Bacterial filamentation as a survival strategy: a goldmine for the discovery of new cell division regulators

Samuel J. Burns

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Certificate of Original Authorship

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

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Samuel J. Burns, August 2016

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Abbreviations

aa	amino acid
AG	arabinogalactan
AGRF	Australian Genome Research Facility
ATc	anhydrotetracycline
ATP	Adenosine 5’ triphosphate
B.	*Bacillus*
BCG	Bacillus Calmette-Guérin
BLAST	basic local alignment search tool
bp	base pair(s)
BP	band pass
BSA	bovine serum albumin
°C	degrees Celsius
chrDNA	chromosomal DNA
cm	centimeters
CCD	charged coupled device
DAPI	4'6-diamidino-2-phenylindole
dATP	deoxyadenosine 5’-triphosphate
dCTP	deoxycytidine 5’-triphosphate
dGTP	deoxyguanosine 5’-triphosphate
DNA	deoxyribonucleic acid

dTTP	deoxythymidine 5'-triphosphate
E.	*Escherichia*
EM	electron microscopy
EsC	environmental sub-clone
et al.	and others
FSC	forward scatter
fts	filamentation temperature sensitive
g	centrifugal force
g	gram(s)
gDNA	genomic DNA
GFP	green fluorescent protein
h	hour(s)
kb	kilo base pair(s) (1000 bp)
kD	kilo Dalton(s)
kg	kilogram
L	litre(s)
LB	Luria Bertani
LIV	leucine, isoleucine and valine
LP	long pass
M.	*Mycobacterium*
m	milli- (10^{-3})
M	moles per litre

MA	mycolic acid
MCS	multi-cloning site
MDR	multi-drug resistant
min	minute(s)
MQW	Milli-Q purified water
Mtb	*Mycobacterium tuberculosis*
n	nano- (10^{-9})
NA	numerical aperture
N/A	not applicable
NaOAc	sodium acetate
NCBI	National Center for Biotechnology Information
NOC	nucleoid occlusion
OD	optical density
OEC	original environmental clone
ORF	open reading frame
P	probability
PBP	penicillin binding protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PG	peptidoglycan
pH	power of Hydrogen
psi	pounds per square inch

RNA	ribonucleic acid
ROS	reactive oxygen species
ROW	reverse osmosis purified water
rpm	revolutions per minute
s	second(s)
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
sp.	species
SSC	side scatter – height (H), width (W)
TAE	tris acetate EDTA
TB	tuberculosis
TBE	tris borate EDTA
TDR	totally-drug resistant
TE	tris-EDTA buffer
tet	tetracycline
Tris	tris(hydroxymethyl)methylamine
U	units (of enzyme)
UV	ultraviolet
V	volt(s)
v/v	volume per volume
W	watt

WE	window extension
WHO	World Health Organisation
WT	wild-type
w/v	weight per volume
XDR	Extremely-drug resistant
µ	micro- (10^{-6})
µF	micro Farad

Abstract

Mycobacterium tuberculosis the causative organism of tuberculosis has been plaguing humanity for centuries. The number of effective antibiotics is dwindling due to the rise of multi-drug resistance within the species and new drugs need to be developed that target essential components of the bacterial life cycle. Bacterial cell division is an essential and highly conserved process across bacteria and new drugs that target this process could have broad-spectrum implications.

Bacilli can survive changes in their environment by forming filamentous cells, where cell division is inhibited while growth and DNA replication continue, giving rise to very long cells (up to 40 μm). Filamentation has been observed in both non-pathogenic and pathogenic bacteria, including *Escherichia coli* and *Mycobacterium tuberculosis* where it has been proposed to be required for replication and persistence within the human host. The process by which filamentation occurs in bacteria is not well understood. However, understanding filamentation can aid in identifying opportunities for new therapeutics and in addition, explore cell division in Mycobacteria as they are missing many of the key cell division genes present in model organisms like *Escherichia coli* and *Bacillus subtilis*.

The overall aim of this work was to use flow cytometry-based cell sorting to identify and characterize novel proteins that regulate cell division in Mycobacteria and allow persistence in mycobacterial disease. This was done by screening expression libraries of *Mycobacterium bovis* BCG genomic DNA (gDNA) hosted in *E. coli* and later *Mycobacterium smegmatis,* to identify clones expressing cell division proteins and regulatory genes via a filamentous phenotype.

The method for flow cytometry screening had to first be verified through the completion of a screen of a library of environmental DNA collected from the marine algae *Ulva australis*. Large environmental DNA inserts were sub-cloned and re-screened using flow cytometry-based cell sorting to identify genes causing filamentation when expressed. One reproducibly filamentous clone contained the Periplasmic Binding Protein Type-1 Superfamily conserved domain and we found that the overexpression of this gene caused a filamentous phenotype, which in turn showed that a single gene causing a filamentous phenotype could be identified with the flow cytometry based cell sorting method.

A library of *M. bovis* BCG gDNA was constructed and hosted in *E. coli*. This library was screened using flow cytometry-based cell sorting but no filamentous clones were found. The host species was then changed to *M. smegmatis* for better expression of heterologous genes and a modified expression vector utilizing the TET-ON/OFF inducible expression system was shown to work for the expression of cloned genes. Unfortunately after repeated attempts, a library of *M. bovis* BCG gDNA was unable to be constructed and screened for mycobacterial cell division genes and regulators.

Bacterial filamentation and cell division are important areas of investigation for clinically relevant bacteria. The information that can be gleaned from these investigations may lead to the next generation of antimicrobials.

Chapter 1

General Introduction

1. General Introduction

There are records of Tuberculosis (TB) since antiquity, with the ancient Greek, Egyptian and Arabian scholars having seen and noted what they saw in patients as the same as what can be seen and noted today; persistent cough, blood stained mucus and fever. It was not until Koch discovered the tubercule bacillus in 1882 that the cause of TB, the bacterium *Mycobacterium tuberculosis*, was elucidated [1].

Even with the development of antibiotics in the 1930's and 1940's TB remained a common disease. In 1993, the incidence of tuberculosis (TB) was declared as being in a state of emergency by the World Health Organisation [2] and it was responsible for 1.4 million deaths in 2010 [3] and 1.5 million deaths in 2014 [4]. Today the incidence of HIV and the resultant suppression of the immune system in these patients, coupled with the outbreak of drug-resistant strains of TB presents a continuing challenge as working treatments for tuberculosis are harder to achieve [1].

Annually TB affects approximately 8.8 million people [3] and unlike other disease-causing organisms, *M. tuberculosis* has both active and latent phases within its human host. People with latent TB are carriers of the organism *M. tuberculosis* but it is in a latent state and causes little to no harm to the patient. It is only when the organism changes to an active state that the patient becomes symptomatic (i.e. blood stained sputum, a persistent cough, fever, etc.) [5]. Approximately one-third of the world's population is latently infected with *M. tuberculosis* [6] and it is this latency which allows the organism to persist for great lengths of time within a host.

With the rise of multi-drug resistant strains of *M. tuberculosis* [7], and more recently, totally-drug resistant strains [8], we can no longer rely on current antibiotics; and so new antibacterial targets need to be discovered and drugs developed.

Cell division is a valuable and viable anti-bacterial target; the targeting of FtsZ, the primary instigator of cell division is one example [9]. Yet little is known about cell division in *Mycobacteria spp.*, as it has not been studied as intensively as it has in model bacteria such as *Escherichia coli* and *Bacillus subtilis*. This oversight is being rectified with a surge in the number of papers being written and released on the subject of cell division and it's regulators in *Mycobacterium spp.*. Hopefully while the cell division process is being studied more intently in *Mycobacterium spp.* new drugs can be developed that target this essential process accurately and effectively to, in time, cure the disease [9].

1.1. The Tuberculosis disease and the problem

Figure 1.1 - Map of countries (in red) that had reported at least one case of XDR-TB by the end of 2011 (WHO 2012)

At the end of 2010, 69 countries had reported at least one case of extensively drug-resistant TB (XDR-TB) [10]; this had jumped to 77 countries by the end of 2011 [11]. Australia was one of those countries.

Figure 1.1 shows that, TB and XDR-TB are not restricted to just the developing world. Much of the developed world is also affected, including the US and much of Europe [11]. Historically TB was more of a problem for developing

countries due to the cost of treatment and the length of time patients needed to be treated along with the concomitant prevalence of HIV in these areas.

Mycobacterium tuberculosis is transmitted person-to-person; that is, an individual with active TB can transfer the bacterium to a new host via aerosols created by the lungs e.g. coughing and sneezing. The aerosolized droplets are inhaled into the large and small airways where an infection can be established [12]. They are then phagocytised by the host's macrophages and dendritic cells, these host immune cells then migrate to lymph nodes and initiate a T-cell response. Lymphocytes and macrophages then move to the site of infection and form a granuloma [12], a hallmark for TB constructed of mostly inflammatory cells and believed to be a physical barrier between the bacterium and the host. When the host's immune system is compromised, the balance between *M. tuberculosis* bacterial load and the body's ability to control the infection is tilted in the bacterium's favor, leading to the active state of the disease [13]. As yet the mechanism or signal that switches a *M. tuberculosis* infection from latent to active is unknown but there is a correlation between patients who are immunocompromised via HIV or some other infection and the active state of the disease [14]. Also unknown is how the tuberculosis cells persist within the host macrophage.

Antibiotic-susceptible strains of TB can be cured over the standard six-month period with a combination of first-line antibiotics like isoniazid and rifampicin. Multi drug-resistant strains of *M. tuberculosis* (MDR-TB) have emerged that are resistant to first-line antibiotics and can be developed over the course of a "normal" TB infection; that is, an antibiotic susceptible TB infection can develop to

a MDR-TB infection [10]. There was an estimated 650,000 cases of MDR-TB in 2010 [3].

As well as MDR-TB, extensively drug-resistant strains of *M. tuberculosis* (XDR-TB) have also developed. It is estimated that there are 25000 new XDR-TB cases each year and this form of TB is resistant to all first-line anti-TB drugs as well as fluoroquinolone and any one of the other second-line antibiotics. XDR-TB also requires a longer treatment plan than the standard six months for the other forms of the disease [10].

Alarmingly, totally drug-resistant strains of *M. tuberculosis* (TDR-TB) have been reported [15]. TDR-TB is defined as being resistant to all first and second-line drugs used in the treatment of TB [8]. This is extremely concerning worldwide as currently there are no drugs or treatment regimens available for TDR-TB.

1.2. Cell division as an antimicrobial target

The identification of novel regulators of bacterial cell division will not only help to better understand the organism but also help to develop better ways to combat the disease. Investigating and extending the understanding of these crucial processes of cell division and its control mechanisms will identify novel targets for antibiotic discovery [9].

Historically, antibiotics target four different but essential processes for bacterial survival; DNA biosynthesis (through inhibiting thymidine 5'-monophosphate), cell-wall synthesis, DNA replication and protein biosynthesis [16]. Cell division, the proteins of the divisome and their regulators are well

characterised in model bacteria like *E. coli* and *B. subtilis*; this being the case there are efforts underway to exploit this knowledge to better develop drugs that target these essential proteins and the process they are involved with i.e. cell division [17]. Drugs in development like trisubstituted benzimidazoles [18].

With the emergence of TDR-TB and the prevalence of multi-drug resistant strains, bacterial cell division which is an essential process needed for the propagation of a bacterial infection has become a target for the development of a new generation of antimicrobials. This need for a new generation of drugs has recently seen cell division and FtsZ (a tubulin homologue and a primary component of the divisome) as a target for antibiotics [19]. With a blockage of cell division, an infection cannot be established and the infecting organism dies [17]. While FtsZ has become a viable target, cell division is a complex process involving many proteins and regulatory mechanisms and these have yet to be investigated as targets. FtsZ is being targeted, as it is essential to the division process and using this particular protein as a target can have far reaching effects as it is very highly conserved across all bacteria even *Mycobacterium spp.*.

