL sample of this culture was placed on a microscope slide and imaged with light microscopy at 1000× magnification using Nixon NIS Elements Viewer Software to monitor the developmental stage of the culture.

2.1.6 Quantifying exploration in wild type and mutant *S. venezuelae* strains

Exploration capabilities of the various *chp* mutants and *chp* regulator mutants were determined by diluting an overnight culture of these strains to an OD_{600} of 0.3. An 8 µL sample of these dilutions was spotted directly onto the centre of a solid YP medium plate and incubated at 30°C for 5-10 days. To account for the fact that exploring colonies produce volatile compounds that impact growth of neighbouring colonies, only one sample was spotted per plate. Photographs of exploring colonies were taken at defined time intervals, and their surface areas were calculated by using ImageJ software (Schneider *et al*., 2012). Specifically, the

image was calibrated to a known measurement (the 10 cm diameter of the petri plate), after which the colony image was outlined and its surface area calculated by the program.

2.2 Molecular biology techniques

2.2.1 Oligonucleotides

Oligonucleotides used in this work are listed in Table 2.3 alongside a brief description of their use.

Table 2.3 Oligonucleotides used in this work

Primer Name	Primer Sequence (5' - 3')	Description or use
Primers used to generate CRISPR mutants (of *chp* and *mtrA* genes)		
chpB LE fwd	CATCATTCTAGAGTTACCCCTGCAAGCACGCC	Used to make editing template for *chpB*-CRISPR construct
chpB LE rev	GCCGGCCGAGAGGGCGAGCGTCAGGCCAGACCACTTCGGCG	
chpB RE fwd	TGACGCTCGCCCTCTCGGCCGGC	
chpB RE rev	CATCATTCTAGACCGCGGTGCGTGCCTCCGTA	
chpB gRNA fwd	ACGCCCCGCCCGTGACCAACAGTG	Guide-RNA for *chpB*-CRISPR
chpB gRNA rev	AAACCACTGTTGGTCACGGGCGGG	
chpC LE fwd	CATCATTCTAGATCGATGACCAACGACGACAAC	Used to make editing template for *chpC*-CRISPR construct
chpC LE rev	TCATCGGCTTCGAAAGAGTGCCAC	
chpC RE fwd	GTGGCACTCTTTCGAAGCCGATGATGCGACAGGTCACGCGC	
chpC RE rev	CATCATTCTAGAGGGACGCACGCTATCCGTAG	
chpC gRNA fwd	ACGCTAGCTTGTTCCCTGCCTGCT	Guide-RNA for *chpC*-CRISPR
chpC gRNA rev	AAACAGCAGGCAGGGAACAAGCTA	
chpD LE fwd	ATATTCTAGACCCCTGACGTGTGAACGTCGTCGC	Used to make editing template for *chpD*-CRISPR construct
chpD LE rev	CGGCCGCGACCTTCTTGCAGTTCAT	
chpD RE fwd	TGAACTGCAAGAAGGTCGCGGCCGAGCCTTCGACGGCAGG	
chpD RE rev	CATCATTCTAGACGCGTACGGCGACTGGTTCG	
chpD gRNA fwd	ACGCCATGACGATTCCGGCGACGA	Guide-RNA for *chpD*-CRISPR
chpD gRNA rev	AAACTCGTCGCCGGAATCGTCATG	
chpE LE fwd	CATCATTCTAGAGGATCGCTCACCGTCAC	Used to make editing template for *chpE*-CRISPR construct
chpE LE rev	TCATGGTCATCGCTGCGGCCGG	
chpE RE fwd	CCGGCCGCAGCGATGACCATGAAACCTCAAGAAGGCCGCTG	
chpE RE rev	ATGATGTCTAGATGGCCATTGTCGGGGAGGAC	
chpE gRNA fwd	ACGCCTTCACTTGGTACATCCTCC	Guide-RNA for

chpE gRNA rev	AAACGGAGGATGTACCAAGTGAAG	*chpE*-CRISPR
chpF LE fwd	CATCATTCTAGAACCGTTTCGAATCCGTCCCA	Used to make editing template for *chpF*-CRISPR construct
chpF LE rev	TCAGGGACATGGGGAAGTGCTCCT	
chpF RE fwd	AGGAGCACTTCCCCATGTCCCTGACTGACCGTGGGTGCCGC	
chpF RE rev	CATCATTCTAGAGAAGCTCGACCGGGGTTTC	
chpF gRNA fwd	ACGCGACAACGGCCTTCGCGATAC	Guide-RNA for *chpF*-CRISPR
chpF gRNA rev	AAACGTATCGCGAAGGCCGTTGTC	
chpG LE fwd	CATCATTCTAGAGCGCGTTGCTGCACACGGGTT	Used to make editing template for *chpG*-CRISPR construct
chpG LE rev	CCCGAGTCGGCGACGGCCATCAGGCACCCGTGCCGGCGAGA	
chpG RE fwd	TGATGGCCGTCGCCGACTCGGG	
chpG RE rev	CATCATTCTAGAGACGCCACGGTCTCCGTCCC	
chpG gRNA fwd	ACGCGCCGGCTCCGCTGAGGGCAA	Guide-RNA for *chpG*-CRISPR
chpG gRNA rev	AAACTTGCCCTCAGCGGAGCCGGC	
chpH LE fwd	CATCATTCTAGAAACGAGAACCTCCGCCTCTA	Used to make editing template for *chpH*-CRISPR construct
chpH LE rev	TCAGGCACCCGCGTCGGCAAC	
chpH RE fwd	CGTTGCCGACGCGGGTGCCTGATGTCCTCTCGGGCAATGTC	
chpH RE rev	CATCATTCTAGAGTACGGCGGTAGATCAGCGA	
chpH gRNA fwd	ACGCGGAACCGATGGCGGCACCCT	Guide-RNA for *chpH*-CRISPR
chpH gRNA rev	AAACAGGGTGCCGCCATCGGTTCC	
mtrA LE fwd	CATCATTCTAGATCAGTCCGAGGAGCTGGAGG	Used to make editing template for *mtrA*-CRISPR construct
mtrA LE rev	TCACGGTGTCGTCGTCGACGACAAG	
mtrA RE fwd	CTTGTCGTCGACGACGACACCGTGAATCGTGCTGCGGGGTGAAGG	
mtrA RE rev	CATCATTCTAGAGGACCTGTCCGATGACGACC	
mtrA gRNA fwd	ACGCGCCGAGCATCTCGGCCAGTG	Guide-RNA for *mtrA*-CRISPR
mtrA gRNA rev	AAACCACTGGCCGAGATGCTCGGC	
pMC600 seq rev	TTTGTGATGCTCGTCAGGGG	To confirm final CRISPR constructs
Primers used to generate ReDirect mutants (select *chp* genes and *mtrA*)		
chpE ReDirect fwd	TGACCGGCCGCAGCGATGACCAGGAGGATGTACCAAGTGATTCCGGGGATCCGTCGACC	Deleting *chpE* via the ReDirect protocol
chpE ReDirect rev	GGGGATCGTCCCGGGGGCCGGCTGGTTCTGGGGGTGTCATGTAGGCTGGAGCTGCTTC	
chpD ReDirect fwd	GAAATGTCTTCCGTCACAGAAAGGATCCAGACGAAAATGATTCCGGGGATCCGTCGACC	Deleting *chpD* via the ReDirect protocol
chpD ReDirect rev	CCGGGGGCCGGCCCGTACCTACCTGCCGTCGAAGGCTCATGTAGGCTGGAGCTGCTTC	
mtrA ReDirect fwd	GCCTTGAGACTGATACGGAAATGGGATGATGTCGATATGATTCCGGGGATCCGTCGACC	Deleting *mtrA* via the ReDirect protocol
mtrA ReDirect rev	TCCCCGGGCTTCGGAGCAGCACTGCCTGTACTCATGTCATGTAGGCTGGAGCTGCTTC	
Primers used to sequence *chp* mutations from genomic loci		
chpB gen chk fwd	ACGTGCCGCATGCCCTGGAG	Binds outside of

chpC gen chk fwd	TGGTCGAGACCGCCCAGAAGGG	CRISPR editing template to ensure genomic region amplified
chpF gen chk fwd	CGGTTTGAGTCGAGCGGGACGC	
chpG gen chk fwd	CGAGCGTAAAACCGGGGCCC	
chpH gen chk fwd	GACCCTCGCGGTCGTACGGG	
Primers used to modify the pCRISPOmyces-2 vector to contain a hygromycin resistance cassette		
Hyg cassette fwd	GTGACACAAGAATCCCTGTTACTT	Used to amplify hyg^R cassette from pIJ82
Hyg cassette rev	CATCATATCGATTCAGGCGCCGGGGGCGG	
pCRISP for hyg fwd	CATCATTCTAGAGGCCAGGAACCG	Amplify a region of pCRISPomyces-2 for use in overlap extension PCR with the above hyg^R cassette
pCRISP for hyg rev	AAGTAACAGGGATTCTTGTGTCACCAGTCGATCATAGCAC GATCAACG	
Primers used to create *chp-gusA* transcriptional fusions		
**chpH* fwd	ATATTCTAGAGTTTCATCGACGCCTGCT	Amplifying the *chpH* promoter
chpH rev	ATATGGTACCCAGCGACGACCTTCTTGA	
**chpE* fwd	ATATTCTAGAACGGCCTCACCCACTGAC	Amplifying the *chpE* promoter
chpE rev	ATATGGTACCTGGTACATCCTCCTGGTCATC	
Primers used to amplify *chpD* coding sequence with a biotin tag		
**Biotin *chpD* fwd	ACGTGCCGATCAACGTCTGC	Amplifying a section of *chpD* CDS
chpD seq rev	GGGCGGCCGCGTACGG	
Primers used to make complementation constructs of select *chp* genes		
chpB comp fwd	CATCATTCTAGACGTTGACGCACACACGAAC	*chpB* complementation
chpB comp rev	CATCATGGATCCTGTCGACACGCCCTACGA	
chpF comp fwd	ATATTCTAGATGGGTGAACGCGCGGCGGG	*chpF* complementation
chpF comp rev	ATATGGATCCCGGTGATGCGGCGCGTGGCC	
chpH comp fwd	ATATTCTAGAGTTTCATCGACGCCTGCT	*chpH* complementation
chpH comp rev	ATATGGATCCAGTGCATGGCACGCACTCC	
Primers used to make *chp-codAS* transcriptional fusions		
chpE for *codAS*	CATCATGGTACCCATGAAGCTGGATCTCGCGGACC	Amplifying the *chpE* promoter
chpE for *codAS* rev	CATCATTCTAGAGCCACCGGCGACCATGGT	
chpH for *codAS* fwd	CATCATGGTACCGTACCCGGCGTGGACGAGCGG	Amplifying the *chpH* promoter
chpH for *codAS* rev	CATCATTCTAGATATTCCTCCTAGTTGGAATGCG	
Primers used to complement mutagenized strains with altered *chp* expression		
7287 fwd	CATCATGGATCCCGTCCTTCTCCTCATGGTGC	*Sven_7287* complementation
7287 rev	CATCATGGATCCATGAGCCCCTTCGACAACG	
3213 fwd	CATCATGGATCCCCGTCGTTCGTCTTCTTCCC	*Sven_3213* complementation
3213 rev	CATCATGGATCCGAGCCACTGCTCGTACACC	
1160 fwd	CATCATGGATCCCTTCGTCGCCTATCTGCTCG	*Sven_1160* complementation
1160 rev	CATCATGGATCCCCAAGCTGATCGTGCACCC	

Primers used to amplify a hygromycin B cassette to replace the ampicillin resistance gene of cosmids		
BlaF	CCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTA	Amplifying *hyg-oriT* from pIJ10700
BlaR	AATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAG	
Primers used to amplify *rdl* genes		
rdl ReDirect fwd	GGGCGGCGGGGCCGCAACCGAGCGGAGCGTCTTCGCTCA	Amplifying all three *rdl* genes
rdl ReDirect rev	GGATGTTCCCTCCGGCCCTGGCGTGCGCGACGCGGATCA	

*These two primers were ordered a second time, with a biotin tag attached to them. The biotin versions of the primers were used to amplify the promoter region of *chpH* and *chpE* (along with "*chpH* rev" and "*chpE* rev" respectively) in order to use these products in biotin-mediated protein pull-downs.
**This primer has a 5' biotin tag

2.2.2 Polymerase chain reaction (PCR)

PCR to generate products for cloning or sequencing was conducted using Phusion® High Fidelity DNA polymerase (New England Biolabs). These reactions were prepared according to the recipe in Table 2.4. PCR products were separated by electrophoresis on a 1% agarose gel, before being purified using the Monarch® DNA Gel Extraction kit (New England Biolabs) or PureLink™ Quick Gel Extraction Kit (Invitrogen). PCR for other applications, such as checking on cloning success through colony PCR (where colonies were used as template DNA), were conducted using Taq DNA polymerase (GeneDirex or New England Biolabs). Taq reactions were prepared according to the recipe seen in Table 2.5.

Table 2.4 Standard recipe for Phusion® PCR

Reagent	Volume (µL)	Final Concentration
Nuclease-free water	15	n/a
DMSO (50%)	2.75	5.5%
5× Phusion GC Buffer	5	1×
dNTPs (10 mM)	0.5	0.2 mM
Forward primer (10 µM)	0.5	0.2 µM
Reverse primer (10 µM)	0.5	0.2 µM
Template DNA (~ 100 ng/µL)	0.5	~2 ng/µL
Phusion® DNA polymerase	0.25	0.02 U/µL
Total	25	n/a

Table 2.5 Standard recipe for Taq PCR

Reagent	Volume (µL)	Final Concentration
Nuclease-free water	16.5	n/a
DMSO (50%)	2.5	5%
10× Taq PCR Buffer	2.5	1×
dNTPS (10 mM)	1	0.4 mM
Forward primer (10 µM)	1	0.4 µM
Reverse primer (10 µM)	1	0.4 µM
Template DNA (from colony)	0.25	na
Taq DNA polymerase	0.25	0.02 U/ µL
Total	25	n/a

2.2.3 Restriction digestion of DNA

Restriction digestions were accomplished by combining 10-20 µL of PCR product or vector (of varying concentrations), with 3 µL of each restriction enzyme (20 U/ µL), and 5 µL of a 10× concentrated digestion buffer (Fast-Digest™ buffer for enzymes supplied from Thermo Scientific or CutSmart™ buffer for enzymes supplied from New England Biolabs). The reactions were brought to 50 µL with nuclease-free water (Thermo Scientific) and incubated at 37°C for 1 h. The restricted DNA was purified using the PureLink™ PCR Purification Kit (Invitrogen).

2.2.4 Dephosphorylation of DNA

Restricted vector DNA was dephosphorylated by adding 3 µL of calf intestinal alkaline phosphatase (CIP; 10 U/µL; New England Biolabs) directly to the digestion reaction. This was incubated in a water bath at 37°C for 1 hr before being purified using the PureLink™ PCR Purification Kit (Invitrogen).

2.2.5 Ligating DNA into vectors

Digested DNA to be ligated into a vector was added in surplus to similarly digested vector DNA. Typically, this entailed adding 5 µL of insert to 1 µL of vector DNA. This mixture was then combined with 10 µL of 2× T4 DNA Ligation Buffer

(Sigma Aldrich) and 1 µL of 5 U/µL T4 DNA Ligase (Sigma Aldrich). The reaction was brought to 20 µL with nuclease-free water and incubated at room temperature (~23°C) for 10 min before being transformed into Subcloning Efficiency™ DH5α competent cells (Invitrogen). As a control, the same ligation procedure was repeated again, but with nuclease-free water in place of the digested insert. This control revealed the number of false positives that should be expected on the ligation plate. Colonies on the ligation plate were PCR-checked with a primer pair that included one insert-specific primer and one vector-specific primer where possible. When not possible, two insert-specific primers were used. Colonies that generated the expected PCR products were grown in selective antibiotics overnight, and their plasmids isolated the next day and sent for sequencing with a vector-specific primer directed towards the cloning site.

2.2.6 Introducing DNA into *E. coli*

Routine cloning was conducted using *E. coli* DH5α, as described above. DNA was introduced into these cells through a heat-shock method, whereby 1-3 µL of ligated plasmid DNA (of varying concentration) was added to a 40 µL aliquot of chemically competent DH5α cells. This mixture was incubated on ice for 20 min, transferred to a 37°C water bath for 45 s, and then placed back on ice for 1 min. Next, 1 mL of LB or SOB liquid medium was added to this mixture before the cell mixture was incubated at 37°C with shaking for approximately 1 h. The incubated cells were then spread on selective LB or SOB solid medium (Sambrook and Russell, 2001)

When introducing DNA into other strains of *E. coli* (*e.g.* ET12567/pUZ8002 or BW25113/pIJ790), the cells were first made electro-competent before being transformed via electroporation (Gust *et al*., 2003). To do this, a 25 mL culture of *E. coli* was grown to an OD_{600} of 0.5-0.8, at which point the cells were pelleted by centrifugation at 3930×*g* for 5 min and then resuspended in 10% glycerol at 4°C. This process was repeated three times, before the cells were resuspended in 200 µL of glycerol. Approximately 1-5 µL of DNA (of varying concentrations) were added to a 40 µL aliquot of washed and resuspended cells, after which the mixture was

subjected to a 2.5 kV pulse from a Micropulser™ (Biorad). Next, 1 mL of nonselective LB or SOB liquid media was added to this mixture, before being incubated at 37°C for approximately 1 h. The cells were then collected by centrifugation, spread on selective LB or SOB solid medium, and incubated overnight.

2.2.7 Isolating DNA from *E. coli*

Plasmid DNA was extracted from *E. coli* strains using the PureLink™ Quick Plasmid Miniprep Kit (Invitrogen), following the manufacturer's instructions.

Cosmid DNA was extracted from *E. coli* strains following the method outlined by Sambrook and Russell (2001). The cell pellet of an overnight culture of *E. coli* was collected and lysed in a 5% sodium dodecyl sulfate (SDS) solution with NaOH added to a final concentration of 200 mM. The lysate was treated with a 49.5% phenol (Bioshop), 49.5% chloroform (Fisher), and 1% isoamylalcohol (Fisher) solution to separate cellular debris into an aqueous and organic phase. The cosmid DNA was precipitated from the aqueous phase by adding a sodium acetate buffer (pH 5.2; Fisher) to a final concentration of 500 mM in 95% ethanol and combining this with the aqueous phase before leaving it at -20°C for at least 1 h. Precipitated nucleic acid was pelleted by centrifuging at 3930×*g* at 4°C for 20 min, after which the supernatant was discarded and the nucleic acid pellet dried, before being resuspended in 50 µL of nuclease-free water. When RNA-free cosmid DNA was desired (*i.e.*, when the cosmid was being used for PCR) this 50 µL sample was then treated with 10 µL of 10 µg/mL of RNase A (Sigma Aldrich), and the reaction brought to 500 µL with nuclease-free water. The mixture was incubated in a water bath at 37°C for 1-2 h to remove contaminating RNA. This solution was then purified by repeating the phenol-chloroform-isoamyl alcohol treatment and subsequent precipitation steps.

2.2.8 Introducing DNA into *Streptomyces*

Plasmid DNA was introduced into *S. venezuelae* via conjugation with the *E. coli* strain ET12567/pUZ8002. This *E. coli* strain is deficient in *dam*-methylation

(MacNeil *et al*., 1992) and contains a helper plasmid, pUZ8002, for conjugal DNA mobilization (Paget *et al*., 1999). Overnight cultures of ET12567/pUZ8002 containing a plasmid of interest were pelleted by centrifugation at 3930×*g* for 5 min. The *E. coli* cell pellet was resuspended in 5 mL of SOB liquid medium and centrifuged again at 3930×*g* for 5 min. This process was repeated three times, after which the final *E. coli* cell pellet was resuspended in 500 µL of SOB liquid medium. *S. venezuelae* spores were added to 500 µL of liquid yeast extract-tryptone (2×YT) medium. The number of *S. venezuelae* spores used for conjugation varied from approximately 1.08×10^{9} (for efficient conjugations) to 2.3×10^{12} (for inefficient conjugations). The *S. venezuelae* and ET12567/pUZ8002 suspensions were mixed, the cells/spores were collected by centrifugation before being plated on MS agar medium (Hobbs *et al*., 1989). These conjugation plates were incubated at 30°C for 6-8 h, after which the plates were overlayed with appropriate antibiotics to select for *S. venezuelae* exconjugants.

2.2.9 Isolating genomic DNA from *Streptomyces*

Genomic DNA was isolated from *S. venezuelae* strains using a Genomic DNA Isolation Kit (Norgen Biotek). In contrast to the kit's instructions, the final genomic DNA was eluted from the column in 50 µL of nuclease-free water.

2.2.10 Accessing genomic DNA from *Streptomyces* to use for colony PCR

When high-quality genomic DNA was not required (*e.g.*, when conducting colony PCR with *S. venezuelae* colonies), a simple genomic DNA extraction was performed: an approximately 2 mm^2-sized *S. venezuelae* colony was removed from a solid MYM medium plate and suspended in 35 µL of water. The suspension was heated at 95°C for 15 min before being centrifuged at 12,175×*g* for 2 min. The supernatant containing genomic DNA was then used as a template for PCR (with 2 µL being added to each 25 µL of PCR volume).

2.3 Directed mutations and mutant complementation

2.3.1 CRISPR-based *chp* deletions

All CRISPR-based deletions were created using a modified pCRISPomyces-2 plasmid (Cobb *et al*., 2014). The 20 nucleotide protospacer (the sequence that guides Cas9 to its target) was chosen by considering protospacer adjacent motifs (three nucleotides immediately 5' to the protospacer that must conform to the consensus NGG) that were positioned as early in the gene as possible. That is to say, protospacer and PAM sequences that occurred at the extreme 5' of the gene were preferentially pursued. Using BLAST analysis, the PAM and first 12 nucleotides of the protospacer were confirmed to be unique relative to any other genomic locus. Double-stranded protospacer DNA was made by annealing two complementary oligonucleotides (labeled "gRNA fwd" or "gRNA rev" in table 2.3) added to 5 μM in a 30 mM HEPES solution at pH 7.5. This solution was heated in a thermocycler to 95°C before cooling to 4°C at a rate of 0.1°C per s. The protospacer was introduced into pCRISPomyces-2 using a Golden Gate reaction with the restriction enzyme BbsI. Correct insertion was confirmed by the loss of LacZ function (with *lacZ* being flanked by BbsI sites in the unmodified plasmid) and by sequencing with the primer 'pMC600 seq rev'.

For CRISPR-based gene disruptions to be created, the pCRISPomyces-2 plasmid must contain a ~2 kb section of the genome around the target site to allow for repair via homologous recombination after cleavage of the protospacer sequence. This 2 kb fragment is called the editing template, and was designed to include 1 kb upstream of the protospacer sequence, and 1 kb downstream. Additionally, this editing template was designed to replace the protospacer sequence with a stop codon. This would incorporate a stop codon into the coding sequence (while disrupting the reading frame). The editing template was introduced into pCRISPomyces-2 in the XbaI restriction site, and proper insertion was confirmed by sequencing with 'pMC600 seq reverse' and the corresponding 'gRNA fwd" primer – which faced the XbaI site in the completed plasmid.