1.3. Mycobacterium tuberculosis cell biology

Generally *Mycobacterium tuberculosis* is classified as Gram-positive bacilli but due to the high lipid content of their cell wall the bacilli only stain very weakly with the Gram's stain [20]. *Mycobacterium tuberculosis* has an unusually thick cell wall made up of a number of components which makes it impermeable to a number of compounds causing the organism to be inherently resistant to some antimicrobials as well as conferring to the bacterium a slight resistance to phagocytosis and in turn the host immune system [20, 21].

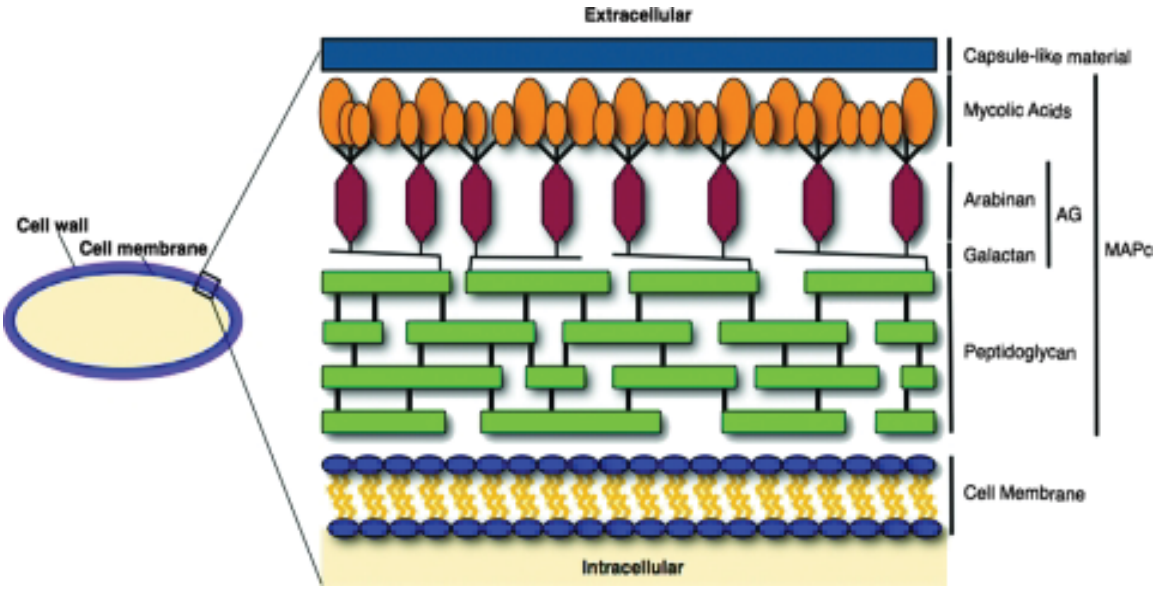

Figure 1.2 - Diagram of the basic components of the *Mycobacterium spp.* cell wall [6]

The cell wall of *Mycobacteria spp.* is composed of an inner layer and an outer which is then surrounded by a plasma membrane [22]. Figure 1.2 shows a basic schematic of the cell wall of a *Mycobacterium spp.* organism; from the extracellular to the intracellular there is the mycolic acid layer attached to arabanogalactin which is in turn attached to the peptidoglycan; this all forms a

complex called the MA-AG-PG complex [22]. The complex and thick cell wall allows for an advantage over stressful conditions such as desiccation and osmotic shock [6].

It is widely held that Mycobacteria do not sporulate as a means of replication but it has been shown in a study that *M. marinum* as well as *M. bovis* can form spores, this has been attributed to adaptations environmental stressors [23]. Sporulation in *Mycobacterium spp.* remains a contentious subject with few experts on the species agreeing one way or another as to whether Mycobacteria sporulate or not [24]. As sporulation leads to the persistence of a species, if *Mycobacterium spp.* does sporulate, this may influence and change the types of treatments we use to treat the tuberculosis disease. It is worth noting that the 16S rRNA gene sequences split *Mycobacterium spp.* into two groups taxonomically, and these two groups have different growth rates [25]. The fast growing species show visible growth within seven days, and are found in aquatic and soil environments (e.g. *Mycobacterium aurum*), while the slow growing species take longer than seven days to show visible growth, and are associated with living hosts (e.g. *Mycobacterium avium*, *Mycobacterium bovis* and *M. tuberculosis*). The faster species were thought to be the more primitive [25] possibly indicating the slower growing species adapted to life within the stressful environment of a host organism. The slower growing species were shown to also have an association with the induction of the dormancy survival regulon, this is associated with a similar response that is seen when *M. tuberculosis* cells are growing within macrophages [6]. As yet there is no concrete evidence to show which genes, if any are used by the organism to regulate the growth speed in the pathogenic members of the Mycobacterial species.

Hopefully through studying and understanding the processes surrounding cell division in Mycobacteria some of the questions can be answered.

1.4. Cell division in bacteria

Much of what we know about bacterial cell division is derived from studies of two model bacteria *Escherichia coli* and *Bacillus subtilis*. Many of the basic processes are highly conserved across taxa, and this knowledge serves as staring point from which to study cell division events in other bacteria.

Tables 1.1 and 1.2 show the main proteins involved in cell division with a comparison between *Escherichia coli*, *Bacillus subtilis* and *Mycobacterium tuberculosis*.

In the most basic sense, bacterial cell division is the splitting of a parent cell into two daughter cells. The division process consists of the separation of the replicated chromosomes into the two poles of the cell, the assembly of the divisome at the division site and then constriction and separation into two identical daughter cells. The divisome consists of the FtsZ protein, which polymerises into a ring structure and recruits downstream proteins to the division site and forms the divisome [26]. The divisome constricts, synthesises peptidoglycan at the new septum and dissolves the residual peptidoglycan joining the two daughter cells, eventually splitting the cell in two [27].

The Z-ring is formed at mid-cell by units of the protein FtsZ. FtsZ is a self-activating GTPase and an analogue of the tubulin protein found in eukaryotes; It is the first protein recruited to the division site [6, 28]; it forms a ring around the middle of the cell, the Z-ring. As well as FtsZ, other proteins are recruited to the Z-

ring. In *E. coli* FtsA and ZipA were found to be needed for proper Z ring assembly, as well as the FtsK protein that aids in the stability of the Z-ring and also helps to segregate the replicated chromosomes near the end of their cycle [29]. Other proteins that comprise the divisome include, FtsB, FtsL and FtsQ, which are thought to be involved in bridging the periplasmic components and the cytoplasmic components of the divisome [30], FtsI and FtsW which are considered to be needed in the synthesis of *de novo* peptidoglycan and lastly FtsN, a protein similar to amidase which binds to peptidoglycan [6]. The amidase and endopeptidase enzymes are recruited late in the piece to hydrolyse the septal peptidoglycan and allow for the separation of the two new daughter cells [6].

Cell division is a critical process, and needs to be tightly regulated to ensure that the divisome is assembled correctly at the division site, that there is a complete replication of the chromosomal DNA for each daughter cell and that both of these events occur at the correct time with regards to each other. There are several known control mechanisms to ensure cell division occurs at mid-cell, for example the Min and the Nucleoid Occlusion (NOC) systems (in *E. coli* and *B. subtilis*; these systems are not in *Mycobacterium spp.*), which inhibit FtsZ polymerisation at the poles and over any nucleoid respectively [23]. This inhibition ensures that FtsZ rings can only form at DNA free regions of the cell, which are not at the pole, hence mid-cell. However, a recent paper showed that a double mutant strain of *B. subtilis* with both the Min and NOC systems removed was still able to position the Z-ring exactly at mid-cell [31]; this shows that there must be another mechanism not influenced by Min or NOC that specifies the mid-cell position for the bacterium. The divisome assists in the final separation of the replicated DNA (via FtsK which is associated to FtsZ in the divisome) [29],

synthesizes peptidoglycan, hydrolyses the middle peptidoglycan layer between the two new daughter cells and separates them [6].

Table 1.1 shows the divisome known components of *E. coli*, *B. subtilis* and *M. tuberculosis* as well as their primary functions if known. Table 1.2 shows the negative regulators for the assembly of the divisome and their primary function. Blacked out cells indicate no annotated homologue or other known protein with functional similarity.

Table 1.1 - Divisome components in *B. subtilis*, *E. coli* and *M. tuberculosis* [6, 32]

E. coli	*B. subtilis*	*M. tuberculosis*	Function
FtsZ	FtsZ	FtsZ	Primary of the Z-ring (tubulin homologue).
FtsA	FtsA		Aids in the organisation of FtsZ units and is needed for the recruitment of downstream components, a principal membrane tether.
ZipA			Membrane associated, positive regulator of FtsZ.
FtsK	SpoIIIE	FtsK	Aids in DNA partitioning
FtsQ	DivIB	FtsQ	Bridges the cytoplasmic with the periplasmic and forms a complex with FtsB and FtsL
FtsB	DivIC		Bridges the cytoplasmic with the periplasmic and forms a complex with FtsL and FtsQ
FtsL	FtsL		Bridges the cytoplasmic with the periplasmic and forms a complex with FtsB and FtsQ
FtsW	SpoVE	FtsW	Multitransmembrane protein, transporter of peptidoglycan precursors, in *M. tuberculosis* attaches Z-ring to cell membrane

FtsI	PBP2B	FtsI	Synthesises septal peptidoglycan
FtsN			Has a weak homology to amidase but otherwise the function is unknown
AmiC	Unknown	AmiC	Hydrolyses septal peptidoglycan to separate daughter cells
EnvC	Unknown	Unknown	Hydrolyses septal peptidoglycan to separate daughter cells
FtsX	Unknown	FtsX	Unknown function but within the ABC transporter family
FtsE	FtsE	FtsE	Unknown function but within the ABC transporter family

Table 1.2 - Negative regulators of FtsZ polymerization [6, 32]

E. coli	*B. subtilis*	*M. tuberculosis*	Action
	EzrA		Negative regulator of FtsZ assembly
MinC	MinC		Negative regulator at the poles of the cell, switches between poles with MinD
MinD	MinD		Moves between the poles with MinC
MinE	DivIVA	DivIVA Has sequence, not functional homology	Oscillates the MinCD complex between the poles of the cell, stimulates the release of MinD from the cell membrane (MinCDE does not oscillate in *B. subtilis*)
SulA	YneA	ChiZ	Induced by the SOS response and is a negative regulator of FtsZ assembly
SepF	SepF	Unknown	Role overlaps with FtsA in the assembly of the Z-ring

SlmA	NOC	Ssd (rv3660c)	Prevents the formation of a septum over a nucleoid
		CrgA	Negative regulator of FtsZ assembly

1.5. Cell growth and division in Mycobacteria

The *Mycobacterium spp.* divisome and its regulators are not as well characterised as *E. coli* and *B. subtilis*. While some homologues of integral proteins exist (see Tables 1.1 and 2.1 above), many do not and there are still a great many questions to be answered as to how these organisms regulate and carry out successful cell division. Currently there are indications that the divisome of *M. tuberculosis* has either fewer proteins involved than model bacteria, or currently unknown proteins that are non-orthologus [9].

1.5.1. Modes of growth

Mycobacteria are very different to the model bacteria, *B. subtilis* and *E. coli* in the way that they grow. Firstly Mycobacteria are slow growers; *M. tuberculosis* has a doubling time of 18 hours [33] and *M. smegmatis*, which is considered relatively fast when compared to the rest of the species has a generation time of 2-3 hours [34]. As described above Mycobacteria also have a thicker cell wall and as is explained below, Mycobacteria grow from the poles of the cell not along their length.

E. coli and *B. subtilis* are known to have two modes of growth; at the division site i.e. the synthesis of new peptidoglycan between two dividing daughter cells and lateral growth, which is along the length of the cell. However, Mycobacteria have been shown to grow from the tips or poles of the cell only [35].

This was shown using vancomycin to stain the D-ala-D-ala residues that are exposed when peptidoglycan is synthesised. Staining was mainly restricted to the poles and septa of the cell where the nascent peptidoglycan was being created and thus the active growth areas of a cell [35]. These investigators also noticed distinctive V shaped cells, which they attributed to the uneven rupture of the multi-layered cell envelope of the cell giving way to the "snapping model" of separation like the snapping of a wooden board and not a lateral separation. A more recent study used electron microscopy to study the process of septation in Mycobacteria and found the outer envelope began to constrict after a distinct septal partition had been formed between the two daughter cells. This is different to the model organisms and a probable reason for this lies in the organisms doubling time. An advantage to this method of division is an increased chance of survival in adverse conditions due to the persistence of the outer layer till the final stage of division [33].

Polar growth has been observed in other bacteria, such as *Agrobacterium tumefaciens* and *Sinorhizobium meliloti* [36], another recent study noticed a peculiarity of polar growth in Mycobacteria [37]. It has been shown that within a clonal population of *M. smegmatis* there is heterogeneity in cell length and elongation rate. This discrepancy was attributed to Mycobacterial cells growing at the cell poles and not along the cell length as is the case with *E. coli* and *B. subtilis* [37]. It was shown that the heterogeneity of growth states could account for different susceptibilities to different classes of drugs, for example, what was found was that drugs that inhibit cell-wall synthesis worked better against the faster growing population of cells and drugs that target RNA polymerase worked better against the slower sub-population of cells [37]. This is significant because it

confers an advantage to a sub-population of the infectious organism depending on what drugs are prescribed, and this advantage could lead to a persistence of infection. This heterogeneity is not found in model organisms and is relevant because drugs targeted to cell division and its regulators neither inhibit cell-wall synthesis nor target RNA polymerase but cell division itself which is needed for all the sub-populations of a bacterial colony.

At a fundamental level, many of the growth systems in *Mycobacterium spp.* are quite different from those of *E. coli* and *B. subtilis*; this could be due to the different environments that each organism requires and or divergent evolution. Regardless of the reason, knowledge of the differences is useful as it can be used to develop narrow spectrum therapeutic agents against Mycobacteria, which do not disturb the body's normal bacteria flora.

1.5.2. The Mycobacterial divisome

Many of the divisome components that are needed for successful cell division in model bacteria have no known homologues in *Mycobacterium spp.*; this can be seen in Table 1 where the known division proteins are compared to those of the model organism's *B. subtilis* and *E. coli*.

As is the case in the model bacteria *E. coli* and *B. subtilis*, as well as nearly all other bacteria, FtsZ is the primary protein for cell division in *Mycobacterium spp.* Similarly as occurs in model bacteria, FtsZ is essential in *Mycobacterium spp.* [9] and polymerises to form a Z-ring at mid-cell [9]. Mycobacteria FtsZ has been shown to interact with other proteins of the divisome including FtsW [38] and through FtsW, FtsI [39]. There are some significant differences between Mycobacteria FtsZ and that of model organisms. For example, In *M. smegmatis ftsZ*

is differentially expressed by four transcripts, as opposed to being constitutively expressed in *E. coli* and *B. subtilis*; being expressed through multiple transcripts has been shown to give the organism an advantage of growth in nutrient deprived and/or hypoxic conditions by being able to produce more FtsZ through its multiple transcripts [40]. As opposed to the model organisms, there are no homologues for FtsA, a protein that anchors the Z-ring to the cell membrane; this is thought to be accomplished by FtsW's interaction with FtsZ, ZipA, FtsB, FtsL and FtsN within the Mycobacterial genome.

During cell division, divisome components and other proteins involved in the process that synthesise peptidoglycan (PG) are of great importance. PG synthesis is important for creating the septa which divides the two daughter cells into two separate compartments. CrgA is a protein that is important in Mycobacterium spp., and is conserved across Actinobacteria but is not found in the model organisms. It is a component of the divisome and is used in peptidoglycan synthesis and the regulation of cell shape [41]. CrgA is recruited late to the division site and helps coordinate the polymerisation of the Z-ring with the proteins involved in cell-wall synthesis and is thought to concentrate peptidoglycan synthesis machinery at the invaginating septa [41].

As PG synthesisers are important, so too are PG hydrolases; proteins involved in the breakdown of PG. Without the ability to breakdown septal PG between two segregated daughter cells, the daughter cells would be unable to separate and in the case of pathogens such as *M. tuberculosis* infect a new host. AmiC and EnvC in *E. coli* are examples of septal hydrolases though EnvC is not present in *Mycobacterium spp.* but *M. tuberculosis* does contain the protein RipA.

RipA interacts with RpfB and this complex has been shown to localise at the division septa, the area at mid-cell where a partition (septum) is being formed between two replicated chromosomes [42] where it is essential for the final stages of bacterial cell division for the separation of two normal daughter cells [43]. Interestingly it was found that only RipA was essential for normal division while *rpfB* could be deleted with no change to vegetative growth. While it is not known yet how this process progresses, it shows an attractive target for anti-Mycobacterial molecules as both RipA an essential protein and RpfB is involved. RpfB stands for resuscitation-promoting factor B; this class of proteins are so named for their role in resuscitating/reactivating growth and division processes in dormant, non-replicating bacteria [43]. Their importance in reactivating M. tuberculosis within a mouse model has been demonstrated [44].

1.5.3. Regulators of cell division

Regulation of the division machinery allows for the correct placement of the septa at midcell, proper segregation of the replicated chromosomes and survival in stressful conditions; regulation also allows for the lengthy if not indefinite latency period attached to *Mycobacterium tuberculosis*.

DNA damage is an example of where negative regulation is important. If DNA is damaged but the chromosome still replicates then the two daughter cells will carry the damaged section of DNA. In the event of DNA damage, *E. coli* transits into the SOS response which induces proteins involved in DNA repair [45]. The protein SulA is also induced; this is a negative regulator of FtsZ polymerisation, which effectively prevents the cell from dividing. When the damage to the DNA is repaired, the SOS response is shut off, SulA is repressed and division can once

again occur [46]. ChiZ is a protein found in the genome of *Mycobacterium spp.* and like SulA in *E. coli* and YneA in *B. subtilis*, was seen to be up regulated when *M. tuberculosis* cells were subjected to DNA damage, ChiZ was then found to inhibit cell division [47]. Although not homologous in protein sequence, ChiZ is similar to SulA in the model organism *E. coli*, in that LexA, a protein integral in the SOS response in *E. coli*, represses its expression. But unlike SulA, ChiZ does not inhibit polymerisation of FtsZ into a Z-ring. Instead ChiZ interacts with FtsI and FtsQ and seems to affect Z-ring stability [48]. While ChiZ is SulA like when up regulated [43], it has other functions at normal levels of regulation [49].

The MinCDE (DivIVA with MinJ acts for MinE in *B. subtilis* [50]) complex is used in *E. coli* and *B. subtilis* to position the Z-ring at mid-cell by creating a concentration gradient highest at the cell poles where FtsZ cannot polymerise and recruit downstream proteins to form the division machinery. ChiZ has also been shown to work with FtsI in *Mycobacterium spp.* to coordinate and regulate cell-wall synthesis and has been shown, due to its hydrolase activity, to constrain the Z-ring at midcell as *M. tuberculosis* cells that have ChiZ deleted have cell division defects during growth within host macrophages [49].

The gene rv3660c in the genome of *M. tuberculosis* has homology to the septum site determination protein (Ssd) [51]. Increased expression of Ssd encoded by rv3660c leads to filamentous cells without septa (no division) and depletion of the protein leads to the formation of minicells (too much division) indicating a negative regulator of FtsZ polymerisation [51]. While the authors did not show any evidence of actual septum site determination (i.e. the positioning of the division site); an interesting thing to note about this protein is that when highly expressed

it up regulated proteins related to dormancy and as such this protein could be one of the regulators involved in the lengthy latency stage of tuberculosis.

While negative regulation of cell division is important in replicating organisms, so too is positive regulation. FtsW, which has been described as a potential functional homologue to ZapA/ZipA in Mycobacteria, is one of the few divisome proteins common to Mycobacteria and the model organisms. FtsW acts to stabilise the Z-ring at cell division by taking over the role of FtsA as in the model organisms and as such it is both a component of the divisome and a positive regulator of cell division in model organisms [52].

Mycobacterial DivIVA, a protein that has orthology to *B. subtilis* DivIVA and is phosphorylated by PknA [53, 54] has been shown to be a regulator of cell shape and cell growth in *Mycobacterium spp.* and determines the growth site and directs the sub-polar addition of new cell wall material [55]. DivIVA is an essential protein in *M. tuberculosis* and while it does not have the same specific function in Mycobacteria as it does in *B. subtilis* (it does not act as a regulator of divisome positioning), when depleted, the organism suffers from severe morphological changes such as one end of the cell becoming round rather that rod shaped [56]. PknA has also been shown to phosphorylate FtsZ which shows a link in Mycobacteria between the controls for cell division and those for cell growth [54, 56]; this makes PknA an attractive target for drug development [57]. Further, *M. smegmatis* DivIVA has been shown to interact with ParA, a protein that coordinates cell growth, division and mediates bacterial chromosome segregation along with ParB [58, 59]. A ParA knockout has been shown to cause problems with growth, septum positioning and chromosome segregation (overexpression of ParA had the

same results) [58]. While Mycobacterial DivIVA does not have the same regulatory role as *B. subtilis* DivIVA in cell division, it is still involved in the division process through the direction of cell wall synthesis and through interacting with other proteins that coordinate chromosome segregation after replication.

1.5.4. Growth under stress

Stress can come in different forms and all bacteria including *Mycobacterium spp.* have developed methods to overcome those stressors. Oxidative stress is the formation of reactive oxygen species (ROS) that can damage the bacterial cell [60]. Oxidative stress which is simulated *in vitro* by treating cells with H_2O_2, and is experienced by cells growing within macrophages *in vivo* is one of the major stressors that needs to be overcome for the survival of *M. tuberculosis* [48].

FipA which stands for **F**tsZ-**i**nteracting **p**rotein **A** has been shown as a protein important for cell division under oxidative stress in *Mycobacteria spp.* FipA has been shown to interact with both FtsZ and FtsQ and forms a ternary complex when phosphorylated again by the kinase PknA [53]. When PknA was knocked out and the cell subjected to oxidative stress, it cannot phosphorylate FipA, which in turn cannot form the complex with FtsZ and FtsQ; this led to miss-positioning of the septa in dividing cells. This is important because FipA is necessary for proper localisation of FtsZ at midcell in situations of oxidative stress, situations such as growth within host macrophages seen *M. tuberculosis* infection models.

ClpX in *M. tuberculosis* is another regulator of cell division and it is utilised during growth within host macrophages and when the host is undergoing antibiotic treatment [61]. ClpX acts directly with FtsZ and stops FtsZ polymerising to form the divisome. The inhibition of the principle recruiter of the downstream

division proteins effectively stops cell division accounting for slower growth and filamentation under those conditions and environments [48].

Knowing and investigating the proteins involved in cell division that overcome oxidative stress is important due to pathogenic strains of *Mycobacteria spp.* such as *Mycobacterium tuberculosis* which can live within the macrophages of the host, an oxidative environment. If we as medical scientists understand this process, drug development to target these proteins could certainly be a viable future prospect.