The pCRISPomyces-2 knockout plasmids were introduced into *Streptomyces* as described in 2.2.8. Exconjugants typically took 8-10 days to appear and were immediately patched onto selective MYM solid medium. Mutations within the gene

of interest were confirmed by colony PCR using a protospacer primer in the reactions, combined with another primer (typically an editing template primer) facing towards it. Successful deletion of this sequence would result in no PCR product being amplified. To ensure that this product was amplifying off the genome, and not the plasmid, a primer combination that would only be possible from the genome was used. For example, since the editing template was inserted into the vector non-directionally it is possible that a primer facing the gRNA sequence in the genome was not facing the gRNA sequencing in the plasmid (Figure 2.1). Since a positive mutant resulted in no PCR product, a possibility that also could have resulted from lack of PCR template, each lysed colony was subjected to a control PCR (a random PCR with primers used in the past that were known to work well).

In order to cure the pCRISPomyces-2 knockout plasmids from the mutants, plasmid-containing cells were grown in liquid MYM medium at 37°C for approximately 24 h to permit plasmid curing (due to the temperature-sensitive origin of replication) before being streaked for single colonies onto solid MYM medium and grown for an additional 24 h at 37°C. Single colonies were patched onto selective (apramycin or hygromycin) or nonselective solid MYM medium, and a spore stock was made of an antibiotic-sensitive colony. Putative CRISPR-based mutants whose plasmids were successfully cured were confirmed one final time by performing colony PCR using a primer that was not complementary to the editing template. This ensured that the gene was amplified from its genomic loci and not from any lingering pCRISPomyces-2 plasmids. This PCR used a forward primer that bound to the genome upstream of where the editing template ends, and a reverse primer that was downstream of the protospacer deletion. The resulting PCR product was sequenced to ensure the expected mutation was present.

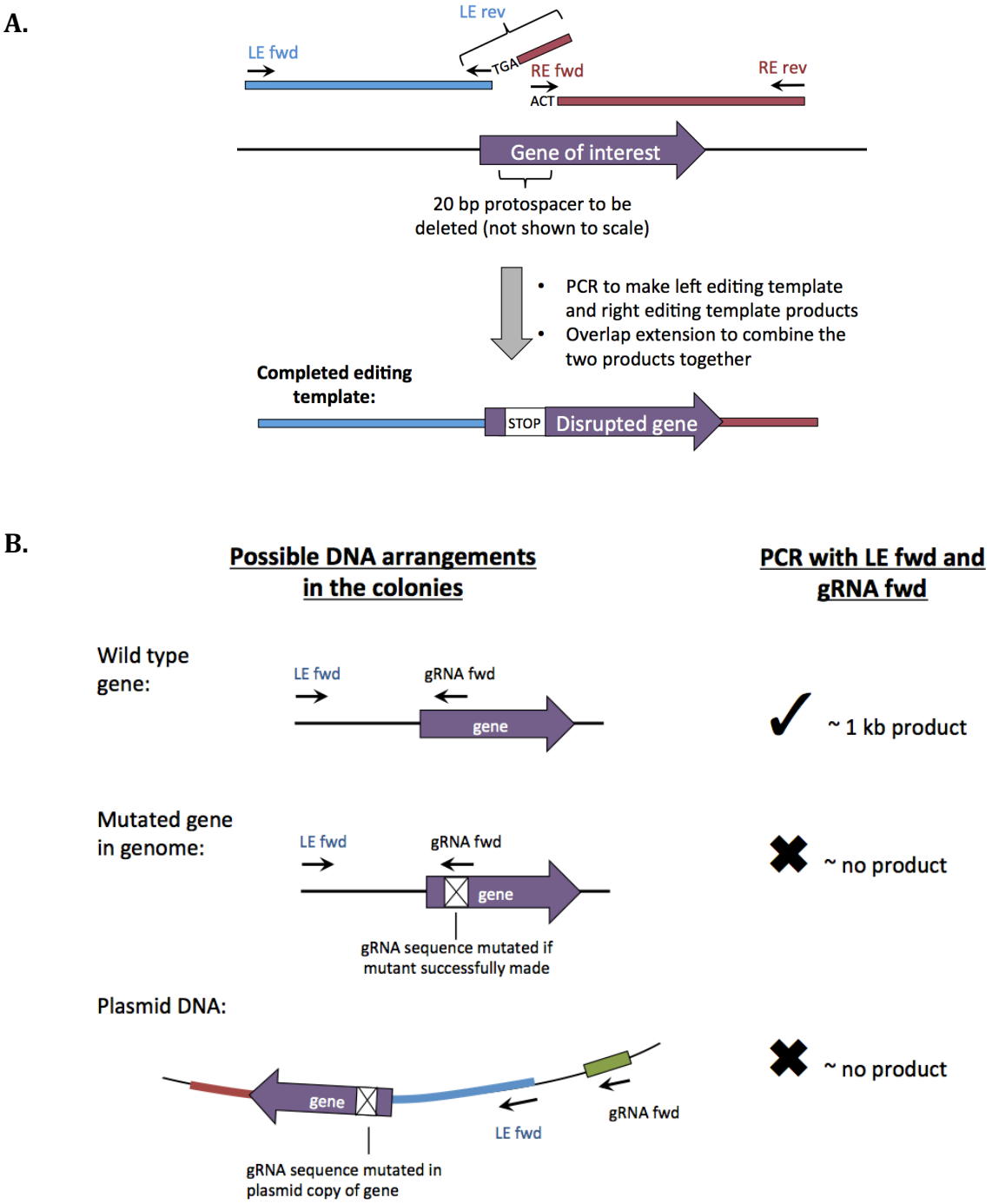


Figure 2.1 CRISPR editing template and schematic of mutation-check PCRs. **(A)** One kb to the left (blue) of the protospacer sequence and 1 kb to the right (red) of the protospacer sequence were amplified individually and then attached together using overlap extension PCR to make the editing template for CRISPR disruptions. **(B)** When checking for mutations, a primer pair was chosen that should only produce a product from wild type DNA, and not from mutant or plasmid DNA. Note: if sequencing (with "pMC600 seq rev") revealed the editing template to be ligated in the other orientation (since this was non-directional cloning), the primer combination used would have instead been gRNA rev and RE rev.

2.3.2 ReDirect-based *chp* and *mtrA* deletions

In instances where CRISPR-based techniques were unsuccessful in yielding a mutant (*chpD*, *chpE*, *mtrA*), the ReDirect PCR-targeting system for streptomycetes (Gust *et al*., 2003) was used to create mutations. To do this, hybrid PCR primers were designed such that their 5' sequence was complementary to the upstream or downstream 39 nucleotides of the gene of interest (including the start and stop codons, respectively), and their 3' ends were complementary to an *oriT*-antibiotic resistance gene-containing cassette. The cassette was amplified using Phusion® PCR (with pIJ773 as template for apramycin cassettes and pIJ10700 as template for hygromycin B cassettes), and were then electroporated into *E. coli* BW25113/pIJ790 harbouring a cosmid containing the gene of interest (Table 2.6). Prior to this, the cosmid-containing BW25113/pIJ790 strain was grown to mid exponential phase with L-arabinose added to 15 mM, where the arabinose induced expression of the lambda-red recombination genes carried on pIJ790. To confirm correct knockout-cosmid construction, diagnostic PCRs were performed using two primer pairs. The first involved amplification using a forward primer that binds upstream of the gene of interest, and a reverse primer that is antibiotic cassette-specific, to confirm the correct positioning of the resistance cassette. The second reaction involved the same forward primer combined with a reverse primer that is complementary to sequences within the gene of interest, allowing for mutant validation. The former PCR should produce a band if the cosmid is successfully mutated, while the latter PCR should produce a band if the cosmid is wild type. As a control to help distinguish between these two options, wild type genomic DNA was also used as template for these PCRs. Additionally, the knockout cosmid was digested with a restriction enzyme (HindIII) that cleaved in multiple locations, and the banding pattern was compared to the wild type cosmid that was similarly digested. Knockout cosmids were introduced into *S. venezuelae* strains through conjugation, and exconjugants were screened to determine if a double homologous crossover had replaced the genomic (wild type) gene with the cassette-disrupted gene from the cosmid. This was determined by the antibiotic resistance profiles of the exconjugants - which should retain the resistance from the cassette that was

flanked by sequences from the gene of interest (hygromycin-B or apramycin), but should have lost the cosmid backbone during a double crossover and therefore be kanamycin sensitive – and also confirmed with the same PCRs mentioned above that were initially used to test for correct knockout cosmid construction.

Table 2.6 Cosmids used or created in this work

Cosmid name	Modification	Purpose
Sv-4-C04	None	Template for *chpB* PCR amplification
3M01	None	Template for *chpC* PCR amplification
Sv-5-A10	*chpD* replaced with hyg-oriT	Template for *chpD* PCR amplification, and for *chpD* ReDirect construction
1-A1	*chpE* replaced with hyg-oriT	Template for *chpE* PCR amplification, and for *chpE* ReDirect construction
Sv-4-H10	None	Template for *chpF* PCR amplification
Sv-4-H10	None	Template for *chpG* PCR amplification
3M01	None	Template for *chpH* PCR amplification
Sv-4-B09	amp^R cassette replaced with hyg-*oriT*	Complement an intergenic mutation near *Sven_6498*
3M16	amp^R cassette replaced with hyg-*oriT*	Complement *Sven_4231*
Sv-4-A05	amp^R cassette replaced with hyg-*oriT*	Complement an intergenic mutation near *Sven_4743*
Sv-2-B03	None (moved into BW25113/pIJ790)	Complement *Sven_5292*
Sv-6-E07	amp^R cassette replaced with hyg-*oriT*	Complement *Sven_4829*
Sv-5-C04	None (moved into BW25113/pIJ790)	Complement *Sven_5165*
5M07	amp^R cassette replaced with hyg-*oriT*	Complement an intergenic mutation near *Sven_4989*
Sv-2_C01	None (moved into BW25113/pIJ790)	Complement *Sven_3958*
Sv-5-B11	None (moved into BW25113/pIJ790)	Complement intergenic mutation
Sv-5-A10	None (moved into BW25113/pIJ790)	Complement *Sven_4622*
Sv-4-C04	amp^R cassette replaced with hyg-*oriT*	Complement *Sven_3148*
Sv-3-E04	amp^R cassette replaced with hyg-*oriT*	Complement *Sven_1239*
Sv-4-E12	amp^R cassette replaced with hyg-*oriT*	Complement *Sven_7050, 7052*
3N12	amp^R cassette replaced with hyg-*oriT*	Complement *Sven_1894*
Sv-4-G08	amp^R cassette replaced with hyg-*oriT*	Complement an intergenic mutation near *Sven_2517*
3J08	amp^R cassette replaced with hyg-*oriT*	Complement *Sven_5397*
Sv-4-G05	amp^R cassette replaced with hyg-*oriT*	Complement *Sven_5329*
Sv-6-F04	amp^R cassette replaced with hyg-*oriT*	Complement *Sven_2965*
Sv-4-C05	amp^R cassette replaced with hyg-*oriT*	Complement *Sven_3134, 3152*
Sv-6-D04	amp^R cassette replaced with hyg-*oriT*	Complement *Sven_5803*
Sv-3-F09	None (moved into BW25113/pIJ790)	Complement *Sven_6712*
Sv-4-E11	None (moved into BW25113/pIJ790)	Complement *Sven_3987*

2.3.3 Cosmid-based complementations

When unsure as to the mutation responsible for a particular phenotype, or in instances where vector-based complementations were unable to fully complement mutant phenotypes, cosmids were used in lieu of cloning specific sequences for the purpose of complementation (*i.e.*, when complementing the various mutations identified by genome sequencing). Similar to the above ReDirect PCR-targeting protocol, cosmids were modified such that a hygromycin B-*oriT* cassette was used to replace the native ampicillin resistance gene (Table 2.6). These modified cosmids were introduced into *S. venezuelae* mutants by conjugation, and positive exconjugants were selected for with hygromycin. Colony PCR with hygromycin B cassette-specific primers was performed to confirm cosmid integration into the chromosome.

2.4 Whole-genome chemical mutagenesis

In an attempt to identify novel *chp* regulators, wild type *S. venezuelae* strains were subjected to chemical mutagenesis using the DNA alkylating agent ethyl methanesulfonate (EMS; Sega, 1984). To begin, spores were washed in a potassium phosphate buffer. Approximately 2.8×10^9 spores were pelleted by centrifuging at 12,175×*g* for 1 min, followed by resuspension in 1.5 mL of a 0.01 M potassium phosphate buffer at pH 7.0. This was repeated three times, with a final resuspension in 1.5 mL of fresh buffer. The mixture was divided into 750 µL aliquots, with 25 µL of EMS (Sigma Aldrich) added to one, and 25 µL of sterile water added to the other as a control. After shaking while incubating for 1 h at room temperature (~23°C), each aliquot was treated with 1 mL of a 5% sodium thiosulfate (Bioshop) solution to deactivate the EMS. The two resulting solutions were serial diluted in order to titer the viable spores within them. The 'water alone' sample had a titer of 4.08×10^8 spores per mL while the EMS-treated sample had a titer of 6.50×10^7 spores per mL. Therefore, the EMS-treated mixture only had 15.9% as many viable spores. The EMS-treated sample was then directly spread onto a lawn of solid MYM medium, and after 1-2 days the biomass was removed, homogenized, and resuspended in 20% glycerol. This entire process was repeated four independent times, to create

four stocks of chemically mutagenized *S. venezuelae*.

2.5 Biotin-streptavidin protein pull downs

One strategy to identify novel *chp* regulators involved affixing the promoters of *chpH* and *chpE* to magnetic beads, and mixing these beads with *S. venezuelae* lysate. Magnetic separation of these beads should theoretically "pull down" any protein that binds with sufficient strength to the promoters of the genes.

2.5.1 Labeling magnetic beads with biotin-tagged *chp* promoter DNA

The promoters of *chpH* (169 bp) and *chpE* (232 bp) were amplified with biotin-tagged primers, alongside non-promoter DNA from the coding sequence of *chpD* (196 bp) as a negative control. The DNA products were purified with the PureLink™ PCR Purification Kit (Invitrogen), and their concentration determined using a NanoDrop spectrophotometer. At the same time, a 250 µL aliquot of Streptavidin Magnetic Particles (Sigma Aldrich) were pelleted by applying a magnetic force to the bottom of the tube. The beads were then washed three times with DNA binding buffer (20 mM Tris-HCl, pH 7.5; 1 mM EDTA; 100 mM NaCl) before finally being resuspended in 250 µL of this buffer. Next, 25 pmol of each PCR product was added to the bead solution before incubating – with light shaking – at room temperature (~23°C) for 1 h. The beads were then washed three additional times with the same buffer as above, prior to resuspending in 250 µL of the DNA binding buffer. Ten microliter aliquots from each wash were collected, and were fractionated on a 1% agarose gel, beside a sample of DNA from the finalized bead-DNA stock [DNA was separated from the beads by adding a 10 µL aliquot of the bead-DNA stock to a 95% formamide solution (Caledon) with 10 mM EDTA, pH 8.2 and heating at 95°C for 5 min] to ensure that biotin-labelled DNA was indeed affixed to the streptavidin beads.

2.5.2 Isolating proteins with *chp* promoter DNA

Five millilitres of an overnight culture of *S. venezuelae* were used to inoculate 500 mL of liquid MYM medium. After 10 h of growth [experimental evidence from

chp transcriptional reporters (see 2.6.2) suggested that this was an optimal time point for isolating proteins involved in *chp* activation], the cells were collected by centrifugation at 3930×*g* for 10 min. The cells were resuspended in 6 mL of lysis buffer [10 mM Tris-HCl pH 7.5, 10 mM KCl, 50 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1 EDTA-free protease inhibitor tablets (Roche)] and sonicated with five to eight rounds of 10 pulses each, at 40% duty, with pulses lasting 30 s. The lysate was centrifuged at 12,175×*g* at 4°C for 20 min and the supernatant decanted. The Streptavidin beads affixed with DNA (prepared in 2.5.1) were pelleted as described previously, and resuspended in 6 mL of 2× binding buffer (40 mM Tris-HCl, pH 8; 2 mM EDTA; 20% glycerol). The beads were combined with 6 mL of cell-free lysate, and the resulting mixture was incubated at 30°C – with shaking at 200 rpm – for 1 h. Next, the magnetic beads were pelleted as described above and sequentially washed with 100 µL of 1× binding buffer containing increasing concentrations of NaCl (100 mM, 150 mM, 200 mM, 250 mM, 400 mM, and 500 mM) so as to dissociate any proteins bound to the DNA.

2.5.3 Silver staining of polyacrylamide gels

From the sequential washes of the bound proteins described above (six different concentrations of NaCl) for each DNA fragment (*chpH* promoter, *chpE* promoter, *chpD* coding sequence), 20 µL of each were combined with 4 µL of 6× SDS loading dye and heated at 95°C for 5 min. The samples were separated on a 12% polyacrylamide gel at 150 V for 45 min before being visualized with silver staining. Silver staining was achieved by subjecting the gels to the following conditions, all involving low speed shaking on a rotary shaker at room temperature (~23°C) unless indicated otherwise, and all solutions made with milli-Q water (volumes given are per one gel): 30 min wash with 150 mL of milli-Q water; 10 min of fixing in 100 mL of 50% ethanol and 10% glacial acetic acid (Fisher); 5 min rinsing with 100 mL of 50% ethanol; 2 min "sensitizing" in 100 mL of 0.02% sodium thiosulfate (Bioshop); 2 min rinsing with 150 mL of milli-Q water; 20 min staining with 150 mL of 0.1% silver nitrate (Fisher) at 4°C; two 1 min washes with 150 mL of milli-Q water; a 1 min introduction to 100 mL of developing solution [made by combining 10 g sodium

carbonate (Bioshop), 200 µL of 37% v/v formaldehyde (Sigma Aldrich), 20 mL of the above "sensitizing" solution, and brought to 500 mL with milli-Q water], followed by replacing with 300 mL of fresh developing solution and manually shaking until sufficient protein visualization was achieved (approximately 5 min; no more than 10 min was permitted). After visualization was achieved, further development was halted by bathing the gels in 100 mL of 1% glacial acetic acid. After 5 min, the gels were transferred to 100 mL of milli-Q water and kept at room temperature (~23°C) for longer storage (typically 1-2 days while the gel bands were visually assessed).

2.6 Gene expression assays

2.6.1 Cytosine deaminase reporter screens

As part of a project aimed at identifying novel *chp* regulators, reporter constructs were designed whereby the promoters of *chpH* and *chpE* were driving the expression of a toxin-encoding gene, *codAS*. CodAS is a cytosine deaminase that converts 5-fluorocytosine (5-FC; Sigma Aldrich) to 5-fluorouracil, a compound that prevents proper protein synthesis (Mullen *et al.* 1992). By incorporating a theophylline-sensitive riboswitch between the promoters and the *codAS* sequence, we generated a strain that would grow normally in the absence of theophylline, but would permit toxin-production when grown with theophylline (the riboswitch represses translation in the absence of theophylline, but permits translation in the presence). Introducing these constructs into chemically mutagenized *S. venezuelae* allowed us to screen for colonies not expressing the toxin – representing mutants impaired in *chpH* promoter activation.

First, the promoter of *chpH* was directionally cloned upstream of a theophylline-sensitive riboswitch, into the KpnI and XbaI restriction sites of pMC600. Next, the entire promoter and riboswitch sequence was excised by digesting the resulting construct with restriction enzymes whose sequences flank the promoter and riboswitch elements (KpnI and SpeI) and subsequently cloned into the same sites upstream of a toxin-encoding *codAS* gene in the vector pMS82,

similarly digested. Optimal concentrations of 5-FC and theophylline were experimentally determined to be 500 μg/mL and 8 mM, respectively. These concentrations, when added to solid MYM medium, resulted in colonies that suffered considerable growth defects compared to a wild type strain (namely, smaller colonies that developed in a significantly delayed manner).

This pMS82-based construct was conjugated into the chemically mutagenized *S. venezuelae* stocks (from 2.4), and the resulting strains were plated for single colonies on MYM plates containing the above concentrations of 5-FC and theophylline. In total, approximately 22,000 colonies were screened, and eight colonies were identified that had near-wild type growth, suggesting reduced *chpH* promoter activity (and therefore, less toxin production in these strains). Spore stocks were made for these strains.

2.6.2 β-glucuronidase reporter screens

To monitor the expression of two different *chp* genes – *chpH* and *chpE* – their promoter regions were amplified with primers that bind ~250 bp upstream of the start site, and ~20 bp downstream of the start site. The promoter DNA was digested with KpnI and XbaI, and directionally cloned into similarly digested pGUS vector, upstream of the reporter gene *gusA*. There were a number of strains that these constructs were introduced into, mainly, select *chp* mutants and the chemically mutagenized strains identified after CodAS activity screening. To assess promoter activity, overnight cultures of the strains were used to inoculate fresh liquid MYM to an OD_{600} of 0.1, and the cultures were incubated at 30°C. Concurrently, wild type strains harbouring the same constructs were used as controls, along with a wild type strain containing pGUS (*gusA*) driven by the constitutive promoter *ermE**, and a wild type strain containing pGUS (*gusA*) with no promoter at all. After typically 10 h of incubation (sometimes shorter incubation times were used – *e.g.*, Figure 4.1), the OD_{600} value of the cultures was recorded, and a 1 mL sample was centrifuged at 12,175×*g* for 5 min to collect the cells. After lysing the cells by a 5 min treatment with 4 mg/mL of lysozyme (Bioshop) and 0.1% triton X-100 (OmniPur), 50 μL of the supernatant was combined with 150 μL of a sodium phosphate buffer at pH 7 with

0.88 mg/mL of p-nitrophenyl-β-D-glucuronide (PNPG; Sigma Aldrich) – the substrate that is cleaved by the product of *gusA* to generate a yellow-coloured molecule. The reactions were incubated at 37°C for 1 h, with OD_{420} values recorded every min. Since the OD_{420} value increases with the level of β-glucuronidase, these values were normalized to the OD_{600} value of the culture at the time of cell harvest. This permitted calculation of enzymatic activity – a direct indication of promoter activity. The formula for this calculation is as follows: [(1000)*(OD_{420} value)]/[(time that recording was taken at in minutes)*(volume of lysate added to reaction in μL)*(OD_{600} value of culture when cell pellet was harvested)].

2.7 Spore outgrowth assays

To assess the impact of *chp* or *chp* regulator mutations on spore germination, spore outgrowth assays were conducted with select strains – a 5-fold *chp* mutant (Δ*chpBCDFG*), a minimal *chp* strain (possessing only *chpC*, *chpH*, and *chpE*), a long *chp* mutant (Δ*chpBC*), and a short *chp* mutant (Δ*chpDFGH*), and a wild type control. This involved inoculating 5×10^7 spores into 1 mL of liquid MYM medium and recording the OD_{600} value every 15 min for up to 255 min.