1.6. Bacterial filamentation as a survival strategy

Bacterial filamentation arises when a bacterial cell continues to grow with a distinct lack of cell division at the appropriate stage of the cell cycle [62]. It is seen in environmental bacteria as a survival strategy to evade phagocytosis by protists and is also seen in strains of uropathogenic *E. coli* that can be seen growing up to 70 μm in length. For uropathogenic *E. coli*, filamentation is essential for pathogenesis therefore a lack of filamentation leads to a lack of infection [62]. It was also postulated by Justice *et al* that bacterial filamentation might aid in the resistance to antimicrobials as filamentous bacteria have been isolated from patients who are being treated with antibiotics [62].

Filamentation is important to study with regard to *M. tuberculosis* because *M. tuberculosis* cells growing in macrophages have been seen as being filamentous and lacking in mid-cell Z-rings [48]. This indicates that a lack of cell division i.e. filamentation within host macrophages could be a survival mechanism characteristic of *M. tuberculosis*, rather than simply a stress-response to DNA damage as had previously been believed. It has been postulated that *M.*

tuberculosis not dividing is as important if not more so than initiating cell division. To establish latency, specific and unique regulatory systems are needed and include signals to initiate a latent phase and other signals to initiate an active stage of disease [6].

The knowledge that can be gained from the study of the regulators involved in filamentation would be useful in the development of novel drug targets for *M. tuberculosis* as these novel approaches could counteract the latency period of the disease which allows for persistence of the infection both to the host and the wider community.

1.7. Flow cytometry based cell sorting as a screening mechanism for bacterial filamentation

Flow cytometry based cell sorting has been used to effectively screen and collect cell populations based on a variety of factors including size, complexity and if fluorescent reporters are used, the presence of specific proteins or components. Through this work we will show that flow cytometry based cell sorting can be used to discriminate between bacterial cell populations based on cell length.

Flow cytometry works by passing bacterial cells that are hydrodynamically focused in a sheath fluid through a laser; the diffracted light is then picked up via detectors and a “pulse” is generated for that event. The pulses are graphed and a scatter-plot output is generated, which separates the events based upon the parameter you are measuring.

Flow cytometry has been used to discriminate between populations of bacterial cells based on their cell lengths without fluorescent reporters. This was done by utilising the side-scatter profile of bacterial cells and the differences between cells that were filamentous and cells that were of a wild-type (WT) phenotype [63].

1.8. Conclusion, objectives and aims

TB is ancient and it is a disease that is still being fought by many worldwide. The early development of antibiotics seemed to stem the tide but what is currently being seen is resurgence. The prevalence of HIV coupled with poverty have made areas of the developing world reservoirs for *M. tuberculosis*; this in tandem with the world becoming smaller due to air travel and emigration means that TB is no longer a problem for the developing world but a problem for the developed world as well. The pipeline has become dry for anti-TB medications and treatments and humanity is at a stage of its existence where there is an emergence of species resistant to our most powerful drugs, MDR-TB, XDR-TB and TDR-TB. Tuberculosis is but one of a growing population of "superbugs" that also include Methicillin-Resistant *Staphlococcus aureus* (MRSA) and Multi-Drug Resistant *Acinetobacter baumannii* (MDRAB). All of these dangerous organisms have one thing in common, they must undergo cell division to maintain infection; and so this leads to the development of novel drug targets, narrow spectrum antibiotics that target the essential process of cell division.

Cell growth and division is different in Mycobacteria when compared to *E. coli* and *B. subtilis*. Mycobacteria grow from the poles of the cell and they have a

thicker cell wall making them inherently resistant organisms. Sub-populations have different susceptibilities to antibiotic classes, cell division itself differs in the number and function of proteins involved as well as the regulation of the division process and *M. tuberculosis* forms filaments within macrophages allowing the organism to persist within the host. Many of the proteins involved in cell division in the model organisms are not annotated in *Mycobacterium spp.* but through the discovery of novel cell division regulators and cell division genes and then developing drugs to act on these targets perhaps it is possible to stem the tide of these emerging "superbugs".

Our hypothesis is that flow cytometry-based cell sorting can be a powerful tool for identifying and investigating cell division regulators and so the first aim for this body of work is to create a sub-clone shotgun DNA expression library of an already screened shotgun expression library of DNA taken from a biofilm on the surface of the marine algae *Ulva australis*. The sub-clone library will be screened using flow cytometry based cell sorting to collect filamentous cells that will then undergo a confirmatory screen using light microscopy. Once a reproducible filamentous clone is isolated, sequencing is done on that clone to identify a gene of interest. This will finish the proof-of-concept experiments to show that a single gene of interest that elicits filamentation when overexpressed can be isolated from a large library of environmental DNA inserts.

Following from the first aim, the hypothesis was that using the flow cytometry-based screening method on an organism in which there is little information on the regulation of cell division could further identify important genes and proteins and further, increase the pace in which novel drugs could be

developed. Therefore the second aim for this work is to construct a shotgun expression library of *M. bovis* BCG gDNA and then screen that library using flow cytometry based cell sorting. A confirmatory screen is then done using light microscopy to isolate clones with a reproducibly filamentous phenotype, and those clones are then sequenced for genes of interest that when induced to high expression levels cause cell division inhibition. This may then lead to the identification of novel cell division regulators in Mycobacteria that cause filamentation and that may aid in an infection scenario.

Chapter 2

Materials and Methods

2. Materials and Methods

2.1. Chemicals, reagents and solutions

Chemicals and reagents used in this work were of analytical reagent (AR) grade and were obtained from Amresco, BDH Chemicals, Sigma Aldrich or Difco unless otherwise specified. Commonly used aqueous buffers and solutions are listed in Table 2.1.

Table 2.1 - Commonly used solutions and buffers

Buffer/solution	*Components* [a]
ADC enrichment	0.85% NaCl (w/v), 5% bovine albumin (w/v), 2% Dextrose (w/v), 0.003% catalase (w/v)
PBS	137 mM NaCl, 10.1 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, pH 7.4
TE	10 mM Tris-HCl, 1 mM EDTA, pH 8.0

[a] All buffers and solutions were made up in Milli-Q® purified water (MQW) and are listed at the working concentration (1×).

2.2. *Escherichia coli* strains and growth conditions

The strains of *E. coli* used in this work are listed in Table 2.2. For a list of the antibiotics used and their working concentrations, refer to Table 2.4.

Table 2.2 - List of *Escherichia coli* strains and plasmids used in this work

Strain	Genotype	Reference/source
DH5α	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	Lab Stock
Epi300	F- λ- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80d*lacZΔM15* Δ*(lac)X74 recA1 endA1 araD139* Δ*(ara, leu)7697 galU galK rpsL* (Str^R) *nupG'* trfA dhfr	Lab Stock
ER1793	F- *fhuA2* Δ*(lacZ)r1 glnV44 e14-(*McrA-*) trp-31 his-1 rpsL104 xyl-7 mtl-2 metB1* Δ*(mcrC-mrr)114::IS10*	I. Duggin
JW3423-1	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*-	CGSC

	, ΔlivK788::kan, *rph-1*, *Δ(rhaD-rhaB)568, hsdR514*	
MG1655	F^- λ^- *ilvG⁻ rfb-50 rph-1*	Lab Stock
Plasmid	Characteristic	Reference/Source
pCC1FOS	Copy-control fosmid (Epicenter) for library production. Arabinose induces high copy number	[64]
pBAD24	Arabinose inducible expression vector	[65]

Table 2.3 - Media used in this work for the growth of *E. coli* and *M. smegmatis*

Medium	*Components* [a]
LB medium	1% tryptone (w/v), 0.5% yeast extract (w/v), 1% NaCl (w/v)
M9 minimal medium	20% 5X M9 Salts [66] (v/v), 0.2% 1 M $MgSO_4$ (v/v), 20% Glycerol (v/v), 0.01% 1 M $CaCl_2$ (v/v)
SOC medium	2% tryptone (w/v), 0.5% yeast extract (w/v), 0.05% NaCl (w/v), 2% 1 M glucose (v/v)

[a] All media were made up in MQW and sterilized by autoclaving or using 0.2 µm filtration for heat labile components.

Table 2.4 - List of different antibiotics used in this study along with the working concentrations for each

Antibiotic	*Working concentration (µg/mL)* [a]
Chloramphenicol	12.5
Kanamycin	25
Ampicillin	100
Hygromycin (*E. coli*)	200
Hygromycin (*M. smegmatis)*	50

[a] Stock solutions were prepared by dissolving antibiotics either in ethanol or MQW and filter sterilizing (0.2 µm filter). Antibiotics were stored at -20°C.

2.2.1. Normal growth of *Escherichia coli*

Unless otherwise indicated, *E. coli* cells were grown in LB broth medium or on LB-Agar supplemented with the appropriate antibiotic for selection. Broth

cultures of *E. coli* were incubated at 37°C in a gyratory water-bath with the shaking speed set to ≤200 rpm (OLS200; Grant Scientific water-baths were used). Growth was monitored through recording the optical density of the culture at 600 nm (OD_{600}) using a spectrophotometer (UV-120-02; Shimadzu).

2.2.2. Storage and revival of bacteria

Overnight broth cultures of bacterial cells were mixed with 20% v/v glycerol then stored at -80°C. To revive bacteria, a portion of the frozen stock was inoculated onto agar plate containing the appropriate antibiotic and incubated overnight at 37°C or used to directly inoculate 20 mL of LB medium with the appropriate antibiotic in a sterilized 250 mL baffled conical flask and incubated overnight in a water-bath shaming at ≤200 rpm.

2.2.3. Growth of *Escherichia coli* Epi300 for induction of the environmental DNA expression libraries contained within pCC1FOS

Cultures of *E. coli* Epi300 were revived from frozen stock as described above. The overnight culture was washed and re-suspended in an equal volume of either LB or M9 minimal medium then a 1:100 dilution made in fresh LB or M9 minimal medium with chloramphenicol. Cells were cultured in the shaking water-bath at 37°C and 200 rpm for 1 hour in a 250 mL baffled conical flask. 20% arabinose inducer was then added to the desired final concentration and the cells cultured for a further 4 hours. Further investigation was then performed.

2.2.4. Growth of *Escherichia coli* strains with cloned genes on the pBAD24 vector

Overnight cultures of *E. coli* cells were inoculated from frozen stock into LB medium with ampicillin and 0.2% (v/v) glucose to repress expression of the

cloned genes and incubated in a water-bath overnight at 37°C and 200 rpm orbital shaking. OD_{600} was read and the overnight culture diluted to an OD_{600} of 0.04 in fresh medium containing 0.2% (v/v) glucose. Cells were then cultured for 3 hours (mid-exponential point) in a shaking water-bath at 37°C and 200 rpm. The OD_{600} was again read and the mid-exponential culture washed and diluted to an OD_{600} of 0.04 in fresh LB medium containing 0.2% arabinose (v/v). The induced culture was then grown for a further 3 hours (approximately 4 generations) in a shaking water-bath at 37°C and 200 rpm. Further investigation was then performed.

2.3. Mycobacterial strains and growth conditions

For a list of mycobacterial strains and plasmids used in this work, refer to Table 2.5. The relevant antibiotic concentrations are listed in Table 2.4.

Table 2.5 - List of *M. smegmatis* strains and plasmids used in this work

Strain	Genotype	Reference/source
mc^2155	Laboratory wild-type strain	J. Triccas
MSTR1	mc^2155 with the *tetR* gene from plasmid pMC2m integrated on the chromosome at the *attB* site.	This study
Plasmid	**Characteristic**	**Reference/source**
pSE100	Expression plasmid with origins of replication for both *M. smegmatis* and *E. coli*.	S. Ehrt
pMC2m	Integrating plasmid containing the *tetR* gene to work in conjunction with the tetracycline inducible promotor	S. Ehrt
pJFR11	Plasmid containing *M. bovis* BCG *ftsZ* gene conjugated to GFP	J. Triccas
pJFR14	Plasmid containing an acetamide inducible GFP	J. Triccas
pSAM1	pSE100 with the addition of a RBS and a *SphI* cloning site between the *SphI* site and the *BamHI* site at the MCS of pSE100	This study
pDHL1029	Plasmid containing the msfGFP	[67]

2.3.1. Growth of Mycobacterium smegmatis

Unless otherwise indicated *M. smegmatis* cells were cultured as follows. Frozen stocks were revived on LB-Agar plates with the appropriate antibiotic and incubated at 37°C until colonies grew. 5 mL of Middlebrook 7H9 (Difco) containing the appropriate antibiotic was then inoculated with a single colony from the plate and incubated at 37°C in a gyratory water-bath set to 200 rpm for 24 hours. If induction was required, the 24-hour culture was diluted to an OD_{600} of 0.04 in fresh pre-warmed Middlebrook 7H9 medium containing the appropriate antibiotic and inducer (For pJFR11 and pJFR14, inducer was 0.2% acetamide (v/v) and for pSAM1 the inducer was 50 ng/mL ATc) and incubated for a further 15 hours (approximately 5 hours). Cells could then be used for further experimentation.

2.4. Construction of a *Mycobacterium smegmatis* growth curve

For effective induction of a cloned expression library to elicit a filamentous phenotype, expression should be induced at the mid-exponential point of a growth cycle. To do this a growth curve must be constructed and the procedure was as follows. Middlebrook 7H9 media was inoculated with a colony revived from frozen stock on an agar plate and incubated at 37°C for 36 hours in a shaking water-bath set to 200 rpm. The culture was then diluted to an OD_{600} of 0.04 and incubated at 37°C and 200 rpm. A small sample was taken every 3 hours and the OD_{60} read, this was done for a total of 30 hours. The data was collected and tabulated using Microsoft Excel and a growth curve graph generated.

2.5. Molecular biology techniques

2.5.1. *Mycobacterium bovis* BCG gDNA preparation

This method was performed by Rachel Pinto in Jamie Triccas' lab at the University of Sydney. 1 mL of cell culture was placed in a screw-cap tube (with O-ring) containing 1 mL of 0.1 mm glass beads. Tube was transferred to a bead-beater and processed at 40 for 20 seconds. Tubes were then spun at 12, 000 g for 5 minutes and the supernatant removed to a new 1.5 mL microfuge tube. 0.1 volume of sodium deoxycholate was added to the tube and incubated at 56°C for 1 hour. 1 volume of phenol:chloroform; chloroform:isoamyl alcohol (25:24:1) was added then vortexed for 1 minute then spun in a microfuge at 12, 000 g for 1 minute. The top layer was removed and added to a new tube with 1 mL chloroform, vortexed for 1 minute and spun in a microfuge at 12, 000 g for 1 minute. This was repeated until the top layer was clear. 0.1 volume of 3 M NaOAc (pH 5.2) and 1 volume of 100% isopropanol were added to the supernatant, mixed and incubated at -80°C for 1 hour. The tube was then spun for 15 minutes at top speed using a microfuge then the supernatant removed. The DNA pellet was then washed with 1 mL 100% ethanol, spun at top speed for 1 minute and the supernatant removed. The pellet was then air-dried, resuspended in 10 mM Tris and stored at -20°C.

2.5.2. Plasmid DNA preperations

Depending upon the amount of plasmid DNA required, plasmid DNA was extracted from stationary phase cultures of *E. coli* using either the GenElute™ Plasmid Miniprep Kit (Sigma), or the Sambrook *et al* 2001 [66] procedure for midipreperations using the Preperation of Plasmid DNA by Alkaline Lysis with SDS method.

2.5.3. Restriction endonuclease digestion

Restriction enzymes were obtained from New England Biolabs and stored at -20°C. Unless otherwise stated within the results section for the relevant chapter, restriction endonuclease digestions were performed as follows. 1 µg of DNA was placed with 5 µL of 10x Buffer and 10 U (1 µL) of restriction enzyme. The solution was made up to 50 µL with MQW and incubated at the appropriate temperature for the restriction enzyme for 1 hour, unless partial digestion is required. The enzyme was then heat-inactivated at the appropriate temperature for the appropriate time and cleaned using the QIAquick® PCR purification kit (Qiagen) according to the manufacturer's instructions.

2.5.4. Shearing and end-repair of DNA using a nebulizer

Shearing of DNA using a nebulizer (Invitrogen) was done as per the manufacturers instructions with the following change. Shearing was done at 10 psi for 80 seconds. The DNA was then checked using agarose gel electrophoresis for appropriate fragmentation of the DNA. As the fragmented DNA would not have compatible ends with the blunt-ended pCC1FOS vector, it had to be end-repaired to create blunt-ends. The sheared DNA was cleaned using the QIAquick® PCR purification kit (Qiagen) according to the manufacturer's instructions. The end-repair was done using the End-It™ DNA End-Repair Kit (Epicenter) as per the manufacturers instructions. Once end-repaired, the fragmented DNA was used for ligation (Section 2.5.4).

2.5.5. Ligation

Ligation of DNA was done using the T4 DNA ligase kit (New England Biolabs). Typically, insert and vector DNA were mixed in a 3:1 molar ratio with 2

µL of 10x ligase buffer and 400 U of ligase enzyme made up to a total volume of 20 µL with MQW. The ligation mixture was then incubated at 16°C overnight and then stored at 4°C.

2.5.6. Oligonucleotides

Oligonucleotide primers for PCR and sequencing were synthesized by Sigma Aldrich or Integrated DNA Technologies (IDT) and supplied in a lyophilized form. When received, oligonucleotides were dissolved in sterile MQW to a final concentration of 100 µM and stored at –20°C until required. Oligonucleotides used in this work are listed in Table 2.5.

Table 2.6 - List of oligonucleotides in this work

Sequence 5' to 3'	*Description*
GCTAGCAGGAGGAATTCACC	Forward primer for PCR and sequencing of the pBAD24 vector
GCCTGCAGGTCGACTCTAG	Reverse primer for PCR and sequencing of the pBAD24 vector
TATCAGTGATAGATAGGCTCTG	Forward primer for PCR and sequencing of the pSE100 and pSAM1 vector
TAAAAAAGGGGACCTCTAGG	Reverse primer for PCR and sequencing of the pSE100 and pSAM1 vector
GGATGTGCTGCAAGGCGATTAAGTTGG	Forward primer for PCR and sequencing of the pCC1FOS vector

CTCGTATGTTGTGTGGAATTGTGAGC	Reverse primer for PCR and sequencing of the pCC1FOS vector
AAGAAGGAGAAGTACCGTGGCATGCG	RBS top strand oligonucleotide
GTACTTCTTCCTCTTCATGGCACCGTACGCCTAG	RBS bottom strand oligonucleotide

2.5.7. Standard Polymerase Chain Reaction (PCR)

PCR was used to amplify specific regions of the *E. coli* chromosome, amplify regions of the environmental library, amplify reigons of the *M. bovis* BCG chromosome and amplify the msfGFP from the plasmd pDHL1029. For checking the efficiency of transformation *Taq* DNA Polymerase (New England Biolabs) was used. If genes were amplified to be used in further experiments such as sequencing or cloning, Phusion DNA Polymerase (New England Biolabs) was used. All PCR reaction mixtures consisted of template DNA (250 ng), 0.5 µM of each primer (see Table 2.6), 1× amplification buffer specific for polymerase (New England Biolabs), 200 µM of dNTP mixture and 1 U of DNA polymerase, made up to 50 µL in sterile MQW. To facilitate amplification, PCR mixtures were subjected to the following temperature cycle using a thermal cycler (Nexus Gradient, Eppendorf): 98°C for 1 minute (initial template denaturation), 30 cycles of 98°C for 10 seconds (template denaturation), X°C for 30 seconds (primer annealing) and 68-72°C for Y minute(s) (extension), followed by a final extension at 68-72°C for a further 10 min [where X = predicted oligonucleotide annealing temperature minus 5 °C, and Y = 1 min per kb to be amplified]. Reaction products were analysed by agarose gel

electrophoresis (Section 2.5.9) and purified using the QIAquick® PCR purification kit (Qiagen) according to the manufacturer's instructions.

2.5.8. Colony PCR

Colony PCR was used to determine the efficiency of transformation during the construction of shotgun DNA expression libraries. The procedure is the same as normal PCR (Section 2.5.7) but with the following modifications. Single colonies were picked with a pipette tip and used to inoculate 19.75 µL of MQW in a 0.2 mL PCR tube. The suspended colony was then placed in the thermo cycler at 95°C for 5 minutes to lyse the cells. A master mix was made with a final concentration of 1x Thermopol buffer, 200 µM dNTPs, 0.2 µM of each primer (Table 2.6). 4 µL of the master mix was added to the lysed cell suspension as well as 0.625 U of *Taq* polymerase then the PCR tube placed in the thermo cycler using the temperature cycles above with the exception of the extension time, which was set to 5 minutes. PCR products then underwent agarose gel electrophoresis (Section 2.5.9) to determine the insertion efficiency.

2.5.9. Agarose gel electrophoresis and quantification of DNA concentration

Agarose gels were prepared on a horizontal slab gel apparatus. Agarose (type 1: low electroendosmosis; Sigma) at 0.6-1% (w/v) was dissolved in either TAE or TBE electrophoresis buffer and gels were cast with a well-forming comb in place. 6× DNA loading buffer (New England Biolabs) was added to DNA samples prior to loading into the pre-cast wells. 1 kb DNA ladder (New England Biolabs) was used for size standards, enabling the size estimation of DNA fragments greater between 0.5 kb and 1 kb. Agarose gels were run submerged under a volume of electrophoresis buffer at 60 V for 1 to 1.5 hours. If the gel was run as a check for

quantification purposes, they contained 60 ng/mL of GelRed® to visualize DNA. If the DNA within the gel was to be extracted (Section 2.5.10) the gel was run without stain. Stained gels were exposed to short-wavelength UV light (254 nm) using a transilluminator (EC-40; UltraLum) and recorded as a digital image using a charge-coupled device (CCD) camera (EDAS 290; Kodak) linked to Carestream MI software (Kodak), which was also able to quantify the concentration of DNA in a band. DNA was also quantified using the NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Instruments) as per the manufacturers instructions.

2.5.10. DNA extraction from agarose gels

Unless otherwise stated in the results section for the relevant chapter fragmented DNA was extracted from agarose gels using the GELsae protocol (Epicenter) as per the manufacturers instructions. Minor modifications were made to the protocol, a cooling step in an ice-bath was added after the enzyme heat inactivation step and 1x TAE was used as the casting and running buffer for the 1% low-melt agarose (Sigma) gel the partially digested DNA was run on (as per section 2.5.9). To visualize the 1 kb DNA ladder (New England Biolabs) and to determine the 2-5 kb region of DNA to be extracted, the gel lanes containing the DNA to be extracted were excised from the agarose gel and covered with cling film. The gel lanes containing the 1 kb ladder and a small aliquot of the digested sample were then immersed in a 3x GelRed® solution in the dark for 30 minutes. The 1 kb ladder and test lanes were then imaged as above (Section2.5.9) and marks were made on the gel to indicate where the 2-5 kb DNA fragments are. The stained and unstained gels were then lined up and the corresponding 2-5 kb region of the un-stained gel excised and processed using the GELase protocol (Epicenter).