2.8 Whole genome sequence analysis of mutant strains

Genomic DNA was harvested from *chp*-expression mutants of interest (identified from *codAS* screening of chemically mutagenized stocks), *chpE* mutants, and escapers as per section 2.2.9. This genomic DNA was sequenced on an Illumina MiSeq instrument (Illumina, San Diego, CA, USA) at 50× coverage with 250 bp paired-end reads. Sequencing data was aligned to the reference *S. venezuelae* genome (StrepDB; http://strepdb.streptomyces.org.uk) using Bowtie 2 software (Langmead and Salzberg, 2012). SAMtools was used to convert the sequencing data to BAM files, and the Pileup and BCFtools functions were used for identification of single nucleotide polymorphisms and insertions or deletions (Li *et al*., 2009). Determining the genomic location and read depth of these SNPs and indels was accomplished with VCFtools (Danecek *et al*., 2011).

Chapter Three:

Creation and Characterization of *S. venezuelae chp* Mutants

3. 1 Introduction

The chaplin proteins have been well studied in the model organism *S. coelicolor*. In this bacterium they are important developmental determinants that both aid the aerial mycelium in its escape from the aqueous growth medium, and polymerize into amyloid fibers that coat the surface of aerial structures and spores and confer hydrophobic properties (Claessen *et al*., 2003, 2004; Elliot *et al*., 2003). As mentioned previously, *S. coelicolor* has some limitations that have meant it has not been possible to address important questions regarding the function of chaplin proteins. One such glaring example is the role of the chaplin proteins in spore formation. Are chaplin-free strains unable to produce spores because of a development hindrance caused by lack of chaplins, or simply because these strains are unable to raise aerial hyphae (and it is within these hyphae that spore chains develop)? In a laboratory setting, *S. venezuelae* is an excellent model species for addressing these types of questions regarding chaplin biology due to its unusual characteristics, including rapid growth, liquid development, and exploratory potential. The goal of this work was to generate a series of *chp* mutants in *S. venezuelae* [single deletions of all of the *chp* genes and defined mutant combinations (*e.g.* short *chp* mutant and a long *chp* mutant)] and use these mutated *S. venezuelae* strains to investigate chaplin function in this species.

3.2 Results

3.2.1 Creating and characterizing single *chp* mutants

The classical *Streptomyces* developmental cycle [forming vegetative mycelium, raising of aerial mycelium, and forming dormant exospores (Figure 1.2)] has been well characterized in other *Streptomyces* species, but has not yet been fully investigated in a model capable of distinct growth and developmental processes. As

such, we set out to investigate how the chaplin proteins contribute to the *S. venezuelae* life cycle. To begin, we attempted to individually disrupt each of the *chp* genes. The *chpB*, *chpC*, *chpF*, *chpG*, and *chpH* coding sequences were all individually disrupted using CRISPR-Cas9 technology, while the coding sequence of *chpD*, for which CRISPR-Cas9 repeatedly failed to yield mutations, was replaced with an apramycin resistance cassette using a PCR-targeting method (Gust *et al*., 2003). Collectively, this allowed us to generate six individual *chp* mutants. We attempted to disrupt *chpE* with CRISPR-Cas9 technology using two different guide RNA sequences, but both repeatedly failed to yield mutants. We next attempted to replace *chpE* with an apramycin resistance cassette, using the PCR-targeting methodology successfully employed for *chpD*. Despite continued screening of exconjugants, none had the correct genetic modification and we suspected the previously-described conditional essentiality of *chpE* (Di Berardo *et al*., 2008) may be responsible for our failed mutational endeavours. To address this, the *chpE* coding sequence along with approximately 200 bp upstream and downstream (to encompass any associated regulatory regions) was cloned into the integrating vector pSET152 and introduced into *S. venezuelae*. We wanted to, again, employ PCR-targeting mutagenesis to attempt to replace *chpE* with an apramyin resistance cassette. At the time of writing, however, the conjugations to introduce this construct into *S. venezuelae* + pSET152::*chpE* are still underway. We hypothesize that we will be able to create a *chpE* mutation in this strain (bearing a second copy of *chpE*), but not an *S. venezuelae* strain containing an empty pSET152 vector.

3.2.1.1 Single *chp* mutants undergo classical development similar to wild type

Because *S. venezuelae* completes its life cycle in liquid, we first sought to determine how single *chp* deletions affected growth and development in liquid medium. To answer this, we grew the mutants and a wild type control in liquid medium and measured their OD_{600} values, while simultaneously monitoring the developmental stage of the cultures over a 32 h time course. The individual *chp* mutants grew almost identically to the wild type strain until approximately 8 h, after which point the strains diverged slightly from each other with no obvious

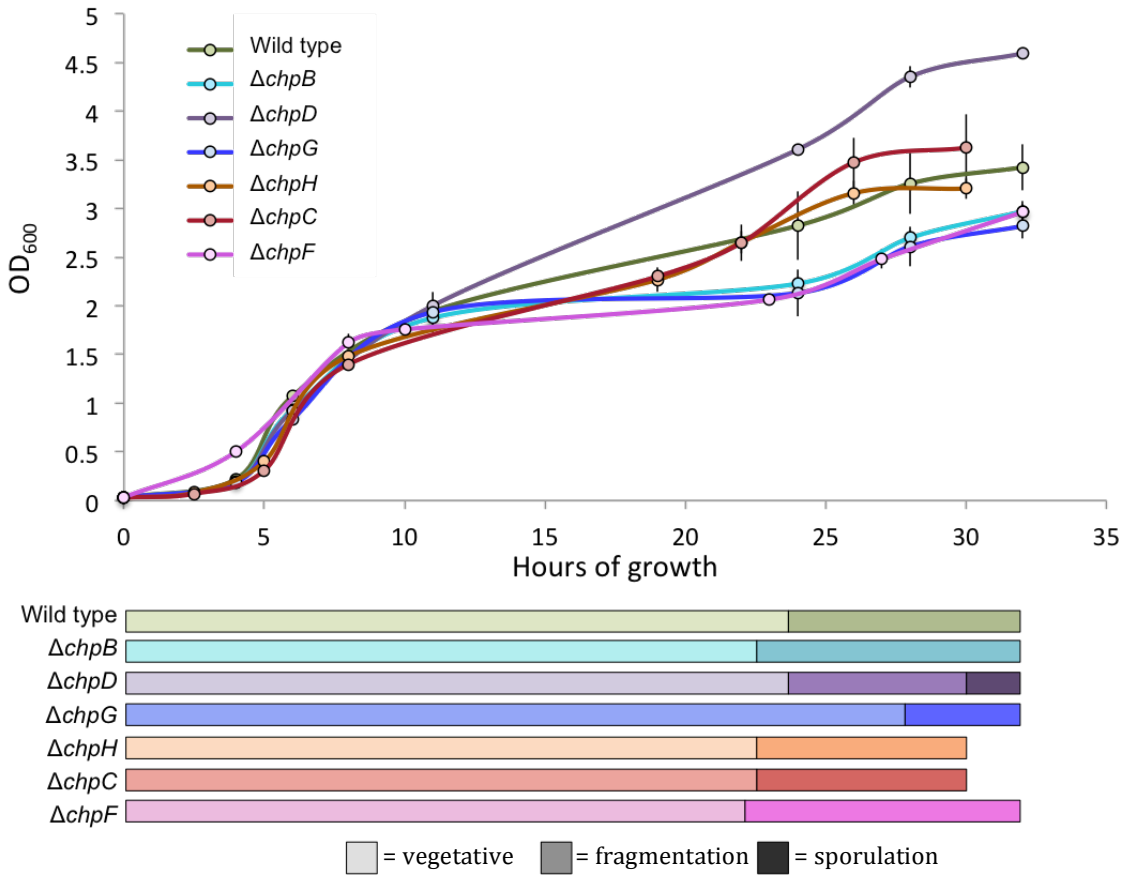


Figure 3.1 Growth and development of single *chp* mutants in liquid medium. Overnight cultures of the single *chp* mutants were used to inoculate liquid MYM medium to an OD_{600} of 0.05. The cultures were left to grow for 32 h (with the exception of Δ*chpH* and Δ*chpC* which were left for only 30 h), with the OD_{600} value of the cultures being recorded at the time points noted on the graph (top). At each time point, samples of the culture were removed and analyzed using light microscopy to determine their developmental stage. The developmental stages are shown by horizontal bars of various hues (bottom), with increasing colour intensity representing the different stages of development. The error bars represent standard error of three biological replicates.

pattern (Figure 3.1). For example, the OD_{600} values of the Δ*chpD* mutant were consistently higher than wild type, whereas the OD_{600} values of the Δ*chpG* mutant were consistently lower. The two mutants lacking long *chp* genes also did not show a consistent trend, with the Δ*chpB* mutants reaching a marginally lower OD_{600} value than wild type, and the Δ*chpC* mutants growing equivalent to wild type. The Δ*chpH*

mutant also grew similarly to wild type, following a growth progression that almost exactly mirrored that of the Δ*chpC* mutant. Light microscopy was used to gauge the developmental stage of the strains, and these were depicted as horizontal bars shown below the growth curves. All of the strains transitioned from vegetative growth to fragmentation around the 23 h time point, with the exception of Δ*chpG*, which did not show fragmented mycelium until 28 h. After 32 h of growth, only the Δ*chpD* strain had begun sporulating, with sparse spore formation being seen. This result suggests that a loss of *chpD* increases growth and accelerates development. This may imply that ChpD has a role in the wild type strain that counteracts or prevents rapid growth. It remains to be determined what this specific role is. The time courses were terminated at 32 h because in previous liquid growth experiments with these strains, the OD_{600} values generally plateaued at this time. However, given that the wild type strain had not yet sporulated, one might consider repeating this experiment in order to assess the relative sporulation times for all the strains.

To complement our liquid growth data, the aforementioned strains were grown on solid MYM medium for 72 h and their development was compared to a wild type *S. venezuelae* strain (Figure 3.2). The single *chp* mutants did not show phenotypic differences from the wild type strain when grown on solid medium. If the Δ*chpD* strain grew and developed more robustly than wild type on solid medium, this difference was subtle enough that it was not observed in our experiment.

3.2.2 Creating and characterizing combinatorial *chp* mutants

To study the effects of multi-chaplin loss, a number of combinatorial *chp* deletions were created. These included a long *chp* mutant (Δ*chpBC*), a 4-fold short *chp* mutant (Δ*chpDFGH*; *chpE* encodes an additional short chaplin and will need to be deleted in this strain to complete the short *chp*-null genotype but we were unable to create the mutation prior to submitting this thesis. The 4-fold short *chp* mutant will be hereafter be referred to as simply the "short *chp* mutant"), a minimal *chp* strain (containing only *chpC*, *chpH*, and *chpE*), and a 5-fold *chp* mutant (Δ*chpBCDFG*;

containing only *chpE* and *chpH*). We recognize the utility of a *chp*-null strain for the purposes of our investigations, but were unable to create a 7-fold *chp* mutant during this time; future work will benefit from the study of this strain once it is complete.

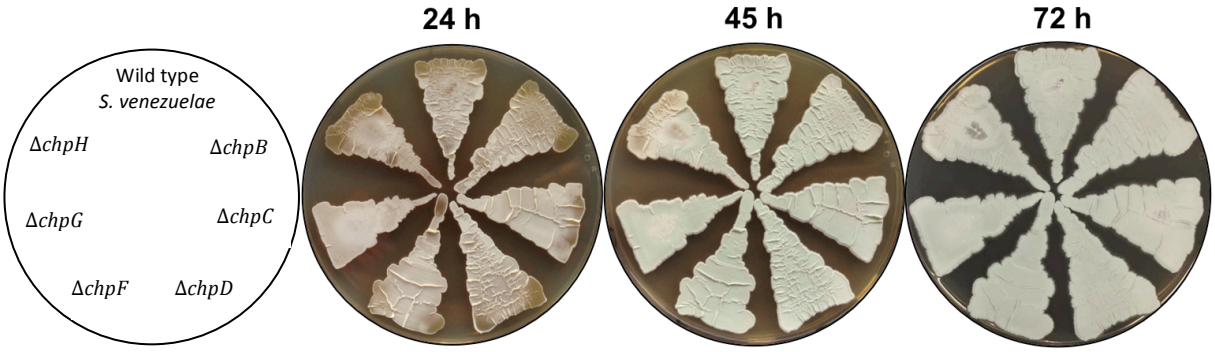


Figure 3.2 Growth and development of *chp* mutants on solid medium. The single *chp* mutants were streaked onto solid MYM medium and left to grow for 72 h. Photos show the development of these mutants compared to wild type *S. venezuelae*. None of the single mutants appeared to exhibit growth or developmental delays. Vegetative-stage colonies were evident by a flat, almost translucent brown layer (see the edges of the triangular colonies in the 24 h image); aerial-stage colonies were evident by a fuzzy looking white to tan-brown colony (see the center of the colonies in the 24 h image); and sporulation was evident by a fuzzy looking green colony (see wild type in the 45 h image).

3.2.2.1 Combinatorial *chp* mutants show differences in their ability to undergo classical development

The combinatorial mutant strains were also grown in liquid MYM with wild type *S. venezuelae* being used as a reference for comparison (Figure 3.3). At the earliest time points (such as 4 h), there were slight differences in the OD_{600} values observed. The short and 5-fold *chp* mutants both had OD_{600} values greater than wild type, but the long *chp* mutant and minimal *chp* strain were approximately equivalent to wild type. At the later time points however, these four strains all exhibited a modest reduction in OD_{600} compared to wild type, with the minimal *chp* strain showing the lowest values, particularly over the first 24 h. The developmental stage of these cultures was determined as described above. The long and short *chp*

mutants showed a delayed onset of fragmentation, with both beginning to fragment approximately 8 h after the wild type strain. However, both strains still progressed to sporulation by 45 h. The 5-fold *chp* mutant was the only strain that did not show any spore formation after 46 h of growth (Figure 3.3). Multiple independent cultures of this strain were left to incubate for a prolonged time period (up to one week), yet still, the colony remained largely fragmented with only the occasional spore being detected. To detect spores, 10 µL was removed from each of three independent cultures and was applied to a microscope slide. Multiple regions of this slide (picked at random) were inspected for spores, but in any given field of view (which may contain ~50 mycelial fragments) only about 0-3 spores could be seen. Cropped images from the various fields of view analyzed are shown in Figure 3.4.

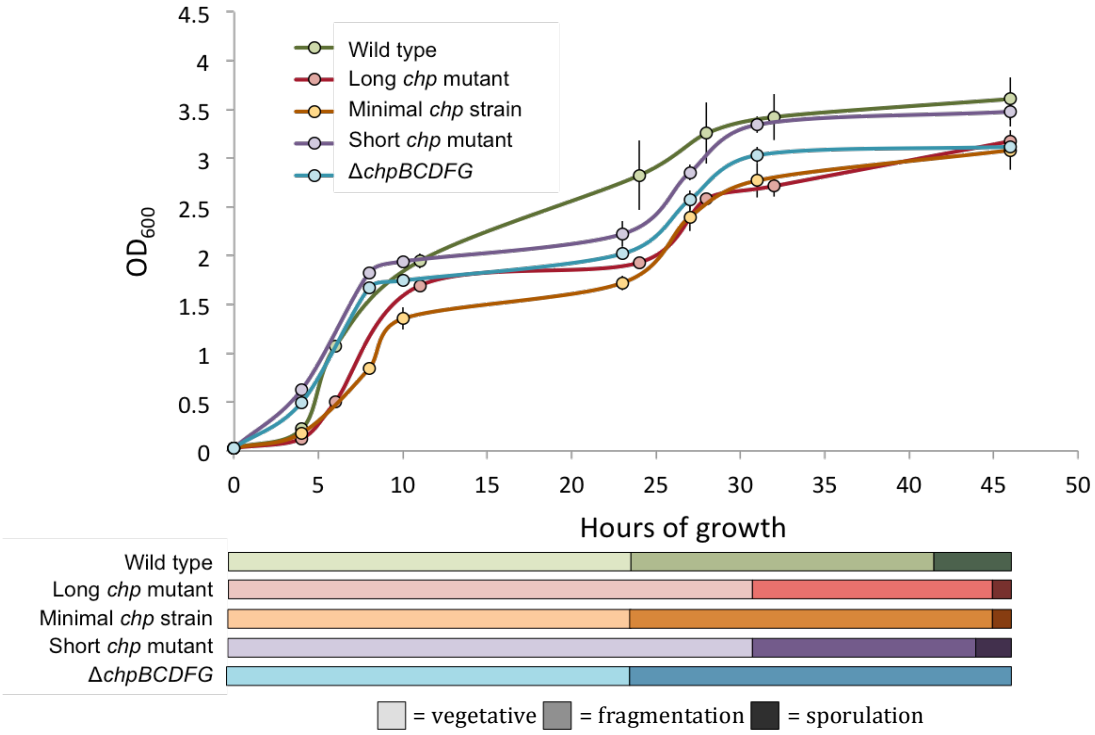


Figure 3.3 Growth and development of combinatorial *chp* mutants in liquid medium. Overnight cultures of the a long *chp* mutant, a short *chp* mutant, a 5-fold *chp* mutant, and a minimal *chp* strain were used to inoculated liquid MYM medium to an OD_{600} of 0.05. They were left to grow for 46 h, with the OD_{600} value of the cultures being recorded at intermediate time points (top). Samples of the culture were removed during these time points and analyzed with light microscopy to

determine which developmental stage they were in. The developmental stages are shown by horizontal bars of various hues (bottom). The error bars represent standard error for three biological replicates.

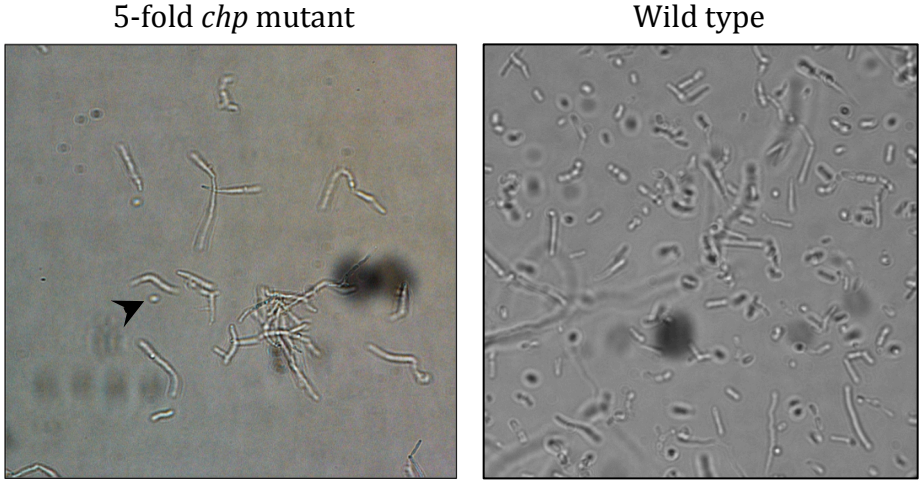


Figure 3.4 The 5-fold *chp* mutant grown in liquid remains mostly as fragmented mycelium even with prolonged incubation. Liquid cultures of the 5-fold *chp* mutant were left to incubate for one week before analyzing the developmental stage of the cultures. The cultures were almost entirely still fragmented, with only sparse spores being seen (arrows), in contrast to wild type, which had mostly sporulated, with only some mycelial fragments remaining.

Growing these combinatorial mutants on solid MYM medium also produced striking and differential phenotypes. The long *chp* mutant was the only strain of the four that developed identically to wild type. The minimal *chp* strain was able to progress through the entire life cycle, but was delayed in raising aerial hyphae and in sporulating. The short and 5-fold *chp* mutants were by far the most affected, with the 5-fold *chp* mutant only entering aerial growth after approximately 35 h of growth, and remaining unable to form mature spores even after incubation for over one week. Microscopy revealed that the 5-fold *chp* mutant could produce spores, however, these were never modified to contain the green pigment seen in wild type spores. The short *chp* mutant remained vegetative for prolonged time points (Figure 3.5), only entering aerial growth and subsequent sporulation after incubation for well over one week.

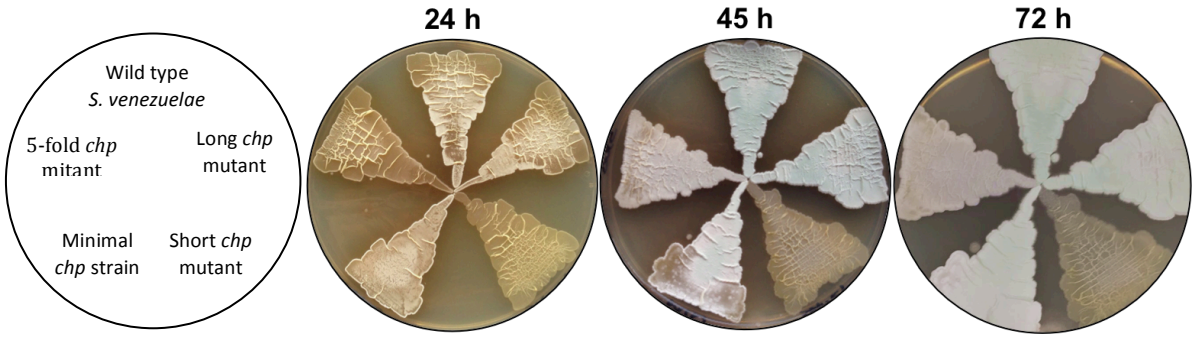


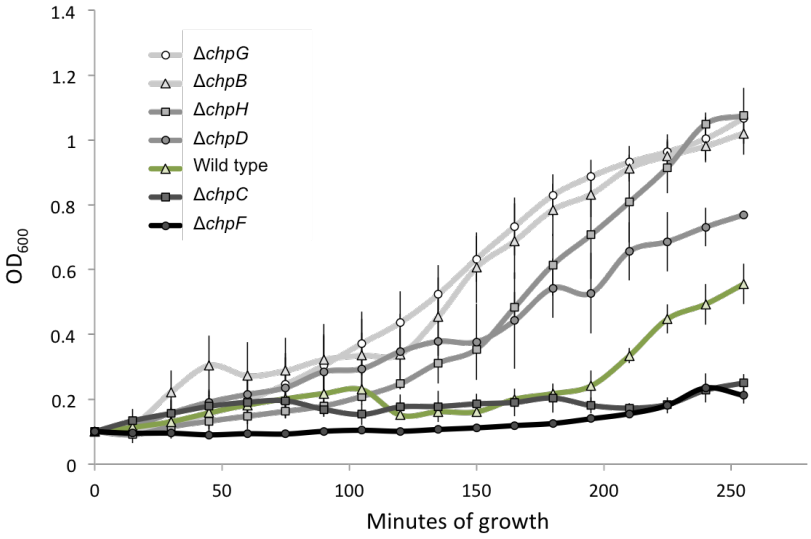
Figure 3.5 Growth and development of combinatorial *chp* mutants on solid medium. The combinatorial *chp* mutants were streaked onto solid MYM medium and left to grow for 72 h. Photos show the development of these mutants compared to wild type *S. venezuelae*. The mutants show delays in development, with the short *chp* mutant and the 5-fold *chp* mutant having the most severe phenotypes. Neither of these two strains had commenced aerial growth by 24 h, while wild type and the other two mutants had (albeit, with the minimal *chp* strain not as uniformly as the wild type). The 5-fold *chp* mutant only produced immature spores, devoid of green pigmentation, and incubation for well over one week was required for the short *chp* mutant to progress to aerial growth and sporulation.