2.6. Cloning of a Ribosome Binding Site (RBS) on to the pSE100 vector

As the pSE100 vector used for expression of cloned genes in *M. smegmatis* did not contain a RBS, one was added for the expression of cloned library genes. Complimentary oligonucleotides were ordered (Sigma) (see Table 2.6), mixed in equal ratios and heated to 95°C in a heat-block for 2 minutes. The power to the heat-block was shut off and the oligonucleotide mixture allowed to come to room temperature very slowly. The concentration of the RBS sample was determined using the NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Instruments). The RBS was ligated to double-digested pSE100 (digested with *SphI* and *BamHI* (New England Biolabs)) for directional cloning and transformed into *E. coli* ER1793. A minipreperation of plasmid DNA was done and the plasmid sequenced by the Australian Genome Research Facility (AGRF) to determine proper insertion.

2.7. Preparation and transformation of electrocompetent *Escherichia coli*

20 mL of LB medium was inoculated with the necessary *E. coli* strain. The culture was incubated at 37°C in a gyratory water-bath set to 200 rpm overnight. A 1:100 dilution was made of the overnight culture in fresh medium and incubated at 37°C and 200 rpm in a shaking water-bath. When an OD_{600} of 0.7 was reached, cultures were transferred to chilled centrifuge tubes and chilled in an ice-bath for 30 minutes. Tubes were then centrifuged at 2,000 g and 4°C for 15 minutes. The supernatant was discarded, the cell pellet washed in 1 volume of ice-cold MQW and centrifuged as before. The supernatant was discarded and the cell pellet washed with a 0.5 volume of ice-cold 10% glycerol then centrifuged as before. The supernatant was carefully decanted, the cell pellet re-suspended with 10%

glycerol in 4% of the starting volume and centrifuged again, the supernatant was carefully decanted until the cell pellet began to move and the cells re-suspended in the residual 10% glycerol. Cells were snap-frozen in liquid nitrogen and stored at -80°C.

Transformation of plasmid DNA into electrocompetent *E. coli* consisted of 50 µL of electrocompetent cells being placed in a 0.2 cm electroporation cuvette (Bio-Rad) with 1 µL of plasmid DNA (1 ng of DNA of positive controls to determine transformation frequency). A Gene Pulser MXcell™ (Bio-Rad) was used for transformation set to 2.5 kV, 200 ohms and 25 µF. After transformation the cells were recovered in SOC medium for 1 hour at 37°C and 100 rpm in a gyratory water-bath. Recovered cells were then plated onto LB-Agar with the appropriate antibiotic and at the appropriate dilution.

2.8. Preparation and transformation of electrocompetent *Mycobacterium smegmatis*

5 mL of Middlebrook 7H9 medium was inoculated with a colony of *M. smegmatis* growing on an agar plate and cultured overnight in a gyratory water-bath at 37°C and 100 rpm. The culture was then diluted 1:100 in fresh, pre-warmed Middlebrook 7H9 medium and incubated as before until an OD_{600} of 0.8 was reached. Cultures were then transferred to centrifugation tubes and chilled on ice for 30 minutes, centrifuged at 3,000 g for 10 minutes and the supernatant discarded. Cells were washed 3x in successive 0.5 volumes of 10% glycerol and finally re-suspended in 1:100 of the original volume. Cells were aliquot into 1.5 mL micro-centrifuge tubes, snap-frozen in liquid nitrogen and stored at -80°C.

To transform the electrocompetent *M. smegmatis* cells the following procedure was used. 1-5 µL of plasmid DNA of a known concentration was added to 200 µL of competent cells thawed on ice and left on ice for 10 minutes. Mixture was then transferred to an ice-cold 0.2 cm electroporation cuvette (Bio-Rad) and pulsed using the following parameters: 2.5 kV, 25 µF and 1,000 ohms. Transformants were mixed with 800 µL of Middlebrook 7H9 medium and left on ice for 10 minutes. The cell suspension was then transferred to a 50 mL Falcon tube and 4.2 mL of fresh Middlebrook 7H9 medium added and incubated in a gyratory water-bath at 37°C and 100 rpm for 3 hours. Suitable dilutions of transformants were plated onto LB-Agar containing the appropriate antibiotic and incubated at 37°C until colonies appeared.

2.9. Fixation of bacterial cells

Bacterial cells were fixed in a 2% (v/v) formaldehyde solution overnight at 4°C. The cells were then pelleted at 12, 000 g for 2 minutes in a centrifuge and washed twice with PBS and finally re-suspended in 200 µL PBS then used for further investigation with either flow cytometry-based cell sorting or wide-field light microscopy.

2.10. Flow cytometry based cell sorting and confirmatory microscopy screen

Unless otherwise indicated in this section or the results section for the relevant chapter, flow cytometry analysis and sorting was done using the Aria II

flow cytometer (BD Biosciences), at the Centenary Research Institute, Sydney, Australia and followed the procedures set out in Burke *et al* 2013 [63]. Prior to analysis and sorting, induced bacterial cells (grown as in Section 2.2.3, 2.2.4 or 2.3.1) and appropriate controls were diluted in filtered growth media to an OD_{600} of 0.1. The induced bacterial cell samples were analysed at 15000–25000 events per second for the first sort with a yield mask, and at 10–50 events per second for re-sorting with a purity mask. PBS was used as the sheath fluid, applied at 70 psi, with a 70 mm nozzle for droplet formation and electrostatic charging for droplet sorting. Events were plotted on a log-scale, with a window extension (WE) setting of 1, and thresholding on forward scatter (FSC) and side scatter (SSC) at 200. Signals for FSC and SSC, area, height and width were recorded and all screens described in results were performed with "Short" and "Long" gates defined on a SSC-H (y axis) vs SSC-W (x axis) dot plot based on the placement of the induced negative control population. The numbers of events screened and collected are set out in the relevant results sections of this work. Events sorted by the "Long" gate were collected into the growth media specific for that experiment and diluted to a concentration of 200 events per 100 µL. The dilution was then plated onto appropriate LB-Agar and plates incubated at 37°C until colonies could be seen (overnight for *E. coli* and 2-3 days for *M. smegmatis*). Colonies that grew were arrayed in 96-well plates, one colony per well with the appropriate growth media and grown overnight in a dry orbital shaking incubator set to 37°C and 100 rpm. 20% glycerol (v/v) was then added to each well and the screened library was then stored at -80°C.

For the confirmatory screen using wide-field light microscopy, clones were revived from the 96-well plate frozen stock using a 96-well plate inoculator into a

new 96-well plate with 150 µL of media per well consisting of the appropriate growth media and antibiotic. This was then incubated overnight at 37°C and 100 rpm in an orbital shaking dry incubator. The overnight cultures were then used to inoculate a new 96-well plate to and OD_{600} of 0.04 that was then grown as in Sections 2.2.3 or 2.2.4.

2.11. Wide-field light microscopy

2.11.1. Slide preparation for fixed cell imaging

Multi-well microscope slides (ICN Biochemicals) were prepared for cell adhesion by incubating the wells with 0.1% solution of poly-L-lysine (Sigma) for 2 minutes, aspirating the wells dry and rinsing twice with MQW. Slides were allowed to dry completely before applying cell samples. Appropriate cell samples (10 µL) were then applied to the respective well on the slide and allowed to fix for 15 minutes. Fixed cells were then washed twice with MQW and the slide was mounted with PBS containing glycerol (50%) and a coverslip. The edges of the coverslip were sealed with nail polish. Slides were stored at 4°C.

2.11.2. Slide preparation for live cell imaging

Live cells of interest were collected and immobilized on agarose coated microscope slides as follows. First, a 1.5 × 1.6 cm rectangular well was created on the surface of the glass slide using a 65 µL Gene Frame (ABgene; slides were obtained from Livingstone). Agarose (type 1; Sigma) was dissolved at 2% (w/v) in the same medium used to culture the cells (including supplements and/or inducers) and 65 µL of this solution was applied to the center of the well. A

coverslip was immediately placed on top and the agarose was allowed to solidify within the well for 5 min, producing a flat 'agarose pad'. To mount cells for live microscopy, the coverslip was gently lifted away and 3 µL of live cell culture was placed on the surface of the agarose pad. The coverslip was then reapplied and the cells were viewed immediately.

2.11.3. Fluorescence and phase-contrast microscopy

The Zeiss Axioplan 2 microscope was equipped with a Plan ApoChromat (100× NA 1.4; Zeiss) objective lens. The light source was a 100 W high-pressure mercury lamp passed through the following filter blocks for visualizing cells expressing msfGFP (Filter set 09, Zeiss; 450 - 490 nm BP excitation filter, 515 nm LP barrier filter). Phase-contrast images were taken as a part of the secondary screen to confirm the filamentous phenotype after the initial screen using flow cytometry. All images were collected using an AxioCam MRm camera. Image processing was performed using AxioVision 4.6 software (Zeiss) and cell length measurements of the images were performed using the same AxioVision software with at least 100 cells measured per sample. Images were exported as TIFF or JPEG files and the cell length data exported in a Microsoft Excel format for further analysis and frequency histogram construction.

Chapter 3

Using flow cytometry to investigate filamentation in environmental bacteria

3. Using flow cytometry to investigate filamentation in environmental bacteria

3.1. Introduction

3.1.1. Filamentation in the environment

The inhibition of bacterial cell division in bacilli (rod-shaped cells) leads to the formation of filamentous cells, cells that continue to elongate but not divide. This filamentous phenotype has been shown to have an advantage in the environment, whether it is in a body of water or our own bodies [62] as outlined in the introductory chapter. The causes of filamentation are numerous and include quorum sensing, anti-microbial signaling, innate immune queues and sensing of predators in the environment [62].

All bacteria must survive and multiply in environments that can be subject to rapid changes. This means that for them to survive they must adapt to their changing surroundings, both quickly and efficiently in a manner which guarantees the continued existence of the species as a whole. Some of the stressors and responses to environmental changes that exist for bacteria include anti-microbials [68], host-associated innate immune cues [69], predatory grazing [70, 71], quorum sensing cues [72], radiation [73], competition with other organisms including competition for nutrients [62] as well as temperature, pH and the presence of toxins. Due to the wide variety of stressors that a microbe can be exposed to and the damage that each of these can cause, the microbe is able to apply different stress responses to combat that which wants to eliminate it. Examples include the production of specific stress response proteins such as antibiotic efflux pumps [74],

the arrest of DNA replication due to DNA damage from either a chemical source or a radiological source or even sensing the presence of a grazing protist [70]. Some stress responses to changing environmental conditions can elicit a filamentous phenotype, such as what is seen when the bacterial SOS response is activated via DNA damage [73].

It has been shown that the environmental bacterium *Flectobacillus sp.* when in the presence of a grazing *Orthochromas sp.* protist, senses the presence of the protist and halts cell division without inhibiting the overall cell growth, meaning that the cell continues to increases its cell length in the absence of cell division. Taken further it was shown that the grazing protist preferentially engulfed and digested the smaller bacterial cells leaving the undivided or filamentous cells to survive. These cells then grew and divided as normal once the presence of the protist, the environmental stressor had disappeared [70].

The example of the grazing protist inducing a filamentous phenotype shows that the bacterial cell, in this case *Flectobacillus sp.* has a great deal of control over its cell cycle and, more importantly, the essential process of cell division. The mechanism for this process is unknown, suggesting that bacteria in the environment contain as yet undiscovered regulators of cell division. Because the cell division process and the proteins involved are relatively well conserved across the different bacterial species, investigating cell division regulation in environmental organisms can give insight into the wider theories of bacterial cell division and its regulation.

3.1.2. Creation of the initial environmental library and method development of flow cytometry based cell sorting for cells having a filamentous phenotype.

The work covered in this thesis starts with completing a proof of principle that the method for creation and overexpression of a shotgun DNA library and the subsequent screening of the library with flow cytometry-based cell sorting works. We had a library of environmental DNA on hand to use as a test for the method, which is relevant, as filamentation has been observed in environmental bacteria. Below are the briefly described theory and methods that were used to build the initial library of environmental DNA, which was used for this chapter of work and are also relevant to the following results chapter (Chapter 4) as well.

A library of bacterial DNA collected from a biofilm on the salt-water algae *Ulva australis* was created [75]. The DNA was collected then packaged into large 40 kb constructs using the CopyControl Fosmid Library Production Kit with the arabinose-inducible pCC1FOS Vector [64]. The fosmid can be induced to a high copy number for functional screening studies (Burke, personal communication).

In the case of this project the environmental DNA library with its large 40 kb inserts were induced to a high copy number and screened for a filamentous phenotype. This system relies on expression from the native promoters of the cloned DNA, with high copy number of the cloned genes (up to 200 copies of the fosmid per cell) providing the basis for increased levels of expression.

Because it was a large library of many different bacterial clones (29 000 fosmid clones), screening individual clones for filamentation would be unfeasible and time consuming using basic bright-field microscopy. Therefore a new

screening method to identify filamentous bacteria was needed; one where thousands of cells could be screened and sorted based on their cell lengths efficiently and quickly.

3.1.3. Flow cytometry based screening of the original library

Prior to the beginning of my work, a method was developed to screen and further sort a filamentous population of cells from a population of cells that have a WT (short cell) phenotype. The new method for screening bacterial cells based on their cell length utilized flow cytometry based cell sorting [63].

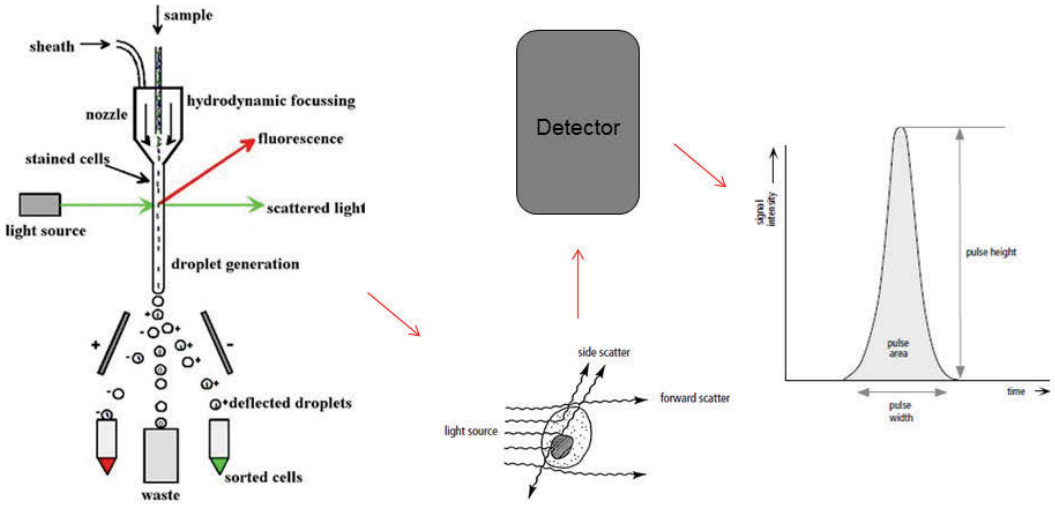

Figure 3.1 - Diagram illustrating the process of flow cytometry. Cells pass through a light source, scattered light is picked up by detectors that record a pulse corresponding to the time taken for the cell to pass through the source corresponding to the cell length.

Figure 3.1 illustrates the use of flow cytometry based cell sorting for the screening and purification of filamentous cells. The cells are pushed through the nozzle where they are hydro-dynamically focused into a single file with gaps between cells. The cells are then passed through a light source where they occlude and scatter the light. The absence of light and the light that is scattered are picked up by detectors, which record the data in the form of a pulse. The pulse width, shown above in Figure 3.1 corresponds to the amount of time that it took for that single event to pass through the light source. This in turn corresponds to the length of the cell, which further corresponds to the placement of that event on a scatter plot.

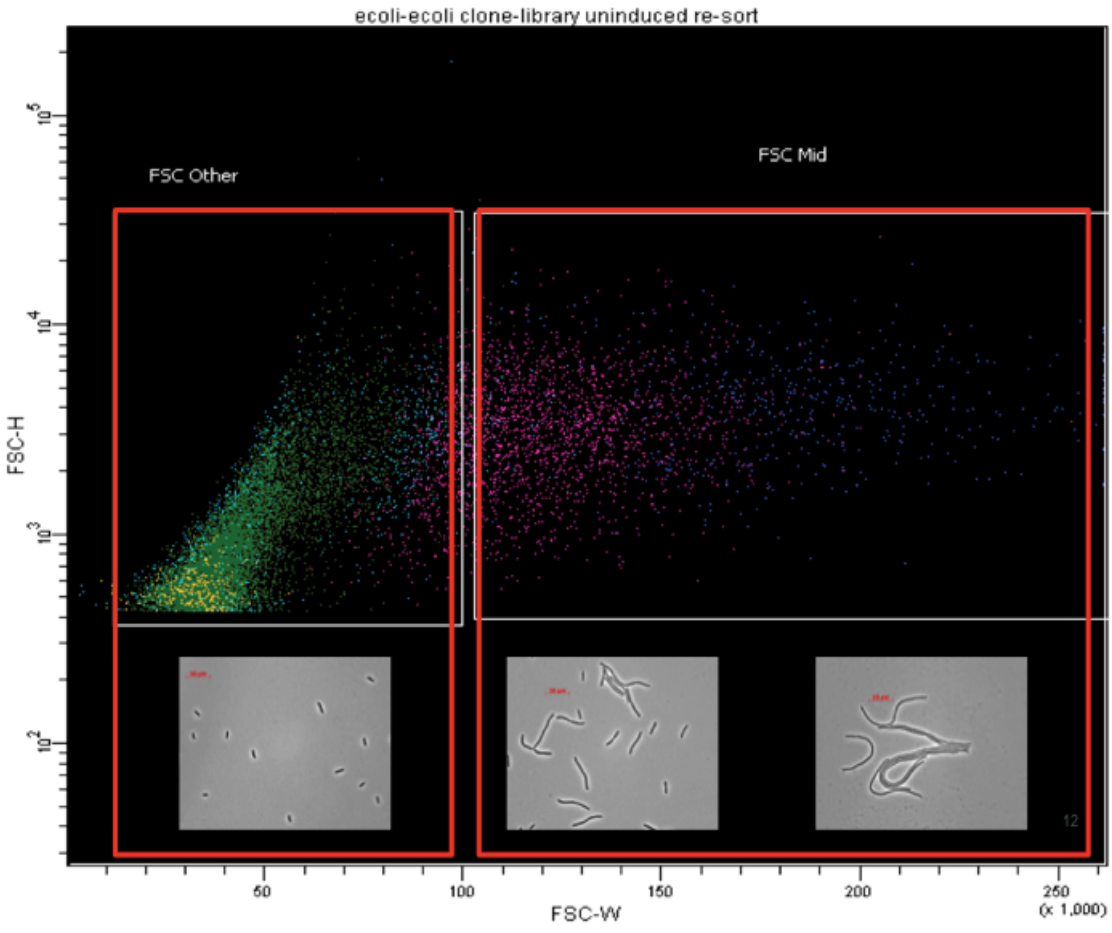

Figure 3.2 - Flow cytometry scatter plot indicating where each of cells with differing cell lengths are recorded on the scatter plot illustrated with phase/contrast images of those cells.

Figure 3.2 above is a flow cytometry scatter plot with representative images of *E. coli* epi300 cells. The images correlate to where the cells would lie on the scatter plot based on their cell length. Using this information we can select a particular population of events from the general population based, for example on their cell lengths and then collect those specific events from the general population.

As part of the development of this flow cytometry based screening method, the environmental library described above was screened as a proof of concept. At the end of this sort, the collected events were cultured and re-screened using wide-field light microscopy to confirm a filamentous phenotype was present. Four clones from the environmental library screened were found to be reproducibly filamentous. These four clones were designated **O**riginal **E**nvironmental **C**lone_1, OEC_2, OEC_3 and OEC_4 (Unpublished work).

The aim of this chapter was to identify a single or group of genes from the OEC clones responsible for eliciting the filamentous phenotype seen with arabinose induction of the 40kb fosmid. This would complete the proof of concept that this screening method using flow cytometry based cell sorting could presumptively identify novel division regulators.

3.2. Results

3.2.1. The Original environmental library screen

Prior to the beginning of my work, the original environmental library was screened with both flow cytometry based cell sorting and then microscopy that resulted in four clones showing a filamentous phenotype.

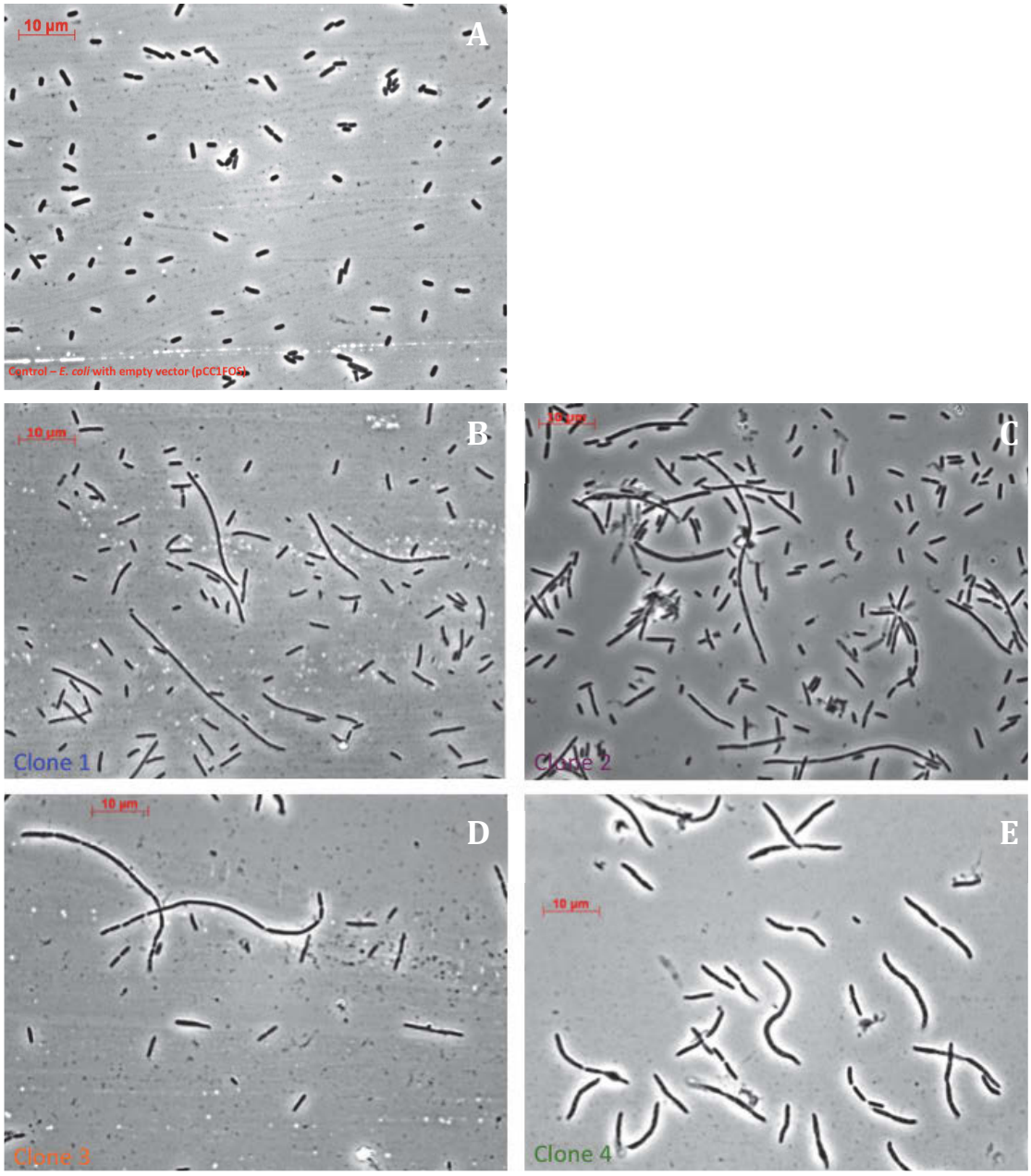

Figure 3.3 - Representative images of each of the filamentous clones isolated from the screen of the environmental library. A) WT Epi300, B) OEC_1, C) OEC_2, D) OEC_3 and E) OEC_4. Scale bars are 10 microns.