3.2.3 Various *chp* mutants show a more rapid spore outgrowth than wild type

We have established that a combinatorial loss of *chp* genes was sufficient to affect the ability of such a strain to raise aerial hyphae and form mature spores (for example, based on the lack of green pigmentation associated with 5-fold *chp* mutant colonies). One question that has not been well addressed in the literature is whether or not the absence of *chp* genes has any effect on the ability of *Streptomyces* spores to germinate. On the one hand, the chaplins drive developmental progression forward, and although this is most important during the formation of aerial structures, they may also impact spore germination. However, as amyloid fibers are reported to be stronger than steel of comparable thickness (Smith *et al.*, 2006), one could imagine that this armoured encasement by chaplins may pose as a barrier that must be degraded or otherwise remodeled before exponential growth can commence. To assess the effect that chaplins have on spore germination, spores were collected from the single and combinatorial mutants (where possible), and an equal number of spores were used to inoculate liquid MYM medium. During the first 4 h of growth, the OD_{600} value for these cultures was recorded every 15 min (Figure

3.6) to determine when each strain began to show an increase in OD_{600} – which we took to represent spore germination and outgrowth. Many of the mutants exhibited an increase in OD_{600} at these early time points compared to wild type, with the most profound difference seen for the minimal *chp* strain which showed an increase in OD_{600} beginning at around 75 min after inoculation (compared to wild type which took approximately 190 min). Interestingly, losing a single *chp* gene seemed sufficient to allow for more-rapid germination, as a number of the single *chp* mutants exhibited an analogous trend (with the exceptions being the Δ*chpC* and Δ*chpF* mutants). It is worth noting that these differences in early spore outgrowth do not seem to affect the culture density at later time points. For example, although the short and 5-fold *chp* mutants show an earlier rise in OD_{600} than wild type, the wild type strain is able to surpass the density of either of these strains by 10 h (Figure 3.3). These findings suggest that spores with impaired chaplin profiles can commence active growth more readily than wild type.

A.



B.

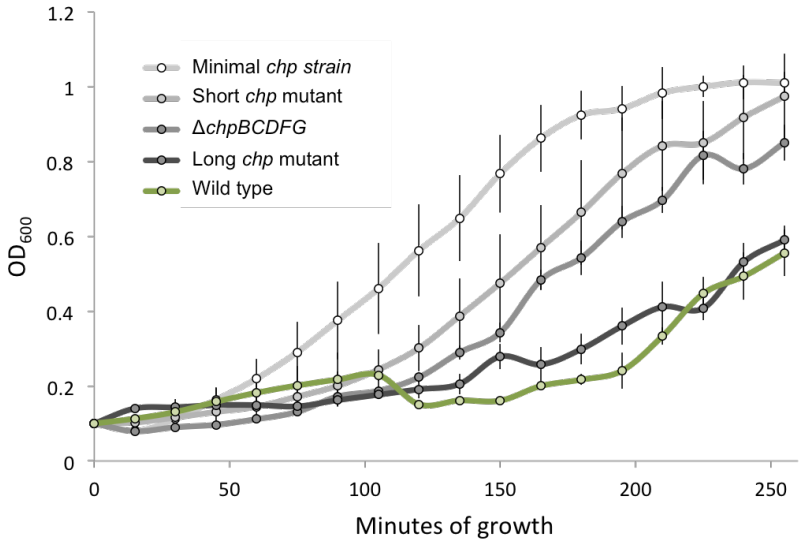


Figure 3.6 Mycelial outgrowth from spores occurs more rapidly in *chp* mutants. Spores were harvested from all single and combinatorial mutants and equal numbers of spores were used to inoculate liquid MYM medium. The OD_{600} values for these cultures were recorded every 15 min, starting immediately after inoculation so as to capture the initiation of spore outgrowth from the various strains. **(A)** Data from the single mutants show that, except for Δ*chpC* and Δ*chpF*, these mutants show increases in OD_{600} value earlier than wild type. **(B)** Data from the combinatorial mutants show that multi-chaplin loss leads to slightly increased rates of spore outgrowth compared to wild type (with the exception of the long *chp* mutant). Error bars represent the standard error of three biological replicates.

3.2.4 The effects of chaplin mutations on exploration

The chaplins are well-established as being critical developmental determinants during the classical developmental cycle. We have now shown that chaplins are also important during a developmental program that does not require aerial structures – liquid growth. This suggests that the roles of chaplins extend beyond their ability to lower surface tension to promote aerial development. As such we next sought to determine if chaplins also affect exploration – a mode of growth accomplished largely through non-branching, hydrophilic, vegetative-like hyphae (which should similarly not require the surface activity of chaplins; Jones *et al.*, 2017). We wanted to determine if our *chp* mutants exhibited any difference in exploration compared with wild type *S. venezuelae*, so all of our *chp* mutants were

spotted in the center of an exploration medium plate (YP agar). After five days of growth, the exploring colonies were imaged, and their surface area calculated (Figure 3.7). Many of the mutants showed exploration expansion that differed from the wild type, with Δ*chpC,* Δ*chpD,* Δ*chpG,* Δ*chpH*, and the 5-fold *chp* mutant achieving surface areas less than wild type, and the Δ*chpF*, short mutant, long mutant, and minimal *chp* strain achieving surface areas greater than wild type. The most extreme phenotype was observed for the short mutant, whose impressive surface area was almost twice the size of the second-largest exploring colony and more than three times the size of wild type.

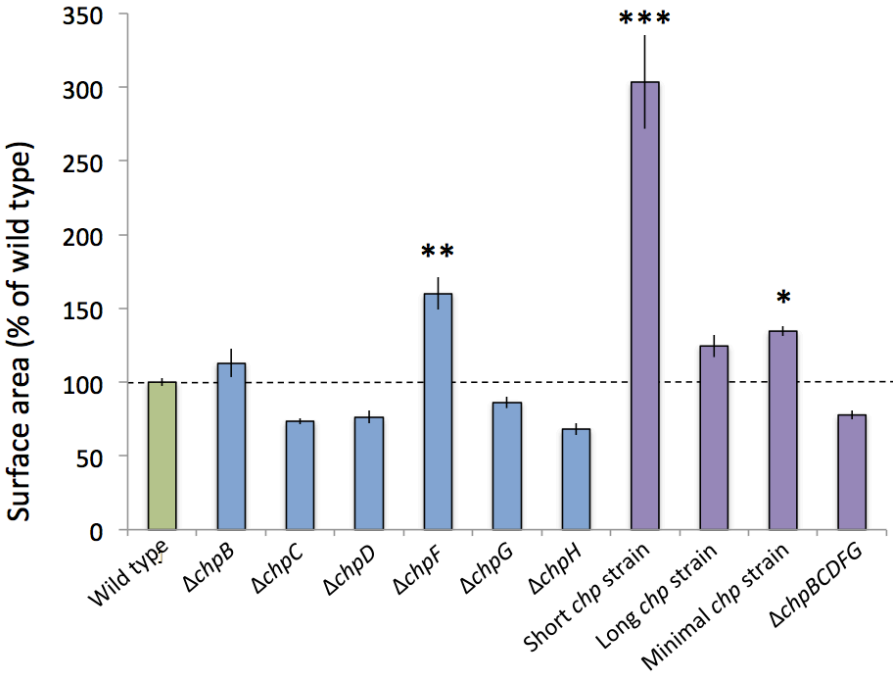


Figure 3.7 Loss of *chp* genes differentially affects exploration. The various *chp* mutants were grown on exploration-promoting medium to assess their exploration capabilities. After 6 days of growth, their surface areas were measured and compared to the surface area of a wild type strain (green). Single mutants (blue) typically displayed slightly reduced surface areas, with the exception of Δ*chpF,* which showed increased levels and Δ*chpB,* which showed wild type-level expansion. Of the combinatorial mutants (violet), the short *chp* strain had the most striking phenotype, achieving a surface area greater than three times that of the wild type. Error bars represent standard error from three biological replicates. A t-test was performed for each mutant compared to wild type. Values 0.005 and below, ***; values 0.005-0.01, **; values 0.01 – 0.05, *.

Amyloid fiber formation is known to be affected by pH (Chiti *et al*., 2001), and *in vitro* work on the *S. coelicolor* chaplin proteins has shown that their polymerization can be impacted by pH, with changes in pH dictating the secondary structure adopted by the amyloid protein (Baldwin *et al*., 2011; Dokouhaki *et al*., 2017a; Dokouhaki *et al*., 2017b). Given that exploring colonies significantly alter medium pH, we sought to determine if this might affect amyloid formation. We calculated the pI and subsequent charge of the mature chaplin peptides of the short chaplins using Prot pi (https://www.protpi.ch/Calculator/ProteinTool). The overall charge of each peptide was predicted to decrease as the pH in the medium rose, but there was no significant difference among the chaplins (Table 3.1). Therefore, variations in amyloid protein characteristics between the chaplin proteins specifically during exploration conditions was unlikely to explain the differences seen in the exploration of the single *chp* mutants. We considered the possibility that chaplins may be differentially required or utilized for exploration. Transcript abundance of all of the *chp* genes was increased in exploring colonies compared to classically growing colonies; this increase was slight for most of the *chp* genes, but was drastic for *chpE* and *chpF* (Jones, RNA-seq). Given the increased transcript abundance observed for *chpF* in exploring colonies, one might predict that ChpF would have an important role in exploration. This was not consistent with the elevated exploration levels seen for the Δ*chpF* mutant.

Table 3.1 Predicted charges of chaplins over a range of pH values. Prot pi was used to predict the pI values and overall charge of the short chaplins over a range of pH values

Protein	pI	Charge at listed pH			
		7	8	9	10
ChpD	5.50	-1.7	-2.9	-4.4	-4.9
ChpE	6.48	-0.6	-1.4	-2	-2.5
ChpF	5.69	-0.9	-1.9	-3.4	-3.9
ChpG	4.98	-3.4	-4.9	-6.6	-7.9
ChpH	5.50	-0.9	-1.9	-3.4	-3.9

As a final consideration, given that the short *chp* mutant showed the most extreme phenotype, we wondered if the phenotype may be due to altered cell wall properties. For example, maybe the chaplins coat the exploring hyphae in a specific way, and disrupting this coating affects their ability to explore. Because the rodlin proteins are predicted to organize the chaplin polymers on the surface of aerial hyphae (Claessen *et al*., 2004), we tested to see whether a rodlin-null strain also hyper-explored. We found the exploration capabilities of rodlin mutant were similar to that of the wild type. Creating a combined *rdl* and *chp* deletion (specifically, a Δ*chpH*Δ*rdlABC* mutant) also did not seem to have any effect: this mutant explored to the same degree as a single Δ*chpH* mutant. This collectively suggested that the exploration effect observed for the short chaplin mutant was due to a combination of factors, that may not encompass the activity of the rodlin proteins.

3.2.5 Identification and characterization of *chpE* mutants

3.2.5.1 Creating three independent *chpE* mutants

As described above, we were initially unable to create a *chpE* mutant, despite extensive efforts. It therefore came as a surprise when three Δ*chpE* mutants were identified after many independent rounds of CRISPR-associated conjugations and screenings (at least 12). This was achieved when we more than doubled the number of spores we would use in a typical conjugation. By way of example: the three Δ*chpE* mutants were identified after conjugating the *chpE* CRISPR-Cas9-containing construct with approximately 1.1×10^{11} spores, whereas just one conjugation of the equivalent *chpC* CRISPR-Cas9-containing construct into only 3.3×10^{9} spores resulted in approximately 25 Δ*chpC* mutants. We hypothesized that these three strains may harbour suppressor mutations enabling the deletion of *chpE* in a background where it was otherwise essential. Hereafter, these three mutants are referred to as Δ*chpE* (1), (2), and (3).

3.2.5.2 Classical development of *chpE* mutants

The three Δ*chpE* mutants identified above were grown both in liquid MYM medium and on solid MYM agar. In liquid medium, growth of the three mutants was similar to that of the wild type, with all four strains achieving a similar culture density at each time point (Figure 3.8A). Interestingly, however, two of the three strains exhibited very different developmental characteristics during liquid culture growth compared with wild type. The mutant Δ*chpE* (1) fragmented at 20 h and had just initiated sporulation by the end of the time course. In contrast to this, wild type and Δ*chpE* (2) did not begin to fragment until approximately 50 h. It is unusual for wild type to take this long to fragment in liquid culture, however, specific medium composition can sometimes be affected by changes in the water supply used to make this medium. When this occurs, developmental delays are commonly seen - that was likely cause of the delay seen for wild type. The mutant Δ*chpE* (3) transitioned from vegetative growth to fragmentation around 20 h, but despite this early transition, did not show similarly accelerated sporulation, and instead maintained a fragmented phenotype for the remainder of the time course (up to 56 h; Figure 3.8B). The length of 52 h was chosen, because, due to unclear reasons, the wild type control required a longer incubation to develop than was typically observed. In contrast, on solid medium, Δ*chpE* (1) and Δ*chpE* (3) both exhibited delayed development relative to wild type, only growing aerially after prolonged incubation (approximately 70 h; Figure 3.9).

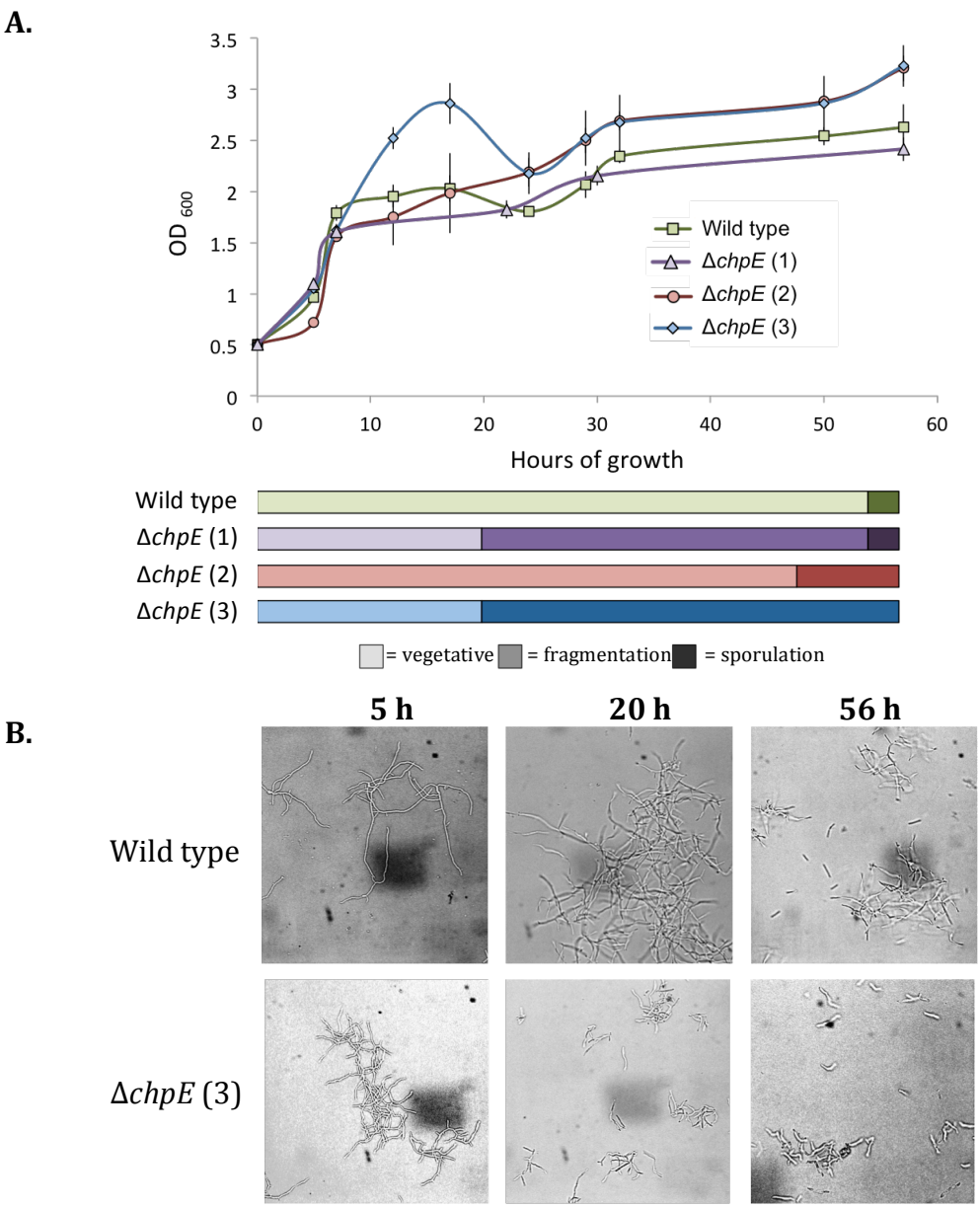


Figure 3.8 The three Δ*chpE* mutants grow comparable to wild type *S. venezuelae* in liquid, but develop differentially. (A) Overnight cultures of the three Δ*chpE* mutants and their wild type parent were used to inoculate liquid MYM medium to an OD_{600} of 0.05, before growing for 56 h. At the points shown in the graph, the OD_{600} value of the culture was recorded and the cultures were analyzed using light microscopy to determine their developmental stage (shown as horizontal bars below the graphs). Error bars represent standard error of three biological replicates. **(B)** Throughout the time-course shown, the Δ*chpE* (2) mutant closely followed the development of wild type, while the Δ*chpE* (3) and Δ*chpE* (1) mutants

entered fragmentation much earlier and maintained this phenotype throughout most of the 56 h duration. Representative images of cultures from (A). The 5 h and 20 h images for wild type show vegetative-stage mycelia, with fragmented mycelium (of much shorter length) shown in the 56 h image. For Δ*chpE* (3), fragmentation is evident as early as 20 h.

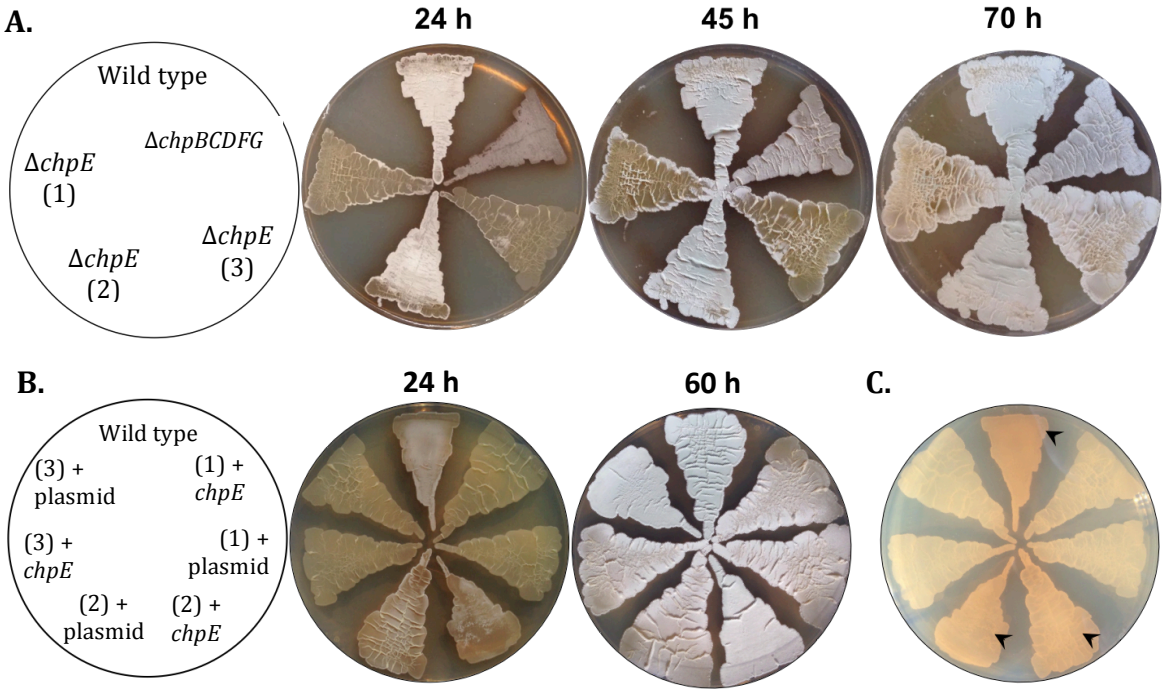


Figure 3.9 The three Δ*chpE* mutants develop differently than wild type *S. venezuelae* and their phenotypes cannot be complemented. (A) The three *chpE* mutants were grown on solid MYM medium next to wild type and a 5-fold *chp* mutant for comparison. The mutants Δ*chpE* (1) and Δ*chpE* (3) had the longest delay before aerial growth, taking more than 4 days to robustly raise aerial hyphae. The wild type and Δ*chpE* (2) strains initiated aerial growth after approximately 18 h, while the 5-fold *chp* mutant began after approximately 40 h. Both the wild type and Δ*chpE* (2) strains had sporulated by 45 h. Despite the delayed development of Δ*chpE* (1) and Δ*chpE* (3), these strains achieved sporulation after approximately 5 days. Sporulation was gauged by the formation of a raised, fuzzy colony, with green pigmentation. **(B)** A wild type *chpE* gene was cloned into the pSET152 plasmid and this construct was introduced into each of the mutants. As a negative control, the empty pSET152 plasmid was separately introduced into these strains. The development of these strains on solid MYM medium was compared to wild type. Complementing the three mutants with *chpE* failed to restore wild type growth (at least for Δ*chpE* (1) and Δ*chpE* (3) which grew less robustly than wild type. **(C)** An image of the underside of the complementation plate shows a lack of melanin

production from Δ*chpE* (1) and Δ*chpE* (3). This phenotype was similarly not complemented through the introduction of wild type *chpE.* Arrows indicate the strains [wild type, Δ*chpE* (2) + plasmid, Δ*chpE* (2) + *chpE*] that are producing melanin.

Because of the differing phenotypes seen for Δ*chpE* (1) and (3) relative to Δ*chpE* (2), it seemed likely that the phenotype observed may be the result of a secondary mutation (and perhaps this mutation may also permit *chpE* deletion). This was confirmed by analyzing the growth and development of the three mutants on solid medium, where complementing with a wild type copy of *chpE* did not restore the wild type phenotype (Figure 3.9B). Previous work has established that loss of the other *chp* genes alleviates the essential nature of *chpE*. Therefore, to determine if a suppressor mutation resulted in reduced expression of the other *chp* genes, and thus enabled *chpE* deletion, we compared the phenotypes of the three *chpE* mutants to a 5-fold *chp* mutant strain. This 5-fold *chp* mutant developed more rapidly than Δ*chpE* (1) and Δ*chpE* (3), although these strains formed mature spores before the 5-fold *chp* mutant. The development of the 5-fold *chp* mutant was delayed in comparison to Δ*chpE* (2). Therefore, it is not likely that *chpE* was able to be mutated in these strains due to a suppressor mutation that impacted the expression of the other *chp* genes. .

3.2.5.3 Exploration of *chpE* mutants

The three *chpE* mutants showed interesting, albeit different, phenotypes when analyzed at all stages of the classical growth cycle. This was true for both growth in liquid and on solid media. We hypothesized that there may be unique exploration phenotypes associated with these different mutants as well, and to assess this, grew the three mutants on exploration-promoting medium. Again, the two most distinctive mutants – Δ*chpE* (1) and Δ*chpE* (3) – showed a remarkable difference in their ability to explore. While the Δ*chpE* (2) mutant exploration was comparable to wild type, the other two mutants hyper-explored (Figure 3.10A). It

appears that this phenotype was also due to secondary mutations, as the complemented strains retained their rapid exploration capabilities (Figure 3.10B).

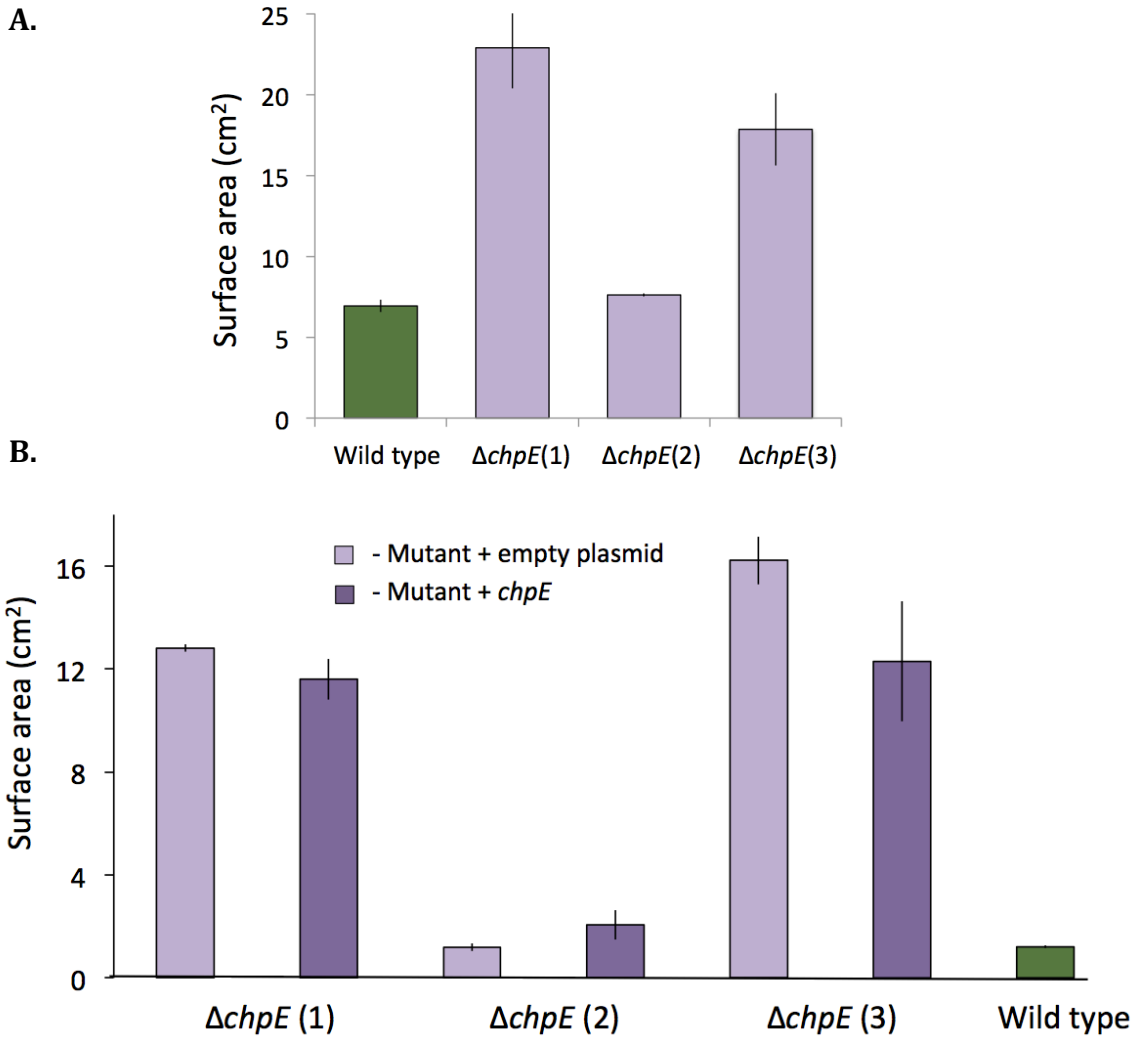


Figure 3.10 Two of the three *chp* mutants display hyper-exploration while the third displays wild type levels of exploration. **(A)** The three Δ*chpE* mutants were grown on exploration medium and their surface area was calculated after 6 days. **(B)** Growing the complemented mutants on exploration medium for 4 days showed that a wild type copy of *chpE* was unable to restore wild type exploration levels. As before, error bars represent standard error of three biological replicates.

3.2.5.4 Identifying suppressor mutations in *chpE* mutant genomes

It has long been established that *chpE* is essential in a wild type *S. coelicolor* background. Work done in *S. coelicolor* revealed that *chpE* can be deleted in *chp*, *rdl*,

or *tat* secretion system mutant backgrounds (Di Berardo *et al*., 2008). Given that our *chpE* mutants did not assume the phenotype of a *chp* null strain or a *tat* mutant strain (not exhibiting sporulation defects similar to that of a 5-fold *chp* mutant, and not hyper-sporulating like a *tat* mutant; Di Berardo *et al*., 2008) and sequencing of the *rdl* genes from these mutants revealed them to be unmutated, we considered the possibility that these *chpE* mutants arose as a result of a novel suppressor mutation(s). We isolated and sequenced the complete genomes of Δ*chpE* (2) and Δ*chpE* (3) in order to identify candidate mutations. Comparing these genomes to a wild type reference genome sequenced at the same time allowed identification of all mutations within the genomes of these strains. The two mutants only harbored two and three mutations (respectively) relative to the wild type strain (table 3.1). A mutated *Sven_1894* was found in both mutants, and a blast search indicated that this gene was conserved among many *Streptomyces* species. An intergenic mutation was found in Δ*chpE* (2). This mutation was between two genes: 207 nt downstream of *Sven_3987* [with *Sven_3987* orientated towards the mutation (*i.e*., on the positive strand of DNA)], and 466 nt upstream of *Sven_3988* [with *Sven_3988* also orientated towards the mutation (*i.e*., on the negative strand of DNA)]. The distance of this mutation from either of those genes suggests that it is likely not affecting their regulation, so to rule out the possibility that the mutation may be affecting an unidentified gene, we consulted transcription start site data (Sherwood, unpublished) but unfortunately this region is not associated with transcription. Another intergenic mutation was identified in Δ*chpE* (3), with this mutation being 14 nucleotides upstream of the start site of *Sven_2517* – possibly affecting the ribosome-binding site.

Table 3.2 Mutations identified in two Δ*chpE* mutants. The genomes of Δ*chpE* (2) and Δ*chpE* (3) were isolated and sequenced. These were compared to a wild type reference genome to identify mutations.

Mutated gene	DNA mutation	Protein mutation	Proposed function of encoded protein
Mutant Δ*chpE* (2)			

Sven_1894	G → C	V171L	Secreted maltose binding protein
Intergenic (between Sven_3987/_3988)	TGCTCAGGGG duplication	n/a	n/a
Mutant Δ*chpE* (3)			
Sven_1894	G → C	V171L	Secreted maltose binding protein
Intergenic	T→C	Upstream of *Sven_2517*	SARP family DNA regulator
Sven_1436	C → T	H120Y	Hypothetical protein

3.3 Discussion

3.3.1 Chaplins and the classical life cycle of *S. venezuelae*

The chaplins are an important family of developmental proteins that collectively play a significant role in *Streptomyces* growth and development. This work has established that individually, the chaplin proteins and their effects on development are largely redundant in nature, although there are intriguing differences, particularly the fact that a loss of *chpD* seems to enhance both growth and development. It will be important to complement this difference by supplying a wild type copy of *chpD* into this mutant to confirm that this deletion is indeed responsible for the observed phenotype. The six single mutants showed development on solid medium that was similar to that of wild type, and development in liquid that differed from wild type only marginally (again, with the exception of Δ*chpD*). This redundancy of individual chaplins is consistent with what has been previously reported for *S. coelicolor* (Di Berardo *et al*., 2008). However, when increasing numbers of *chp* genes were deleted, the resulting effects on classical *S. venezuelae* development were profound. The most chaplin-devoid strains were the 5-fold *chp* mutant closely followed by the short *chp* mutant (a 4-fold *chp* mutant). Both of these strains exhibited delays in aerial development, and although the 5-fold *chp* mutant grew aerially and progressed to sporulation, it was unable to form mature spores even after extended incubation. Lack of spore pigmentation is typically associated with *whi* mutants, as the final steps in spore maturation

involves modifying the spore wall and incorporating a pigmented polyketide (Flärdh & Buttner, 2009). Given that the 5-fold *chp* mutant was unable to produce mature spores suggested that either I) these chaplins serve an additional developmental role, possibly indirectly affecting the downstream stages of development (beyond the transition to aerial growth), or II) a spore coat lacking chaplins is not amenable to the final stages of spore modification. As for the phenotype associated with the short *chp* mutant, a possible explanation is that this strain does not produce sufficient chaplin proteins to effectively lower the surface tension of the medium-air interface. As such, this strain would remain severely affected in its ability to breach this interface and grow aerially.

As stated previously, the ability of *S. venezuelae* to develop in liquid provides a unique opportunity to assess the roles that chaplins play in development during liquid growth. If the chaplins are strictly required for aerial growth, then growth in liquid culture (where lowering of the surface tension to grow aerially is not a factor) should not impact strain development. This was indeed the case for the single *chp* mutants, which showed both growth rates and developmental transitions similar to wild type, with the exception of the *ΔchpD* mutant. Similarly, this was also the case for the minimal *chp* strain, the long *chp* mutant, and the short *chp* mutant. However, the 5-fold *chp* mutant displayed a striking difference. In liquid, although this mutant was able to progress to fragmentation around the same time as wild type, the fragmented mycelia failed to differentiate into spores, except upon extended incubation (*e.g.* after a week, only a few spores could be detected; Figure 3.4). This strongly suggested that the chaplins likely have additional roles in development beyond simply promoting the raising of aerial hyphae. The abysmal development of this mutant may be because *S. venezuelae* requires a minimal number of total *chp* genes to effectively promote development, and a 5-fold deletion brings this strain below that threshold.

The germination rates of the various *chp* mutants were assessed by recording the OD_{600} values of freshly inoculated cultures immediately after incubation began. Our results suggested that chaplin fibers may impart a slight, yet consistent, obstacle for a germinating spore. For a dormant spore to resume active

growth, considerable modifications to the thickened cell wall must first be completed – an example being through cleavage from the muralytic enzymes resuscitation-promoting factors (Sexton *et al*., 2015). Accordingly, one can imagine that the hydrophobic sheath of chaplin and rodlin proteins would pose an additional barrier that would need to be physically penetrated in order for germination to occur. Our data further support this, as many of the *chp* mutants showed an increase in OD_{600} occurring before the wild type strain. This is consistent with previous findings that disrupting of the spore wall by ultrasonication results in increased germination rates of *Streptomyces antibioticus* (Miguez *et al*., 1993).

3.3.2 Chaplins and their roles in exploration

In addition to contributing to the different classical stages of development, it was exciting to discover that the chaplin proteins also contribute to *Streptomyces* exploration. In general, many of the single *chp* mutants showed a slightly reduced ability to explore. The exception to this was the Δ*chpB* mutant, which explored equivalently to wild type, and the Δ*chpF* mutant, which explored more robustly than wild type. Important future steps of this work include attempting to complement these phenotypes by re-introducing a copy of their respective wild type genes in order to confirm that these phenotypes are a direct result of the mutated *chp* genes. The 5-fold *chp* mutant also had slightly reduced exploratory potential. The most striking phenotype of all, however, came from the short *chp* mutant. Its exploratory potential was more than three times greater than the wild type strain. Our efforts to explain the exploration phenotypes of the various *chp* mutants in terms of chaplin abundance or amyloid properties failed to yield a cohesive model. However, given that exploring colonies surround themselves in a matrix of as-yet determined composition (Sexton, unpublished), it is possible that chaplins do not affect exploration through the formation of specific amyloid fibers, but rather, their peptides contribute to matrix formation in ways that are not yet fully understood. Perhaps the viscosity of this matrix is reduced in the absence of the short chaplins, and the colony is spread thinner by virtue of this less-structured matrix. This is reminiscent of what is seen in *Bacillus* models, where the amyloid constituent

(TasA) of biofilms is a major factor dictating colony morphology and architecture. In these colonies, mutants unable to produce TasA remain flat and architecture-free (Romero *et al*., 2010). From our exploring colonies, while it is true that the short *chp* mutant looks flatter than wild type, it is also associated with larger wrinkles that radiate outwards from the core. Future efforts to address this possibility will be required for definitive conclusions to be drawn.

3.3.3 Secondary mutations within Δ*chpE* strains

It is generally accepted that *chpE*, at least in *S. coelicolor*, is conditionally essential. It was therefore an exciting discovery when we confirmed three *chpE* mutants after extensive efforts. Because two of these mutants [Δ*chpE* (1) and Δ*chpE* (3)] behaved similarly in all phenotypic assays, they possibly harbour the same mutation; whether through randomness or through being clonally related. Since Δ*chpE* (2) grew and developed in a wild type manner, and since the observed phenotypes from Δ*chpE* (1) and Δ*chpE* (3) were not complemented by the addition of a wild type copy of *chpE*, it is likely that loss of ChpE, in itself, does not have significant effects on development. It seems that the major role of ChpE is to somehow regulate the other chaplin proteins, and its deletion was potentially made possible due to a suppressor mutation that compensated for its conditionally essential role. Nonetheless, the phenotypes of these two Δ*chpE* mutants are interesting, particularly given both their significantly altered development, and their increased exploration capabilities.

The genomic sequencing of two of these mutants highlighted only one common mutation– a mutation in a gene (*Sven_1894*) encoding a maltose binding protein. The gene *Sven_1894* encodes MalE of the *mal* operon, which is an operon dedicated to the uptake of maltodextrins (Schlo *et al*., 2018; Wezel *et al*., 1997). The amino acid sequence of Sven_1894 was analyzed with SignalIP (http://www.cbs.dtu.dk/services/SignalP/) to confirm that this protein was likely secreted. Because there is an unclear connection between *tat* mutants, and the ability to delete *chpE*, we further analyzed the amino acid sequence of *Sven_1894* with TatP (http://www.cbs.dtu.dk/services/TatP/), which suggested that this

protein is not a substrate of the tat secretion system, although it does have a twin arginine motif. Without knowing the exact reason why ChpE is conditionally essential, it is difficult to speculate how a mutated protein could compensate for this function. However, one theory is that ChpE controls or regulates the polymerization of the other chaplins by functioning as a 'capping factor' that interacts with the chaplins and prevents uncontrolled polymerization. If this was indeed the case, it is possible that Sven_1894, which contains a secretion signal, is serving a similar role, and is perhaps now able to interact with the chaplins. One potential way this could be determined is by conducting a targeted bacterial two-hybrid screen between the mutated Sven_1894 from the Δ*chpE* strains and other chaplin proteins. If un-mutated Sven_1894 was used as a control, then an interaction between the former pair but not the latter pair would support this idea. It is likely that one or both of the other two mutations identified in Δ*chpE* (3) are responsible for the odd phenotype exhibited by this strain (lack of melanin, impaired development, hyper-exploration) and complementation experiments will need to be conducted in order to determine which mutations are causing these effects. Once these follow-up suggestions have been completed, we will have a much better grasp of the reasons behind the conditional essentiality of *chpE*, and we will have additional candidate genes to investigate for their roles in exploration.

Given ChpE essentiality – and the precarious nature of amyloid proteins in general – it is possible that ChpE has a role to play in maintaining the other chaplins in a functional, not harmful, state. Collectively, this work has generated important preliminary data for understanding how this regulation may be achieved. Hopefully future investigations will take advantage of these findings and elucidate the reasons behind why these mutations are permitting *chpE* deletion, and by doing so, make strides in understanding the elusive role of ChpE for this functional amyloid system.

Chapter Four

Characterizing the regulation of *chp* genes in *S. venezuelae*

4. 1 Introduction

Based on phenotypic analyses of the assortment of *chp* mutants generated in chapter three, it appears that the chaplins of *S. venezuelae* may not be entirely redundant, as is the case with the chaplins of *S. coelicolor* (Di Berardo *et al*., 2008). Our data, and data from exploring *Streptomyces* (Jones, RNA-seq) suggest that *chp* genes may be subject to differential regulation. Therefore, in an effort to better understand how these proteins are controlled, we sought to investigate the regulation of the *chp* genes. As described previously, there are two regulators known to directly bind to the promoters of the *chp* genes: σ^{BldN} and MtrA. σ^{BldN} mediates the expression of all *chp* genes (how it impacts *chpD* expression is currently unknown, as it was not identified until after this work was published) in *S. venezuelae*, with their expression uniformly activating after 10 h of growth, but plateauing at different levels at later time points such as 16 h – 20 h (Bibb *et al*., 2012). MtrA, a two-component response regulator, binds the promoters of the short *chp* genes in *S. venezuelae*, but affects the expression of both short and long *chp* genes in *S. coelicolor*. Chromatin immunoprecipitation experiments in *S. venezuelae* with tagged MtrA revealed the *chp* genes to be most enriched after 20 h of growth (Som *et al*., 2017), with *chpH* being the most highly enriched, and *chpE* being the least (Zhang *et al*., 2017).

Work conducted in *S. coelicolor* has shown that *chpH* and *chpE* are unique in that they, along with *chpC*, are the most strictly conserved *chp* genes from many sequenced *Streptomyces* genomes, and their expression initiates during vegetative growth and is maintained at a high level throughout development, in contrast to the others, which are expressed most highly during aerial growth and sporulation (Claessen *et al*., 2003; Elliot *et al*., 2003). Furthermore, *chpE* is also intriguing given its conditionally essential nature. It was determined that *chpE* could not be deleted in a wild type genetic background, and this deletion was only possible in *chp*, *rdl*, or

tat mutant backgrounds (Di Berardo *et al*., 2008). Given this, we have focused our attention on *chpH* and *chpE* in *S. venezuelae*. We began this work before the effects of MtrA on *chp* genes was known, therefore we were unable to go into more depth on how MtrA effects the life cycles of *S. venezuelae*. Work done by Bibb *et al*., has suggested that additional *chp* regulators likely remain undiscovered, so this section is focused with identifying candidate genes whose products may influence *chp* expression (Bibb *et al*., 2012).

4.2 Results

4.2.1 σ^{BldN} does not appear to be activated by extracellular chaplin signals

The signal that liberates the *chp*-activating σ^{BldN} from sequestration by its cognate antisigma factor RsbN has yet to be identified. Given that RsbN most likely responds to an extracellular signal, one possibility is that secreted chaplins may serve as this signal. If this was the case, basal-level *chp* expression could eventually result in greater accumulations of chaplin proteins, which could in turn drive large-scale upregulation, propelling *Streptomyces* development towards the aerial stage by indirectly causing targets of σ^{BldN} to be activated. To address this possibility, P*chpH* and P*chpE* transcriptional fusions to the reporter gene *gusA* were introduced into wild type *S. venezuelae,* and into a three-fold c*hp* mutant, Δ*chpCFG* (the strain in which the greatest number of *chp* mutations had been generated at the time of this experiment). The expression levels of the reporter gene were monitored in both of these strains at 9 h and 24 h time points [corresponding to vegetative growth and aerial growth (which in liquid medium is indicated by hyphal fragmentation) respectively]. Compared to wild type levels, neither *chpH* nor *chpE* expression were affected in the Δ*chpCFG* mutant (Figure 4.1A). Choosing to look at even earlier time points, the *chpE* reporter strain (in wild type and the Δ*chpCFG* mutant) was analyzed at 3, 6, 8, and 10 h time points. The rational for going forward with just the *chpE* reporter is because the 9 h expression value in the Δ*chpCFG* mutant looked slightly greater than in wild type. However, unexpectedly, this analysis showed the opposite trend. It appears that the expression values of *chpE* were slightly reduced

in the Δ*chpCFG* mutant at these early time points, with the difference becoming most pronounced around 10 h (Figure 4.1B).

4.2.2 Identifying novel *chp* regulators

In 2012, work done by Bibb *et al*. suggested that σ^{BldN}, while important for *chp* activation, was alone not sufficient. *In vitro* transcription with σ^{BldN} as the sigma factor was uniquely unable to promote *chp* gene transcription, whereas another target gene (*bldM)* was transcribed effectively; therefore, it was proposed that, *in vivo*, *S. venezuelae* must harbour additional regulators that work in conjunction with σ^{BldN} to activate transcription from *chp* promoters (Bibb *et al*., 2012).

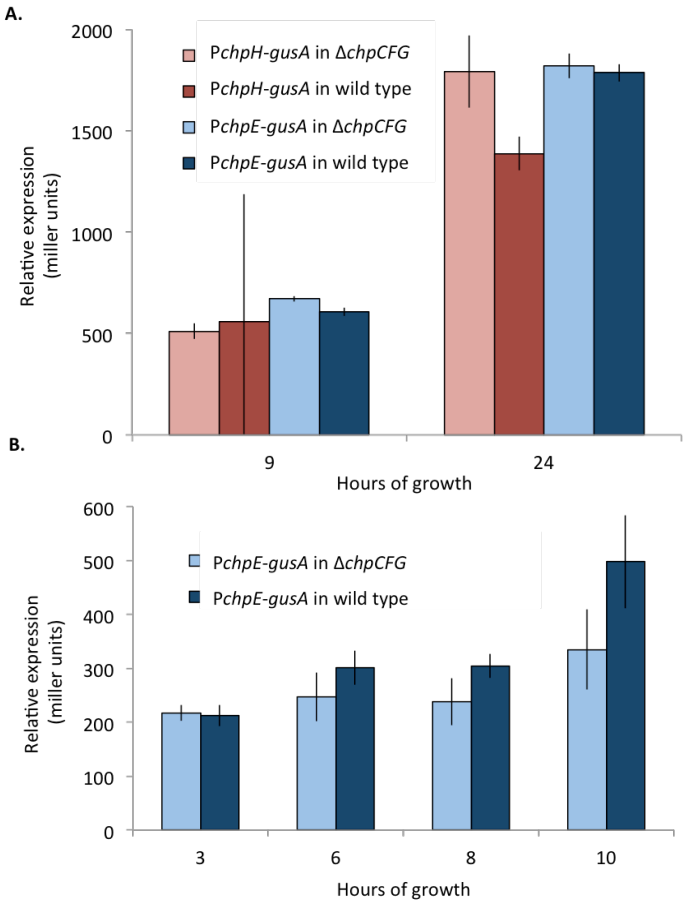


Figure 4.1 Expression of *chp* genes is not compromised in a multiple-*chp* mutant strain. Transcriptional reporters of *chpH* and *chpE* to the *gusA* gene were introduced into wild type *S. venezuelae*, and a 3-fold, Δ*chpCFG*, mutant. Levels of

reporter activity from both reporter strains were monitored at 9 and 24 h **(A)**, and at 3, 6, 8, and 10 h just for the *chpE* reporter **(B)**.

4.2.2.1 Protein pull downs with fragments upstream of *chpH* and *chpE* did not identify novel interacting partners

To identify proteins that may be involved in regulating *chp* gene expression, the promoters of *chpH* and *chpE* were used in pull-down experiments. Approximately 200 bp upstream of the start site were amplified from both of these genes, with the upstream primer being affixed to a biotin tag. These PCR products were attached to magnetic beads complexed with streptavidin as a way to enable future purification of the promoter DNA and any interacting proteins. As a negative control, approximately 200 bp of non-promoter DNA (*chpD* coding sequence) were similarly prepared. By comparing the proteins that bound either of the two promoters to the proteins that bound non-promoter DNA, it would be possible to discriminate between specific regulatory proteins, and proteins having promiscuous DNA binding.

Chaplin expression data from liquid-grown *S. venezuelae* cultures showed increased expression of all *chp* genes beginning at approximately 10 h (Bibb *et al*., 2012). Therefore, a 500 mL liquid culture of *S. venezuelae* was harvested at 10 h, and a portion of the corresponding cell lysate was combined with each of the three magnetic bead-PCR product preparations (*chpH* promoter, *chpE* promoter; *chpD* coding sequence). After sequential washing with buffers of increasing sodium chloride content (ranging from 50 mM to 500 mM), an aliquot from each wash was boiled in SDS and separated on a polyacrylamide gel. To ensure protein samples remained amenable to mass spectrometry, the gels were stained with silver to visualize protein bands. Multiple attempts were made to pull down unique proteins associated with the *chpH/E* promoter regions (with the extent of silver staining being modulated each time in an effort to identify protein bands that may not be visible due to over- or under-exposure); however, the associated proteins were consistently identical to those from the coding sequence DNA (Figure 4.2).

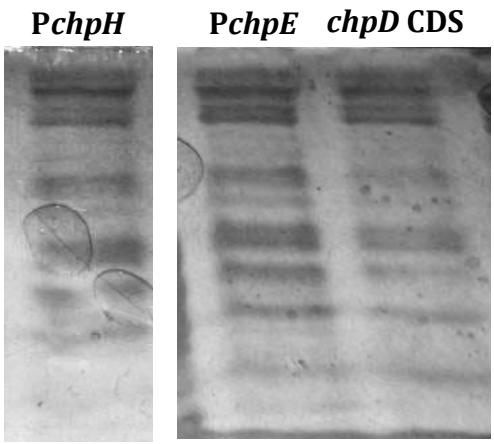


Figure 4.2 Proteins isolated from P*chpH* and P*chpE* pull downs. The promoters of *chpH* and *chpE* were PCR amplified using a biotinylated primer, and the resulting product was then incubated with *S. venezuelae* lysate. Streptavidin-coated beads were used to pull down the DNA, and high salt buffer was used to elute bound proteins. The proteins associated with the two promoters are shown here (stained with silver) alongside the proteins that were pulled down from a *chpD* coding sequence negative control.

4.2.2.2 Using *chpH* and *chpE* transcriptional reporters in chemically mutagenized spores to identify novel *chp*-regulator mutants

A parallel approach was taken to identify novel chaplin regulators, making use of the same transcriptional reporters (P*chpH-gusA* or P*chpE-gusA*) described above, coupled with chemical mutagenesis. Each reporter construct (shown in Figure 4.3A) was conjugated into four independently created *S. venezuelae* stocks that had been randomly mutagenized with the mutagen EMS. Reporter constructs were introduced into the mutagenized *S. venezuelae* stocks after EMS treatment to ensure that the promoter sequence remained unmutated. Because the reporter protein GusA catalyzes the conversion of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium (X-gluc) into a blue coloured compound, we predicted that changes in the amount of blue pigment could be used to identify mutants with altered promoter activation. Additionally, as a control, each construct was also introduced into the non-mutagenized wild type *S. venezuelae* strain. The non-mutagenized strains were plated for single colonies on solid medium

containing X-gluc. The single colonies grew and developed exactly as wild type *S. venezuelae*, and after approximately 24 h, had generated enough blue pigment to be distinguished with the naked eye. This resulted in a halo of blue around the colony, slightly eclipsing the colony perimeter (Figure 4.3B, middle). With a baseline established for the amount of blue pigment expected from wild type *S. venezuelae*, the mutagenized strains were next screened on solid medium with X-gluc, and colonies producing more blue pigment (mutation enhancing *chp* expression) or less blue pigment (mutation reducing *chp* expression) than wild type were identified (Figure 4.3B, left and right).

In total, approximately 21,000 colonies were screened, and from these, four colonies were identified that produced less blue than wild type. Oddly, hundreds of colonies were identified that produced more blue than wild type; these colonies were smaller than their wild type counterparts, and were almost exclusively bald in phenotype, and produced a large zone of blue around the colony (Figure 4.3B, right). This phenotype represented approximately 1% of all screened colonies.

A.

B.

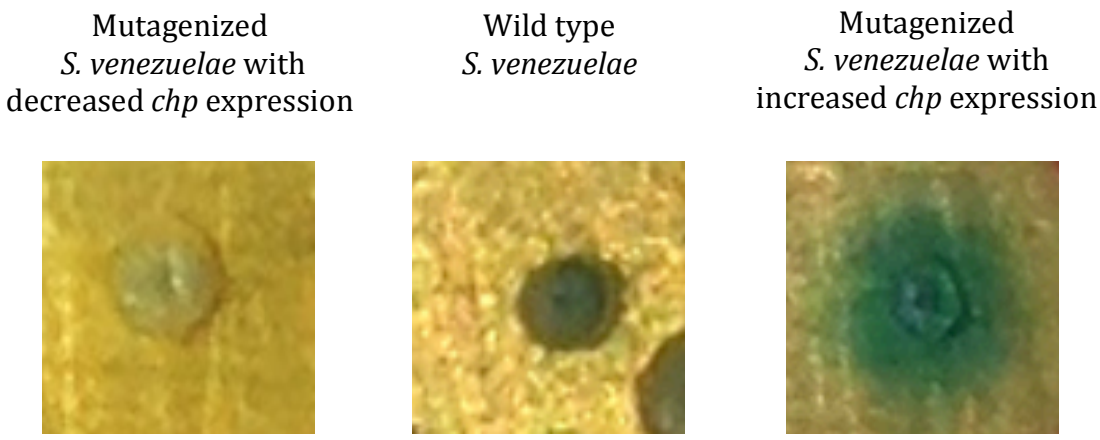
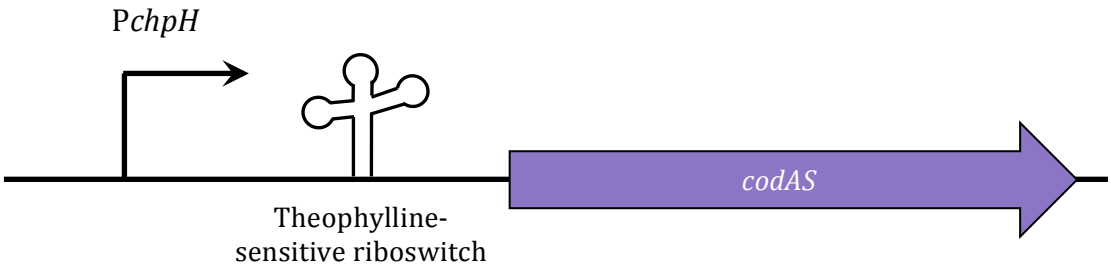


Figure 4.3 Phenotypic outcomes of screens for altered *chpH* promoter activity using *gusA* reporter constructs in chemically mutagenized *S. venezuelae*. (A) Reporter strains containing P*chpH*- or P*chpE-gusA* were designed and constructed in accordance with the schematic shown. These were conjugated into spore stocks mutagenized with ethyl methanesulfonate (EMS). Mutagenized spores were plated on solid MYM medium embedded with 2-Nitrophenyl β–D-galactopyranoside and the production of blue pigment monitored. **(B)** As expected, the vast majority of colonies had wild type levels of promoter activity (middle). We identified a small number of colonies that had lost promoter activity (left), and many colonies that had increased promoter activity (right).

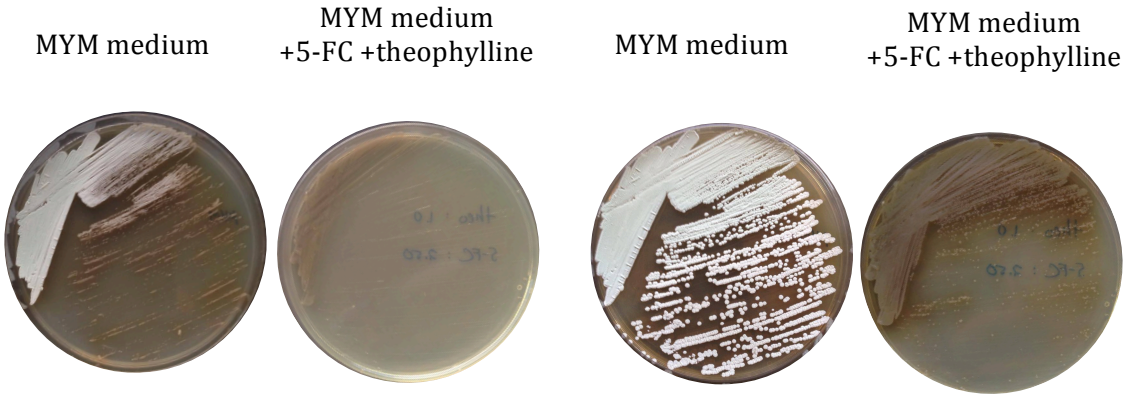
Because the P*chpH*- and P*chpE-gusA* reporter screens produced incongruous results (the drastic over-representation of small, hyper-blue, bald colonies), a different approach was taken to identify novel *chp* regulators. This approach made use of a toxin-encoding gene known as *codA*, which had been modified for efficient expression in *Streptomyces* (*codAS*). CodA is toxic due to its ability to convert 5-fluorocytosine to 5-fluorouracil, a nucleoside analog that disrupts DNA and RNA synthesis (Dubeau *et al*., 2009). The same promoter fragment used for the P*chpH-gusA* reporters was cloned upstream of *codAS*, together with a theophylline-sensitive riboswitch cloned between the promoter and the *codAS* gene (Figure 4.4A). This allowed for efficient cloning of the *codAS* reporter gene, and growth of the reporter-containing strains in the absence of theophylline (resulting in translation attenuation and no toxin production). Prior to initiating screening of our mutant library, the reporter construct was introduced into unmutated wild type *S. venezuelae*. This wild type, reporter-containing strain was grown on solid medium containing a gradient of theophylline (to relieve the repression of the riboswitch and allow for translation of the reporter gene) and 5-FC to determine optimal concentrations of each of these compounds that would result in maximal toxicity. The most effective concentrations were 8 mM of theophylline and 600 μg/mL 5-FC. With these medium conditions, wild type *S. venezuelae* with the reporter strain exhibited considerable growth and developmental delays, with colonies remaining small and bald, even after 48 h. When the same strain was grown on un-supplemented medium, it grew robustly and had formed sporulating colonies by

this time point, indicating that there were no phenotypic consequences to having the construct in *S. venezuelae* (Figure 4.4B). The reporter constructs were introduced into the mutagenized spore stocks to allow for screening to identify colonies with abolished or diminished promoter activation (which would phenotypically manifest as restoration of rapid development).

A.



B.



C.

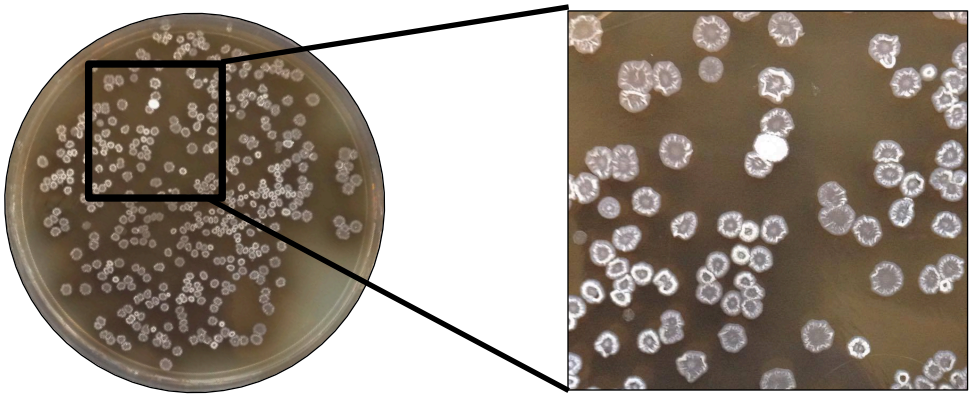


Figure 4.4 Construction and application of toxin-reporter constructs for screening chemically mutagenized *S. venezuelae* stocks. **(A)** Reporter strains containing P*chpH* controlling the expression of *codAS* (a toxin-encoding gene) were designed and constructed in accordance with the schematic shown. A theophylline-sensitive riboswitch was included between the promoters and the *codAS* gene. These reporter constructs were conjugated into spore stocks mutagenized with EMS. **(B)** To determine the optimal concentrations of theophylline (to enable production of CodAS) and 5-fluorocytosine (5-FC; the substrate converted to a toxin by CodAS), these constructs were introduced into wild type *S. venezuelae*, and the resulting strains plated on a gradient of 5-FC and theophylline. Concentrations ranging from 2 mM – 8 mM of theophylline, and from 150 μg/mL – 600 μg/mL of 5-FC were considered. No combination of the two reagents resulted in total cell death, but 8 mM of theophylline in combination with 600 μg/mL of 5-FC severely impeded proper development of *S. venezuelae*. After 24 h, colonies expressing *codAS* on a solid medium plate had just started to appear and were still in early vegetative growth, whereas colonies without expression were growing robustly and had already entered late vegetative growth. This difference in growth and development between the two conditions remained just as contrasting at 48 h. **(C)** To identify mutants impaired in chaplin expression, the mutagenized spore stocks harbouring the *codAS* constructs were plated on 8 mM of theophylline with 600 μg/mL 5-FC, and screened for colonies showing robust growth (indicating lessened toxin production and therefore lessened promoter activation). An example of a mutant identified through this method is shown. In total, eight mutants were identified that developed at wild type or near-wild type levels when grown on this medium.

The four independently mutagenized spore stocks were screened on medium supplemented with theophylline and 5-FC, and in total, eight colonies were identified that grew and developed at near-wild type levels. Hereafter, the colonies will be referred to as 1A, 1B, 1C, 2A, 2B, 3A, 3B, 3C, where the numbers indicate which mutagenized spore stocks the colonies came from and the letters indicate separately identified colonies (for example, colonies 1A and 1B were both identified from spore stock one, and colonies 2A and 2B were both identified from spore stock two).

4.2.2.3 Confirming altered *chp* expression in mutants

To ensure that the eight identified mutants from 4.2.2.2 were indeed impaired in chaplin expression, the *gusA* reporter constructs described in early 4.2.2.2 (P*chpH* or P*chpE* controlling expression of *gusA*) were each introduced into these strains. The resulting strains were grown in liquid MYM medium, alongside wild type *S. venezuelae* carrying the same reporter constructs. After 10 h of growth, the cellular biomass was collected and lysed, and the levels of GusA activity were monitored. From the eight mutants (1A, 1B, 1C, 2A, 2B, 3A, 3B, 3C), 1B and 3C were the only two that showed reduced *chp* expression. Specifically, these two mutants showed reduced reporter activity relative to the wild type control only for the P*chpH* reporter, not the P*chpE* reporter, with GusA levels comparable to wild type for the latter. Interestingly, compared to the wild type control, mutants 1A, 2A, and

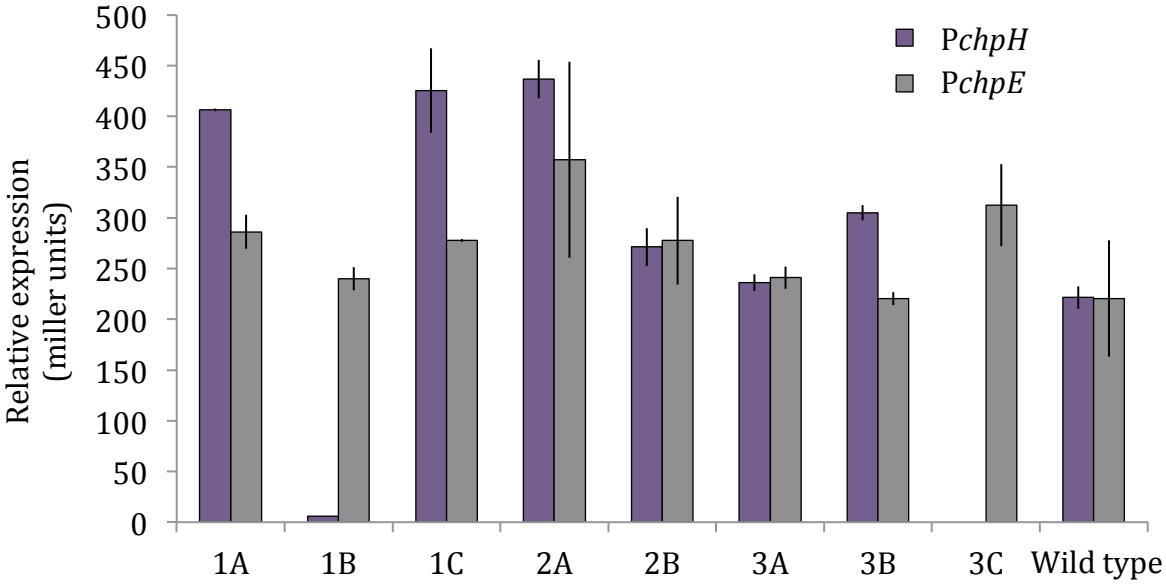


Figure 4.5 Expression of *chpH* and *chpE* from chemically mutagenized mutants of interest. The P*chpH*- or P*chpE-gusA* reporter constructs were introduced into each of the mutants identified in the toxin reporter screens that had showed robust development. The strains were grown in liquid MYM medium for 10 h before monitoring reporter activity, and comparing it with the levels seen for the wild type strain. Mutants 1B and 3C showed drastically reduced levels of *chpH* – a trend congruent with the toxin screening results. Data from technical triplicates was averaged for each strain, and strains were analyzed in biological triplicates. Error bars represent the standard error of the three biological triplicate averages.

1C showed increased reporter activity only when driven by P*chpH*. The other mutants did not have any striking difference in reporter activity (of either reporter) from the wild type controls (Figure 4.5). This assay was repeated to ensure the results were reproducible, and the same general trends in expression levels compared to wild type were seen.

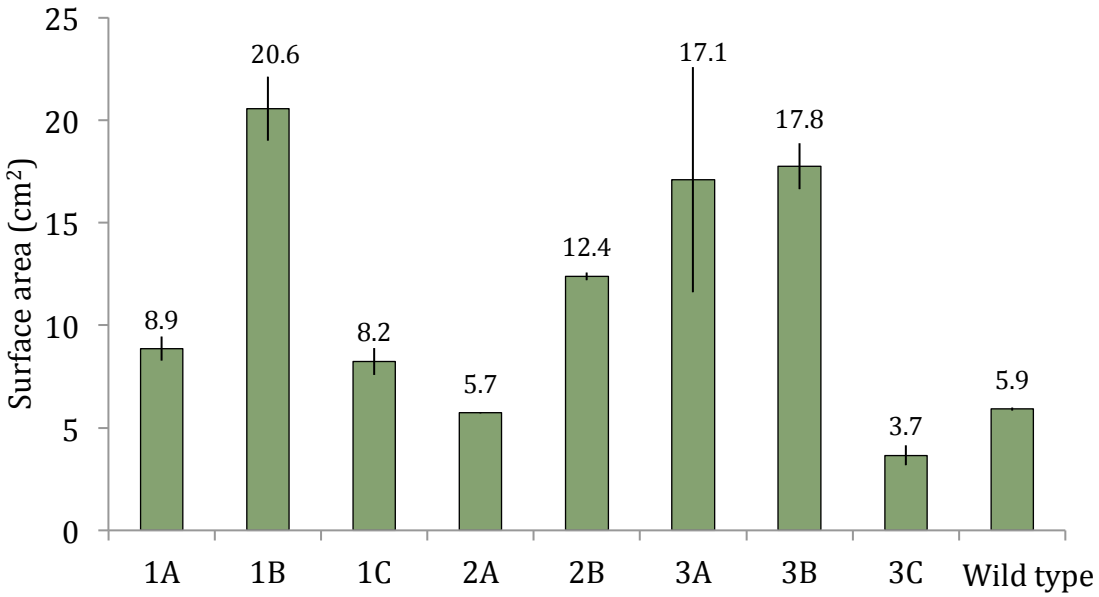
Based on the reporter gene activity of the *chpH* and *chpE* transcriptional fusions in the mutants of interest, none seemed to have impaired *chpE* activation, but a number of mutants showed either increased or decreased *chpH* expression compared to wild type levels. To validate these findings, and narrow down the list of meaningful mutants (that is, those that reliably demonstrate defects in *chpH* expression) the eight mutants were tested for their exploration capabilities, as our results are suggesting that Δ*chpH* mutants are compromised in their ability to explore. We grew these mutants on exploration-promoting solid YP agar for 5 days, at which point their surface area was calculated and compared to a wild type strain. Mutants defective in *chpH* expression would be expected to show the same reduced exploration ability exhibited by the Δ*chpH* strain. Of the eight mutants tested, two showed a reduced surface area compared to wild type (2A: 5.7 cm^2, 3C: 3.7 cm^2, wild type: 5.9 cm^2), while the other six showed exploration potential greater than wild type (with average surface areas of these strains ranging from 8.2 cm^2 to 20.6 cm^2). It is worth noting that this wild type does not have empty plasmids (pIJ82 or pGUS) integrated in its genome, so we recognized that this control was not entirely ideal. Mutants 1B and 3C both showed a drastic reduction in *chpH* expression, but interestingly, only 3C had an exploration potential comparable to that of the Δ*chpH* strain, with 1B reaching a surface area more than three times larger than the wild type strain (Figure 4.6).

4.2.2.4 Identifying genes involved in *chp* regulation

The complete genomes of mutants 1A and 3C were isolated and sequenced, as was that of the wild type strain used in the initial mutagenesis experiments. Mutant 3C was sequenced because the collective data from the toxin screens, P*chpH-gusA* reporter expression assay, and exploration potential strongly suggested that

this mutant was compromised in *chpH* expression. Mutant 1A was chosen because the P*chpH-gusA* reporter expression assay and the exploration potential implied

A.



B.

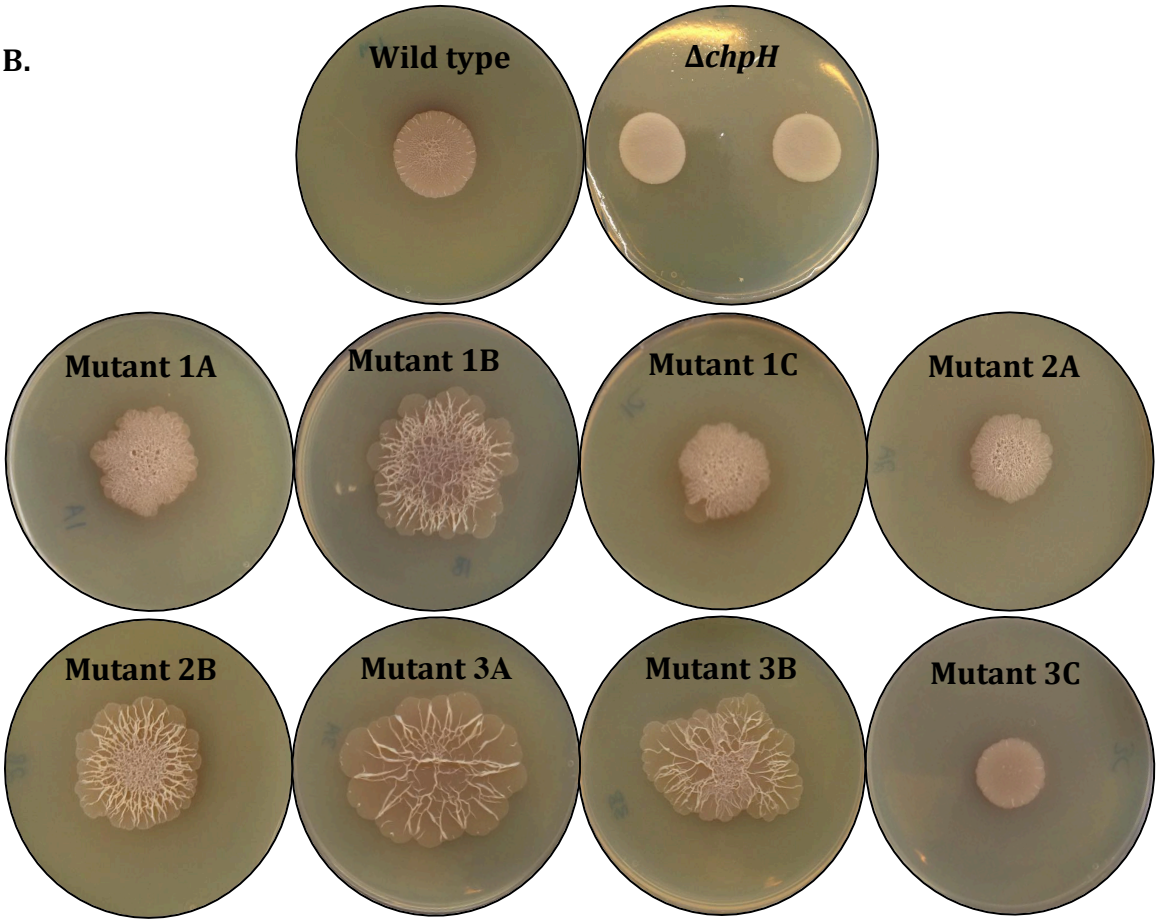


Figure 4.6 Exploration of chemically mutagenized mutants of interest. The mutants identified in the toxin reporter screens that showed robust development were spotted onto solid YP medium. **(A)** After 5 days of growth, the surface area of the colony was measured for each strain, along with that of a wild type control. Error bars represent standard error from three biological replicates. **(B)** Representative images of the exploring mutants are shown, along with that of wild type *S. venezuelae* and the Δ*chpH* strain.

the exact opposite: this strain may have a mutation that increases *chpH* expression and leads to enhanced exploration when grown on exploration-conducive medium.

The sequences of both mutants were compared to the wild type reference genome (sequenced at the same time), and point mutations were identified. In total, mutant 1A had 22 missense mutations, and mutant 3C had two missense mutations. Of the 22 mutations in 1A, 18 were intragenic and four were intergenic. Both mutations in 3C were intragenic (Table 4.1). Interestingly, both genes that were mutated in 3C were also mutated in 1A, with each strain containing the same point mutation in the peptide sequence (Sven_7287, Q259K; Sven_7050, P310S). These mutations are the same two that were identified in another strain that similarly had its genome sequenced for an unrelated experiment (see Chapter 6). This seems odd, because the most likely explanation for this might be that they arose in a wild type progenitor, however, it is then inconsistent why they would not be identified from all genomes sequenced, as all strains ultimately came from this same wild type stock. To elucidate which of the mutations were responsible for the observed phenotypes and expression level changes, *S. venezuelae* cosmids that carried wild type copies of the mutated genes of interest were modified to contain an origin of transfer so they could be conjugated into the mutants. For those mutated genes that were not associated with an available cosmid library, these were to be complemented using a PCR-amplified 6 – 8 kb region encompassing both the gene of interest and flanking chromosomal regions, cloned into a conjugatable plasmid (the larger construct will be created because typical complementation requires plasmid integration, but both commonly used plasmid integration sites have been filled in these mutants, and consequently these will need to be integrated at their native

locs; an additional benefit is that these construct could later be modified to enable a ReDirect-based deletion of the gene).

Table 4.1 Identified mutations within the genomes of chemically mutagenized mutants of interest. The genomes of mutants 1A and 3C were sequenced and compared to that of a wild type reference genome. The genomic loci that differ between the mutants and the wild type are listed, along with the predicted function of the protein encoded by the mutated gene. Asterisks indicate genes that are not represented on available cosmids, and will be complemented by cloning a 6 – 8 kb region into a vector.

Mutated gene	DNA mutation	Protein mutation	Proposed function of encoded protein
		Mutant 1A	
Sven_3134	G → A	G72A	Oxidoreductase
Intergenic	G → A	Downstream of *Sven_6498*	Subtilisin protease
Sven_4231	C →T	Q272stop	Cyclohexene-carboxylate:CoA ligase
Sven_4156	C → T	A376V	Possible S/T kinase
Intergenic	C → T	Upstream of *Sven_4743*	Transcriptional regulator
Sven_2422	C→ T	A116V	Transmembrane protein (HPP family)
Sven_5292	C → T	P195S	Signal peptidase
Sven_4829	G → A	G25E	Possible proteinase
Sven_3987	C → T	A224V	Hypothetical protein
Sven_5165	G → A	A437T	Possible phosphodiesterase
Sven_3148	C → T	T71M	Amino acid permease membrane protein
Intergenic	G → T	Upstream of *Sven_4989*	Secreted protein
Sven_3958	C → T	P5S	Phosphoglycerate mutase
**Sven_3213*	G → A	G201R	Membrane protein
Sven_3990	G → A	R4H	Possible endonuclease
Sven_4622	G → A	G226S	Possible S/T kinase
Sven_3152	C → T	P98S	Hypothetical protein
Intergenic	C → T	Upstream of *Sven_1246*	DNA-binding response regulator
Sven_5803	C → T	R119C	Acid-responsive transcriptional regulator
Sven_6712	C → T	P3S	Cellulase
**Sven_7287*	C → A	Q259K	Poly depolymerase
Sven_7050	C → T	P310S	Fe^{3+}-siderophore permease
		Mutant 3C	
**Sven_7287*	C → A	Q259K	Poly depolymerase
Sven_7050	C → T	P310S	Fe^{3+}-siderophore permease

At the time of writing, many of the modified cosmids have been made and cloning to create the other complementation constructs is underway. After all complementations have been completed, it will be important to confirm complementation by performing additional P*chpH-gusA* expression assays to determine which mutations were responsible for the observed effects.

4.3 Discussion

This work has laid the foundation for expanding our understanding of *chp* regulation, allowing for both better characterization of known *chp* regulators, and for identifying new genes that may be important for the activation of *chpH* or *chpE*.

4.3.1 Characterizing known *chp* regulators in more depth

To date, only two proteins have been shown to directly bind the promoter regions of the *chp* genes, σ^{BldN} and MtrA (Bibb *et al*., 2012; Som *et al*., 2017). σ^{BldN} is the better characterized of these two, with its role as a bald regulator in the *Streptomyces* developmental cascade being well established. In liquid-grown cultures, σ^{BldN} was determined to regulate *chp* gene expression by comparing *chp* transcriptional profiles from a Δ*bldN* strain to those of a wild type strain. The abolished transcript levels were coupled with chromatin immunoprecipitation of a tagged σ^{BldN} protein, which revealed σ^{BldN} to directly bind to the promoter regions of the *chp* genes (Bibb *et al*., 2012). It is known that RsbN [itself under control of σ^{BldN} and BldD regulation (Bibb *et al*., 2012; Schumacher *et al*., 2018)], is an anti-σ factor that sequesters σ^{BldN}. RsbN contains an extracellular sensing domain, a transmembrane region, and an intracellular domain that adopts three α-helices crucial for the binding to σ^{BldN}. These helices are conserved across multiple *Streptomyces*. Recent work has determined that RsbN binds and inhibits σ^{BldN} in a manner not consistent with any other ECF anti-σ factors (Schumacher *et al*., 2018). Moreover, the sequence of RsbN is unique compared with other characterized anti-σ factors. Given this lack of similarity, it is not possible to use sequence conservation or structural models to predict the signal that causes σ^{BldN} liberation from RsbN (Schumacher *et al*., 2018). Because chaplins are secreted to the extracellular space

and their involvement in a positive feed-forward loop would make sense biologically, we probed the possibility that they may serve as the signal recognized by RsbN. Our work suggests that chaplin proteins do not function in this capacity, mainly because the expression of *chp* genes was not significantly altered in a three-fold *chp* mutant strain. It is worth noting that this type of approach does not directly assess the liberation of σ^{BldN}, as it only considers *chp* expression level changes. A more-directed experiment may include a bacterial two-hybrid analysis of the extracellular domain of RsbN against the chaplin proteins. Alternatively, RsbN could be incubated with purified chaplins, and then tested to see whether RsbN was still capable of inhibiting σ^{BldN} -directed *in vitro* transcription (Bibb *et al*., 2012). In *S. coelicolor*, the expression of *chpH* and *chpE* are activated at earlier time points than the other *chp* genes, so it is tempting to speculate that the role of this earlier activation is to initiate the positive feedback loop of *chp* expression. This striking temporal difference in *chp* activation was not seen in *S. venezuelae* liquid cultures however (with the *chp* genes all showing expression at 10 h; Bibb *et al*., 2012). It is possible that *chp* expression may differ between liquid-grown and solid-grown colonies, so it is worth noting that this hypothesis has not been definitively ruled out by this data. Nevertheless, future steps to address the reasoning behind this earlier expression could entail repeating the experiments conducted in this work, but including a mutant that is lacking more than three *chp* genes, or examining a mutant that is devoid of *chpH* and/or *chpE* (to determine if their earlier activation is indeed to initiate this feedback loop). However, given the non-compelling nature of our data and lack of appropriate strains at the time, we did not pursue this hypothesis further. Once *chpE* has been deleted in the 4-fold short *chp* mutant, the resulting short *chp*-null strain may be appropriate to use for this experiment.

4.3.2 Identifying novel *chp* regulators

Identifying protein-DNA interactions via isolation by biotin-tagged DNA is a well established method for identifying unknown proteins, and has successfully been used to identify unknown proteins for microbial and human investigations alike (Leblond-Francillard *et al*., 1987; Mojsin *et al*., 2006). Our inability to identify

novel *chp* regulators through this technique may be due to certain limitations inherent in this approach. For example, although data show that *chp* expression commences 10 h after inoculation in liquid medium, additional regulatory proteins, possibly endowing *chp*-specific regulation for discrete roles, are not necessarily expressed at this time point; it is possible that "fine-tuning" regulatory proteins may be expressed earlier or later. This may be the reason why transcript abundance between *chp* genes was not found to be consistent at time points later than 10 h (Bibb *et al*., 2012). This is not uncommon in the regulatory pathways of *Streptomyces*. The sporulation-specific *whi* gene *whiA,* for instance, is constitutively expressed, with its activity being modulated hours after growth begins, only when an additional *whi* gene, *whiB*, is expressed (Bush *et al*., 2013). Additionally, the pull-down technique is stated to work best with high-affinity interactions (Jutras *et al*., 2012), so it is also possible that novel *chp* regulators did not bind strongly enough to the promoter DNA sequences used in these techniques to be present in high enough values to be visualized by silver staining. Additionally, it is also possible that a novel regulator might simply have been present in concentrations too low to be detected by this method.

Mutagenesis coupled with reporter-based screening proved much more successful in identifying potential genes involved, either directly or indirectly, in *chp* regulation. This approach initially led to the identification of eight potential mutant strains of interest. The CodAS toxin delayed growth and development, which is, in one way, ideal as it permitted screening for colonies of interest. Conversely, this phenotype may have limited the potential hits that were identified. Based on the phenotypes of various *chp* mutants (Chapter 3), the developmental delay caused by CodAS would still have demarcated mutants devoid in only *chpH* expression – which develop similar to wild type – from the rest of the colonies that had full *codAS* expression. However, a short *chp* mutant and the mutants missing at least five *chp* genes also suffered developmental delays. It is therefore possible that a colony with a mutation affecting expression levels of all or most *chp* genes would have been identical to those expressing the toxin and therefore not identified in the screens. It is possible that this was the case, as the reporter expression data suggest that the

mutations do not affect *chpH* and *chpE* expression equally, so it seems as though our screen selectively identified mutations that do not affect all *chp* genes. Further, it is also possible that colonies may have been identified due to an increase in *chp* expression (or positive regulation at another stage). Within these cells, toxin expression would be hampering growth and development, however, concurrent elevated levels of a developmental determinant could be driving development forward. It is conceivable that aerial development could occur at wild type levels in these mutants. This provides an explanation for the reporter expression levels seen in mutants 1A, 1C, and 2A, and is why mutant 1A was chosen along with 3C for sequencing.

Complementation of all the mutations of interest is currently being conducted, and it is exciting to consider which mutations may be responsible for the observed phenotypes. A number of transcriptional regulators were identified among these mutants: *Sven_4743*, *Sven_1246*, and *Sven_5803*, of the GntR, LuxR, and unknown family of regulators, respectively, all of which appear to be conserved among sequenced *Streptomyces* species. Analyzing the amino acid sequence of *Sven_5803* using InterPro (https://www.ebi.ac.uk/interpro/) suggests that the encoded protein may be a member of the PaaX family of regulators. Given their DNA binding ability, it is possible that these regulators may also bind to *chp* promoters or to the promoters of genes whose products govern *chp* expression. In wild type *S. venezuelae*, *Sven_4743* is expressed at high levels during all stages of growth (in liquid); *Sven_1246* is expressed to a lesser degree, but is highest during late vegetative growth; and *Sven_5803* is consistently lowly expressed during all stages of growth (Sherwood, unpublished). However, for the first two listed, the identified mutations are likely in the promoters, so it is possible that their expression patterns are altered in the mutant.

Thinking about mutations from an RsbN and σ^{BldN} perspective and considering mutations that may affect the release of σ^{BldN} from RsbN, four genes from our list are predicted to be secreted (Petersen *et al*., 2011): *Sven_4829*, *Sven_4989*, and *Sven_6712*, and *Sven_7287*, which encode a proteinase, a hypothetical protein, a cellulase, and a polydepolymerase respectively. Of these,

Sven_4989 is the most highly expressed in wild type, but still at low levels, with its expression peaking during aerial growth/fragmentation in liquid (Sherwood, unpublished). Given that *Sven_7287* – which encodes a depolymerase that is predicted to be specific for polyhydroxybutyrate – is mutated in both identified mutants (1A and 3C), this may be an excellent candidate gene for further investigation. Investigations into the soil bacterium *Methylobacterium rhodesianum* have shown that they produce polyhydroxybutyrate during stresses such as low carbon, nitrogen, or phosphate sources (Ackermann *et al*., 1995). Similarly, one report found that 80% of soil-isolated *Streptomyces* could produce polyhydroxybutyrate (Ugur & Sahin, 2002). It would be interesting if the cleaved oligomers and monomers from the depolymerase reaction by Sven_7287 were the signal that affected *chp* expression. Given that *Streptomyces* have been predicted to raise aerial hyphae during stress (Hopwood *et al*., 1970), it would be fascinating if this was partially due to stress signals produced by themselves or through cross-talk with other soil microbes in their natural habitat, that then indirectly lead to *chp* upregulation.

The differences in exploration potential observed for mutants 1A and 3C may well be due to altered *chp* expression. Indeed, the exploring 3C colony closely resembled that of an exploring Δ*chpH* colony (Figure 4.6). However, it is also possible that these strains harbour mutations that affect exploration through a chaplin-independent route. For example, ongoing work in our lab has shown that exploring colonies produce an extracellular matrix (Sexton, unpublished). In many extracellular matrices, polysaccharides are a major constituent, and cellulose is one such polysaccharide (Hung *et al*., 2013). It is possible that the loss of gross-architectural features in mutant 1A were due to mutation of its cellulase-encoding *Sven_6712* gene. We briefly wondered if the mutation in *Sven_7050* (which both strains possess) might be affecting exploration given that the predicted function of its encoded protein is in iron acquisition because exploring *Streptomyces* rely on iron for wild type-exploration levels (Jones, unpublished). However, this gene is poorly expressed in exploring colonies (Jones, RNA-seq) so it seems unlikely to impact exploration.

Future work will require an in-depth look into these mutated genes in order to determine which mutations are responsible for the observed phenotypes and how they are impacting protein function. Complementation of the different mutant phenotypes with wild type genes will be a mandatory first step before beginning any directed investigations.

Chapter 5:
"Escapers"

5. 1 Introduction

The mechanisms underlying *Streptomyces* exploration are poorly understood. Initially discovered because of fungal influences on medium composition, exploration was revealed to occur by local depletion of glucose. Upon screening a library of mutated fungi, it was determined that fungal mutants that accumulate acidic compounds (due to mutations in metabolic pathways) were unable to trigger exploration. This lead to the finding that exploration requires a high pH environment. Lastly, by screening randomly mutagenized *S. venezuelae* for mutants unable to explore, it was found that mutations in the *Sven_3713-3716* operon abolished exploratory potential. This operon is predicted to encode a cytochrome *bd* oxidase complex and was thought to contribute to exploration through its roles in alkaline stress response (Jones *et al*., 2017). Since the initial description of *Streptomyces* exploration in 2017, it has additionally been found that exploration is enhanced in low oxygen environments and requires a steady supply of iron, achieved through siderophore upregulation (Jones *et al*., unpublished). While characterizing the exploration of a number of our *chp* mutant strains (Chapter 3) a unexpected – but common – trend was observed. Occasionally, from foci situated at the extreme periphery of the colony, one or more sub-sections would grow outwards at a rate far greater than that of the rest of the colony (Figure 5.1). It is unclear why exploring *Streptomyces* would be prone to producing these hyper-exploring sub-sections, but this no doubt speaks to the complexity of exploration as a whole. Of the many unknown factors regarding exploration, these hyper-exploring sub-sections provide a valuable avenue for better understanding this mode of growth. Indeed, understanding the mechanisms underlying their enhanced exploration will undoubtedly help reveal what factors are important for wild type exploration. Here, we determine that the emergence of these hyper-exploring sub-

sections can likely be explained by both stochastic changes in the medium and underlying genetic factors. We have termed these outgrowths “escapers”.

5.2 Results

5.2.1 Identifying and isolating escapers

When *S. venezuelae* grow as an exploratory colony, the biomass spreads outwards from the central core as a thin, slightly wrinkled sheet of what appear to be vegetative hyphae (Jones *et al.*, 2017). Characteristics of exploring colonies include a more-developed core (occasionally showing aerial growth) and a slightly wrinkled architecture radiating outwards from this core. While analyzing the ability of our *chp* mutants to explore, our efforts were regularly impeded by outgrowths from the exploring colonies that displayed hyper-exploration (for an example, see the 7 o’clock position of “Mutant 1C” in Figure 4.6). These outgrowths often contained a slightly altered architecture relative to the core parental strain they were derived from, but not consistently so. For example, some would show equal or more architecture than their parental colony, while others would show significantly less (Figure 5.1).

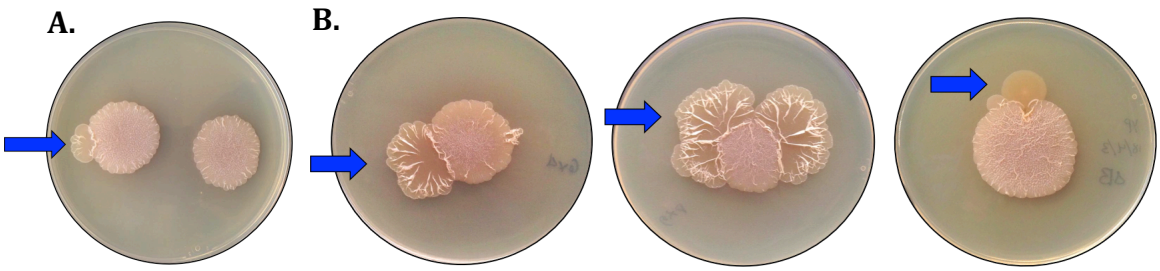


Figure 5.1 “Escaper”: a rapidly growing sub-section of an exploring colony. A commonly identified phenomenon associated with exploring *Streptomyces* were peripheral outgrowths that explored at a more-rapid pace than the rest of the colony. These rapid outgrowths were termed escapers, and are indicated with blue arrows above. **(A)** An escaper (indicated with arrow) growing outwards from a Δ*chpH* mutant with the pIJ82 plasmid integrated in its genome. **(B)** The morphology of escapers was not always consistent. The two centre images show escapers that

have a more wrinkled colony structure than their core parental strains, while the right image shows an escaper with a much flatter appearance than its core parental strain.

These outgrowths were prevalent among many exploring *Streptomyces* strains – not exclusively *chp* mutants – and they were termed "escapers" due to their escaping of the otherwise starkly defined colony border. The occurrence of escapers seemed random, with the only identified trends being that their presence was exacerbated in the 5-fold *chp* mutant strain (Figure 5.2), and in strains harbouring an integrated plasmid in the ΦC31 integration site (specifically, the plasmid pIJ82). This integration site is located around the center of the *S. venezuelae* linear chromosome (*i.e.*, near the 4 Mb mark) within a gene encoding a pirin-like protein. By comparing the assortment of escapers we have encountered, it is clear that they do not all explore to the same degree; while it is true that they are all hyper-explorers, the extent of this varies considerably among different escapers.

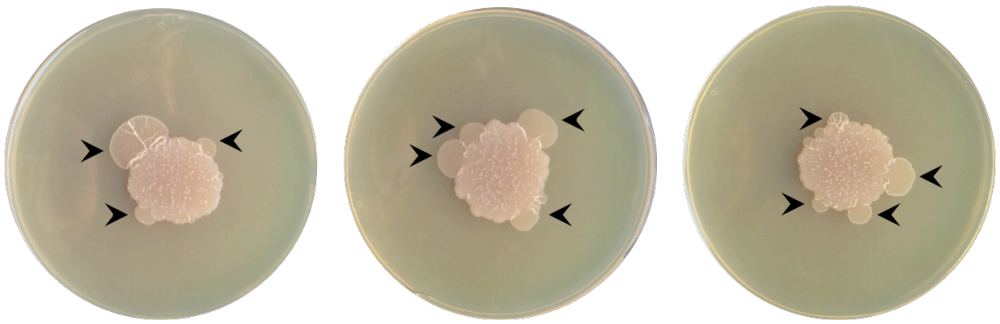


Figure 5.2 The 5-fold *chp* mutant yields escapers at high frequency. Images show the prevalence of escapers from the 5-fold *chp* mutant (Δ*chpBCDFG*). This frequency of escapers is typical for an exploring 5-fold *chp* mutant colony. Arrowheads indicate the escapers.

5.2.2 Identifying mutations in escapers

To address the genetic differences between escapers and their parent colonies, we sought to isolate escapers having a reproducibly enhanced exploration phenotype and to determine the genetic differences between them and wild type *S. venezuelae*. When an escaper appeared, both it and its parent colony were grown in

liquid medium overnight and were spotted onto the centre of an exploration medium plate the next day. This was to ensure that the escaper phenotype was reproducible and not due to stochastic effects. Confirmed escapers were those colonies that, after a defined number of days of growth after re-spotting (typically 6 days), had achieved a larger surface area than their parent colony. The genome of three confirmed escapers (arising from three distinct strains) were isolated and sequenced. The escapers came from a wild type colony, a Δ*chpH* mutant with an integrated pIJ82 plasmid, and a Δ*chpFG* mutant colony. Polymorphisms, insertions and deletions were identified by comparing these sequences to a parental wild type reference genome sequenced at the same time.

Table 5.1 Mutations identified in three independent escapers relative to our wild type strain. Escaper colonies that stemmed from three distinct strains (wild type, Δ*chpH* + pIJ82, and Δ*chpFG*) had their genomes isolated and sequenced. These were compared to a wild type reference genome and mutations were identified. The insertion mutations in *Sven_3001* and *Sven_5329* caused a frameshift in the protein, denoted by "fr".

Mutated gene	DNA mutation	Protein mutation	Proposed function of encoded protein
Escaper from wild type *S. venezuelae*			
Sven_7287	C → A	Q259K	Poly-depolymerase
Sven_7050	C → T	P310S	Fe^{3+} siderophore transporter
Escaper from Δ*chpH* mutant +pIJ82			
Sven_7323	G → C	R392P	Esterase or thioesterase
Sven_1160	G → C	V138L	Integral membrane protein
Escaper from Δ*chpFG* mutant			
Sven_5397	G → A	R116H	Two-component system response regulator
Sven_1160	G → C	V138L	Integral membrane protein
Sven_1239	G → A	D460N	M-pup ligase
Sven_3001	Insertion (G)	L133fr	DNA regulator (TetR family)
Sven_5329	Insertion (G)	T3fr	Homolog of swarming motility protein T42A6.7
Sven_7052	A → G	E193G	Siderophore binding protein
Sven_2965	G → C	A373P	Possible S/T kinase
Sven_2965	C → G	P374A	Possible S/T kinase

5.2.3 Determining which escaper mutations affect exploration

To complement the mutations identified in the escapers, cosmids containing the genes of interest were modified to include an origin of transfer, before being conjugated into the escapers (like in Chapter 4). Genes that were not located within any available cosmids (*Sven_1160*, *Sven_7323*) were complemented by PCR amplifying these genes (along with flanking upstream and downstream sequences), and cloning them into the pSET152 integrating plasmid and conjugating the resulting construct into the escaper strains. These complemented strains were grown on exploration medium and their surface areas were compared to the escaper and the parent strain to see if parental-strain exploration was restored. At the time of writing, neither of the two mutations identified in the Δ*chpH* mutant +pIJ82 strain were complemented (that is, the wild type counterpart of those genes had not yet been introduced into the escaper). However, we were able to introduce the wild type counterpart of *Sven_7050* into the escaper from wild type and the wild type counterpart of seven of the eight mutations into the escaper from Δ*chpFG.* None of these genes were able to restore parental-strain exploration except for *Sven_1160*, which was able to partially restore parental-strain exploration in the Δ*chpFG* escaper (Figure 5.3).

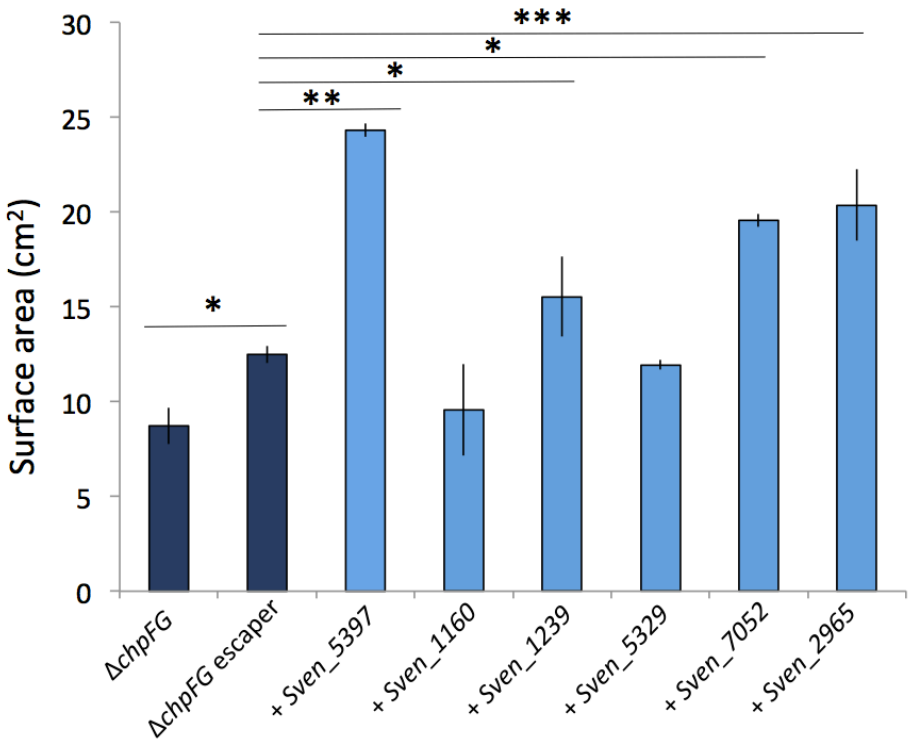


Figure 5.3 *Sven_1160* is involved in the enhanced exploration demonstrated by escapers. Escapers were complemented with wild type versions of the mutated genes identified by genome sequencing (shown in light blue on the graph). The exploration potential of these complemented escapers was compared to the non-complemented escapers and the parent strain that the escaper originated from (both shown in navy blue on the graph). The addition of wild type *Sven_1160* was able to partially restore the escaper to parental-strain exploration. Error bars represent standard error of three biological replicates. A t-test was performed for the escaper compared to the parent strain, and each complemented strain compared to the non-complemented escaper. Values 0.005 and below, ***; values 0.005-0.01, **; values 0.01 – 0.05, *.

5.3 Discussion

The growth and development of *Streptomyces* bacteria has been studied for decades (Hopwood *et al.*, 1970), and the classical life cycle is reasonably well understood. A layer of complexity was added in 2017, when *Streptomyces* exploration was first described by Jones *et al.*, whereby the classical *Streptomyces*

developmental cycle was waived in favour of the rapid outward expansion of what appears to be vegetative hyphae (Jones *et al*., 2017). The work presented here further adds to this complexity by describing the frequent appearance of hyper-exploring colony sectors (escapers).

Because escapers arose commonly, we initially wondered if these colony sub-sections might represent spontaneous mutator strains. The predicted frequency of mutator strains varies significantly, with predicted values as high as 3×10^{-5} for *E. coli* (Boe *et al*., 2000), or as low as 1×10^{-6} for *Salmonella typhimurium* (LeClerc *et al*., 1998). It is conceivable that a mutator strain could result in the mutation of a gene involved in normal exploration, exacerbating the exploratory potential of the mutant. A related possibility is that the escapers were the result of a spontaneous mutation occurring in an exploration-impacting gene. The fact that escapers were commonly seen may suggest that these hypotheses are unlikely; although, in other bacterial models, it has been determined that mutation rate is elevated with increases in pH (Eckert & Kunkels, 1993). Given that exploring *Streptomyces* drastically increase medium pH (to as high as 9.5; Jones *et al*., 2017), it is possible that they have naturally higher mutation rates than would be seen for a classically growing *Streptomyces* colony. We were able to address the mutator hypothesis through our genome sequencing of the three escapers. None of the escapers had mutations in any gene known to be involved in DNA repair or replication fidelity, either suggesting that one or more of the mutated genes' products assist in these tasks but has not yet been identified, or that escapers are not mutator strains. The mutations identified by genome sequencing did, however, reveal a number of candidate genes that might logically affect exploration.

The ability of a *Streptomyces* colony to explore is impacted by many factors, one of which involves iron availability (Jones *et al*., unpublished). Colonies that are iron starved show an increased rate of exploration. It was therefore interesting to note that two of the three escapers had mutations in genes involved in iron acquisition (*Sven_7050* and *Sven_7052*). While complementing the escapers to determine "meaningful" mutations, we initially hypothesized that these genes would be involved in the escaper phenotype, and that addition of the wild type genes

would likely reduce exploration vigor by restoring intracellular iron levels. However, consistent with our assumptions (in Chapter 4) that these genes are expressed too lowly in exploring colonies to be of importance, supplying the respective escaper with wild type copies of either gene was not sufficient to ablate the escaper phenotype.

When conducting complementation experiments, one must always consider the fact that dominant mutations will not be complementable. If our efforts to identify mutations from the escapers due to classical complementation are unsuccessful, it may potentially be ascribed to dominant mutations. However, we were able to successfully complement one gene so far: the addition of wild type *Sven_1160* into its escaper was able to partially restore parental strain-exploration. The amino acid sequence encoded by this gene was examined for conserved protein domains using ExPASy (https://prosite.expasy.org/) and InterPro (https://www.ebi.ac.uk/interpro/), both of which predicted that there are no identifiable domains in this sequence. Future investigations into the connection between the escaper phenotype and a mutated *Sven_1160* gene are required in order to elucidate the function of Sven_1160 and how the V138L mutation is able to increase exploration. The connection between plasmid integration in ΦC31 and an increased production in escapers is also interesting when considering the genetic causes of escapers. Integration into ΦC31 disrupts *Sven_3565*, a gene that encodes a pirin-like protein known to have broad effects on metabolic regulation. In fact, it was recently found that disrupting this pirin-encoding gene in *Streptomyces ambofaciens* caused pleiotropic dysregulation of metabolism. This resulted in impaired fat metabolism, a shift to oxidative metabolism, increased oxidative stress, and a decreased ability to produce certain secondary metabolites (Tala *et al*., 2018). It is very likely that the increased exploration of colonies growing in low oxygen conditions (Jones *et al*., unpublished) is due to physiological effects from oxidative stress. Therefore, the prediction that pirin disruption (*i.e*., by ΦC31 integration) may affect oxidative stress states, is compatible with our findings that plasmid integration at this site similarly increases exploration. This will be an important

factor to keep in mind for future studies on *Streptomyces* exploration and it is recommended that these studies avoid integrating plasmids that target this site.

There are still many unanswered questions regarding *Streptomyces* exploration, and this work on the "escaper" phenomenon provides an important preliminary steps forward in better understanding exploration as a whole. Next steps in this work will be to complete the complementation experiments and identify which additional genes are involved in the escaper phenomenon.

An obvious question arising from this work is why escapers from exploring colonies are so abundant. In other systems, it is not uncommon to detect colony isolates that display increased motility relative to the core colony. In *E. coli* biofilms, it is possible to isolate individual members with increased or decreased motility. This is generally due to differences in flagella regulation or activity (Horne *et al*., 2016; Wang & Wood, 2011). Interestingly, the activation of genes that contribute to flagella and amyloid production are often inversely correlated in *E. coli*, with a 2008 study showing that the production of these are mutually exclusive events (Pesavento *et al*., 2008). The findings that amyloid proteins are inversely correlated with motility are consistent with our findings of increased escapers in the 5-fold *chp* mutant – albeit with the obvious and important distinction between these examples being that exploring *Streptomyces* do not utilize flagella. However, perhaps in the absence of chaplins, exploring cultures are able to glide outwards more rapidly.

Considering the prevalence of escapers from a different perspective, they may be caused by mutations stemming from an underlying issue associated with the increased DNA replication that is assumed to be necessary to support such rapid *Streptomyces* growth (the rate of chromosome duplication have not yet been studied in exploring *Streptomyces*). Alternatively, perhaps the chaplins may make important contributions to the architecture and extracellular biology of exploring colonies. Exploring colonies are encased in a matrix of similar composition to a biofilm (Sexton, unpublished) and it is possible that this matrix aids in structuring and restraining the colony. Changes in its composition may allow subsections of the colony to break free. Speculative support for this includes the fact that escapers were commonly identified (often up to three escapers per exploring colony) from

the 5-fold *chp* mutant (Figure 5.3). If chaplins play a role in promoting matrix integrity, their loss could explain why escapers were rampant in this strain. This may be similar to what has been seen in *Pseudomonas aeruginosa*, whereby certain members of the biofilm coordinate increased motility with decreased matrix production. Although in such cases, the decreased matrix component is a polysaccharide and not an amyloid (Wang *et al*., 2014). Nonetheless, biofilm heterogeneity due to matrix disruptions has also been shown in *Bacillus*, where a perforated matrix results in differential exposure to medium components (Houry *et al*., 2012). Similarly, maybe a less reliable matrix is what causes the heterogeneity seen in exploring *Streptomyces* colonies. An exploring *Streptomyces* colony represents a distinct form of bacterial growth from the aforementioned examples, and so it is likely that the explanation for escaper production will differ from these examples. Also, it still remains to be seen if the matrix produced by exploring *Streptomyces* if functionally similar to biofilm matrix and can be considered in the same context. Therefore, additional work is required to determine if there is a connection between matrix composition and escapers, and if so, what factors link this connection. One caveat to this "matrix and escapers" hypothesis is that it does not address why the morphology of individual escapers differ. Future studies illuminating these causes and better-describing the biology of escapers in general will no doubt have important implications for understanding exploration as a whole, and could prove to be a valuable tool in dissecting out the components that contribute to – and enhance - exploration

Chapter Six:
Summary and Future Directions

6. 1 Summary

The life cycle of *Streptomyces* is a complicated process, and despite having been studied for decades, there are still features and components of this life cycle that remain uncharacterized. Of the many reasons why the development of *Streptomyces* is intriguing, their ability to exploit potentially detrimental amyloid proteins for a functional purpose is possibly one of the most fascinating. Amyloid proteins have such capacity for cellular harm, that their conservation and use in biological systems remains an exciting area of research. As such, there is still significant potential for better understanding the chaplin proteins of *Streptomyces* and, specifically, how the negation of their potential detriment is achieved so that these proteins can fulfill their roles in development. In this work we have studied the chaplin proteins of *S. venezuelae* in order to address the importance of chaplins to rapid growth, development in liquid culture, and exploratory behaviour – circumstances not possible when using other *Streptomyces* species. We have also begun preliminarily work into further understanding how *chp* genes are regulated, and how mutated versions of genes can compensate for the conditional essentiality of *chpE*. By examining the exploration of our *chp* mutants, we were also able to describe the escaper phenomenon of exploration and begin to address the underlying genetic causes of their occurrence.

We have determined that the chaplin proteins affect all stages of the classical life cycle of *S. venezuelae*. In terms of germination, the hydrophobic sheath of chaplin proteins seems to have slight effects on germ tube outgrowth from the spore. Admittedly, these effects do not have drastic consequences on the overall density of a culture achieved in more long-term analyses [for example, although the 5-fold *chp* mutant begins outgrowth approximately 50 min before wild type (Figure 3.3), the wild type strain reaches culture density of equal or greater value at later time points (Figure 3.2)]. However, the effects of chaplins on the next stage of

development (aerial growth), are quite profound. Deleting *chp* genes from *S. venezuelae* to make a 5-fold *chp* mutant and a short *chp* mutant resulted in strains that had delayed aerial growth. As for the final stage of development, sporulation, it appears that chaplins impact this process as well, as the 5-fold *chp* mutant was unable to form mature spores on solid medium and formed only occasional spores in liquid medium. This last finding is significant as it shows that the effects of chaplins extend beyond simply lowering the surface tension. Indeed, this work has shown that chaplins are critical for normal *S. venezuelae* development in all conditions considered.

When we started this work, chaplins were known to be directly regulated only by σ^{BldN}; however, evidence suggested that this could not be the only *chp* regulator that exists in *S. venezuelae* (Bibb *et al*., 2012). Our mutagenesis experiment coupled with toxin reporters driven by *chp* promoters was an important first step towards elucidating the genes whose products may be involved in *chp* regulation. Using this combined strategy, we were ultimately able to identify a number of candidate genes that may be important for direct or indirect *chp* regulation.

Collectively, these studies revealed the chaplin proteins to play a significant role in the classical developmental life cycle of *S. venezuelae*. Additionally, the newly discovered alternative life cycle trajectory of *S. venezuelae* – exploration – was similarly found to rely on chaplins for proper progression. We showed that the short chaplins have considerable effects on exploration, as our short *chp* mutant demonstrated a striking hyper-exploration phenotype. Furthermore, our investigations into exploration led us to the fascinating discovery of escapers, and our work has uncovered that a mutated *Sven_1160* gene is capable of contributing to this escaper phenotype. We have also generated a list of additional mutations that can be prioritized for follow-up investigation to see if they similarly affect the escaper phenomenon.

6. 2 Future Directions

6.2.1 Understanding how chaplins affect the development of *S. venezuelae*

This work has shown that chaplin proteins have broad effects on *S. venezuelae* development – both classical and exploratory. The chaplin proteins are clearly very important for development in ways not yet fully understood, given their contributions to development in liquid environments and exploration conditions (where the amphipathic properties of chaplins should not be required). Short-term future directions of this work will be to create a complete chaplin-null strain (a 7-fold *chp* mutant) and determine the phenotypic effects of a chaplin-free existence. The long-term goals of this work are two-fold. The first is to uncover how chaplins affect the development of *S. venezuelae*. Our work suggests that there are novel mechanisms by which chaplins promote development, and illuminating these mechanisms may help to explain why chaplins are still required for development in an entirely-aqueous environment. The second is to understand the essential nature of *chpE*. We intend to follow up our investigations into the second site suppressor mutations identified in our *chpE* mutants and determine which of these are allowing an otherwise essential gene to be deleted. Particularly, it will be interesting to see if *Sven_1894*, a mutated gene common to both Δ*chpE* mutants, is permitting the loss of *chpE*. This will likely have important ramifications for understanding how these amyloid proteins – whose effects are known to range from lethal to functionally important – are maintained and utilized as beneficial developmental determinants. Addressing these two long-term goals will provide a more comprehensive understanding of this family of important developmental determinants, and enable a better understanding of how these functional amyloid proteins are maintained in a harmless state so as to facilitate *S. venezuelae* development.

6.2.2 Understanding the regulation of *chp* genes

We have successfully identified a number of genes whose products may contribute to the regulation of the *chp* genes. Experimental evidence collected from classically growing *S. venezuelae* indicates that there are likely additional regulatory factors that have remained elusive over the years. Whether these additional regulators will broadly affect all seven *chp* genes (like BldN; Bibb *et al*., 2012), or if

there are regulators that modulate the expression of specific *chp* genes remains to be seen. This latter possibility is seeming more likely, as we identified mutants that differentially expressed *chpH* and *chpE* (Figure 4.5). RNA-seq experiments conducted on exploring *Streptomyces* further suggested an ability to differentially regulate *chp* genes (as the transcript abundance of *chpF* was significantly greater than other *chp* genes; Jones, RNA-seq). Future directions of this work include identifying the gene(s) responsible for the dysregulation of *chpH* in our mutants 1A and 3C. Complementation constructs (*oriT*-containing cosmids) have been made for eight of the 22 mutations identified in mutant 1A, and for one of the two mutations identified in mutant 3C. After these constructs have been introduced into the respective mutants and constructs for the remaining mutations have been finished, *gusA* assays will be conducted on these strains to determine which wild type genes are able to complement and increase *chpH* expression to a wild type-level. If the mutation directly or indirectly affects a regulatory protein, it will then be important to better characterize its regulon. Does the regulator bind directly to chaplin promoters? Is the regulator *chpH* specific or broad-acting? What interplay, if any, is there between this regulator and other *bld* gene products? Electrophoretic mobility shift assays could be effectively used to gauge the binding specificity of a regulator to *chp* promoters, and for a more comprehensive view, chromatin immunoprecipitation using a tagged regulator would nicely delineate the regulon of any identified regulators. These will be critical questions to answer if we are to accurately map the network responsible for regulating the *chp* genes.

6.2.3 Characterizing and understanding escapers

Streptomyces exploration is an exciting and novel developmental option that has just recently begun to be characterized. As such, there are many unexplored features of this life cycle that will need to be better understood before we can fully appreciate how exploration occurs. One of these features is the commonly seen phenomenon of escapers. We have identified a number of escapers, and have determined which genes within them are mutated. This has allowed us to identify a candidate gene (*Sven_1160*) that, when mutated, causes exacerbated exploration.

Continuing to investigate mutations within escapers will reveal the genetic basis for the escaper phenomenon and in turn provide insight into the factors that are important for promoting exploration. It is conceivable that escapers emerge as a result of genetic factors or stochastic factors related to exploration (some escapers were not "confirmed" after re-spotting overnight cultures of escapers and their parental strains), or a combination of both. The relationship between colony morphology and the presence of escapers should also be considered (perhaps in the context of chaplins), as matrix production and/or the differential architectures of various exploring strains may influence the emergence of escapers. Hopefully, by understanding how escapers arise and explore, we will be able to make strides in our understanding of exploration as a whole.

6.3 Conclusion

Overall, this research has enhanced our understanding of chaplin proteins, and has illuminated how they affect *Streptomyces* development in conditions that have never before been analyzed. Functional amyloid systems are still in the process of being fully understood and appreciated, and this knowledge about a family of functional amyloids that collectively make broad contributions to development is therefore a meaningful offering. There are still many unanswered questions about chaplin proteins, but this work has shown that their influence on *Streptomyces* development is considerable and multifaceted. *Streptomyces* bacteria have captivated researchers for decades because of their fascinating and complex developmental cycles; it seems fitting that their use of functional amyloid proteins for these cycles is similarly intriguing and intricate.

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