Figure 3.3 above shows representative images of the four filamentous clones that came from the flow cytometry screen of the environmental library against a negative control for filamentation (the *E. coli* strain epi300 containing the empty vector). Images for each of the clones were analysed and cell length measurements were taken for 100 cells per clone. These cell lengths were then binned in two-micron increments and graphed as shown in Figure 3.4.

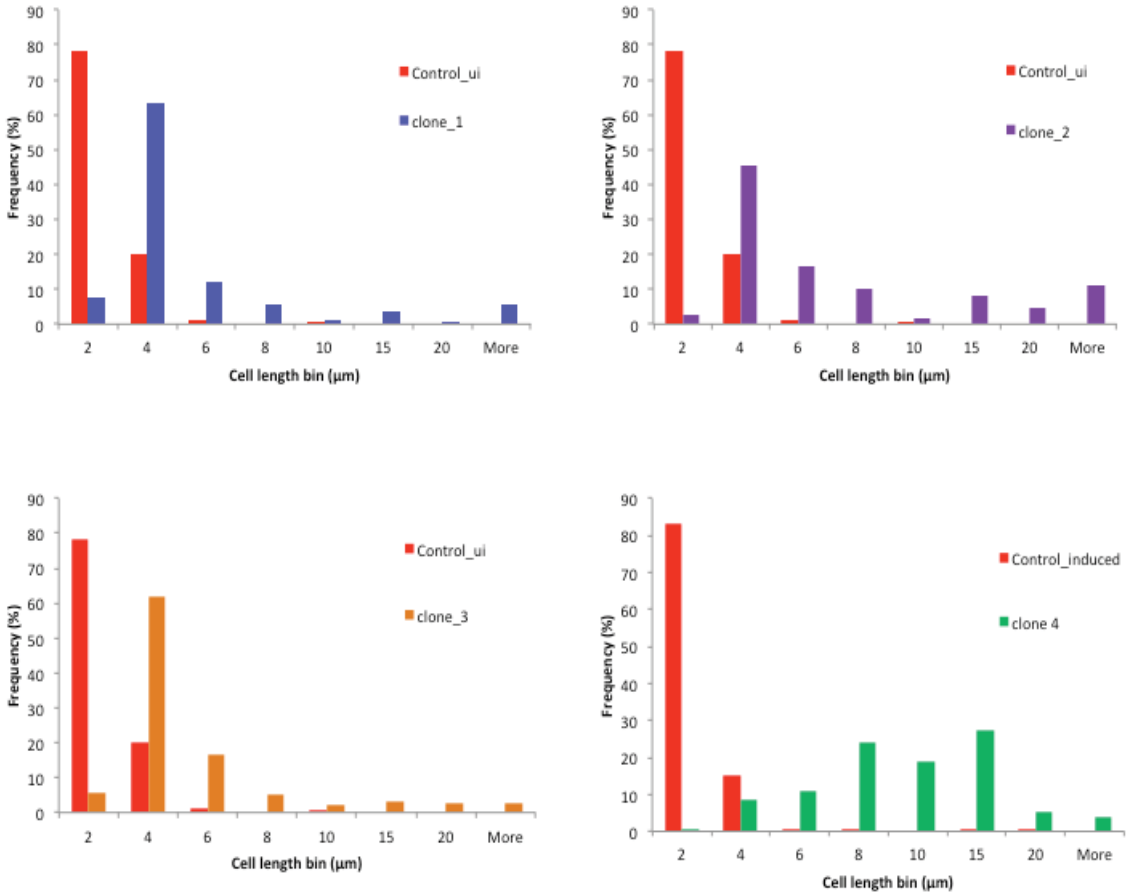

Figure 3.4 - histograms of cell length distributions of each of the OECs compared to the vector only control (red).

The above Figure 3.4 shows the cell length distribution of each of the four clones against the distribution of the vector only control cells in red. As can be seen, from the figure, there is a distinct difference between the two populations of cells where each of the environmental clones have distributions of cell lengths which outperform those of the control cells. Each of the original environmental clones

were sequenced so that a presumptive annotation could be applied to elucidate the function of any candidate genes that may be causing filamentation.

Once the four filamentous clones from the original environmental library were confirmed to have a filamentous phenotype, my project begun and I was tasked with following through the proof of concept where we could identify a single or set of genes which when overexpressed inhibited the cell division of the bacterial cell.

3.2.2. Creating the sub-clone library

The flow cytometry screen originally performed by Dr. Catherine Burke was done using a library of environmental DNA taken from an algal associated bacterial biofilm community, containing at least 70 different bacterial species. It was constructed from approximately 40 kb of environmental DNA packaged into the pCC1FOS fosmid copy-control vector (Epicenter). Because 40 kb of DNA can carry a great number of genes (approximately 20-40 genes), the gene or genes responsible for the filamentous phenotype of the four filamentous clones was investigated. To do this each clone was sub-cloned into smaller DNA fragments, approximately 1-3 kb.

The fosmid DNA of the four filamentous clones was extracted then sheared to 1-3 kb fragments using a nebulizer (refer to methods section 2.5.4). Their size distribution as confirmed using agarose gel electrophoresis. These fragments were then end-repaired to make them blunt-ended and ligated to cut pCC1FOS vector

(refer to methods section 2.5). The ligation reaction was then transformed into the *E. coli* strain Epi300 and recovered on agar plates with chloramphenicol selection.

The desired number of clones was calculated using the equation **N = ln (1-P) / ln (1-f)** [66]. **P** is the desired probability (99%); **f** is the proportion of the genome contained in a single clone (used 2 kb over 40 kb, yielding a value of 0.05); and **N** is the required number of fosmid clones. At least 92 sub-clones were required for each of the original environmental clones.

Table 3.1 indicates the number of sub-clones that were pooled together from each of the original environmental clones. Each of the pooled libraries were named according to the OEC the library came from, e.g. Environmental sub-clone library_1 (EsC_1) came from OEC_1. The number of subclones greatly exceeded the 92 clones for each that was required for 99% coverage and each of these sub-clone libraries were then screened for filamentation when overexpressed using flow cytometry and sorted.

Table 3.1 - Numbers of clones in sub-clone libraries for each of the original environmental clones

	OEC_1	OEC_2	OEC_3	OEC_4
Number of clones	694	3,654	881	1,145

3.2.3. Flow cytometry screen of the sub-clone library

Prior to being sorted using flow cytometry the empty vector Epi300 control and the environmental sub-clone libraries of each of the original environmental clones were grown under conditions to induce the fosmid to a high copy number

(refer to method section 2.2.3). The control and each of the libraries were also grown under non-inducing conditions for comparison.

The environmental sub-clone libraries were sorted using flow cytometry (refer to methods section 2.10). Table 3.2 shows the number of events that were analysed. Each of the sub-clone libraries were screened both under induced and un-induced conditions but filamentous events were only collected from the induced sub-clone libraries. The screen and sort were done in two stages as shown in Figure 3.5 below. First, dot plots were gated based on side scatter to define long and short cell populations, and the long cell population was sorted and collected from the induced sub-clone library. This first-pass long cell sorted population was then re-sorted and events from the long cell gate collected to enrich the proportion of filamentous cells in that population.

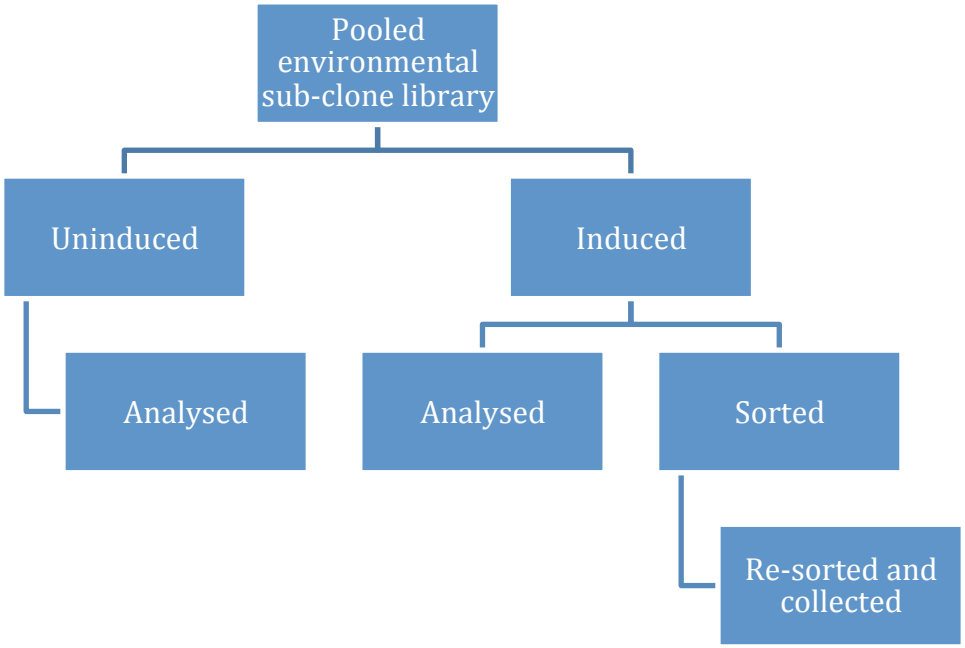

Figure 3.5 - Diagram of the flow cytometry screening process

Examples of the flow cytometry dot plots from the screen of each OEC sub clone libraries are arranged in Figure 3.6, which shows a comparison between the vector control and EsC_1. There is little difference in proportions of events in the "Long" gate (gate determined by the position of 99.9% of the "short" vector control population) between the vector and control but when the induced EsC_1 library is re-sorted there is an increase in the number of long events of 6.9%. Table 2 further illustrates that in the first sort there was no increase in events in the long gate between each of the induced sub-clone libraries and the vector control. When the collected events from each of the induced sub-clone libraries were re-sorted, the number of events encompassed by the long gate greatly increased.

Table 3.2 – Number of events analysed in each of the samples and the percentage of events falling within the long gate for each of the sub-clone libraries and the vector control for each condition.

	Vector Control		EsC_1		EsC_2	
	Total number of events analysed	Percentage of events in "long" gate	Total number of events analysed	Percentage of events in "long" gate	Total number of events analysed	Percentage of events in "long" gate
Uninduced	40,291	0.04	40,000	0.03	40,000	0.05
Induced	39,689	0.05	64,629	0.04	40,000	0.02
Induced re-sort	N/A	N/A	5,522	7.3	5,070	8.01

	EsC_3		EsC_4	
	Total number of events analysed	Percentage of events in "long" gate	Total number of events analysed	Percentage of events in "long" gate
Uninduced	40,000	0.03	40,000	0.06
Induced	40,000	0.05	40,000	0.03
Induced re-sort	4,455	9.16	5,385	7.74

Table 3.2 above, indicates the percentages of events falling within the "long" cell gate on the flow cytometer as well as the overall number of events that were analysed by the flow cytometer. The induced re-sorted population shows a large increase in long events showing enrichment for filamentous cells.

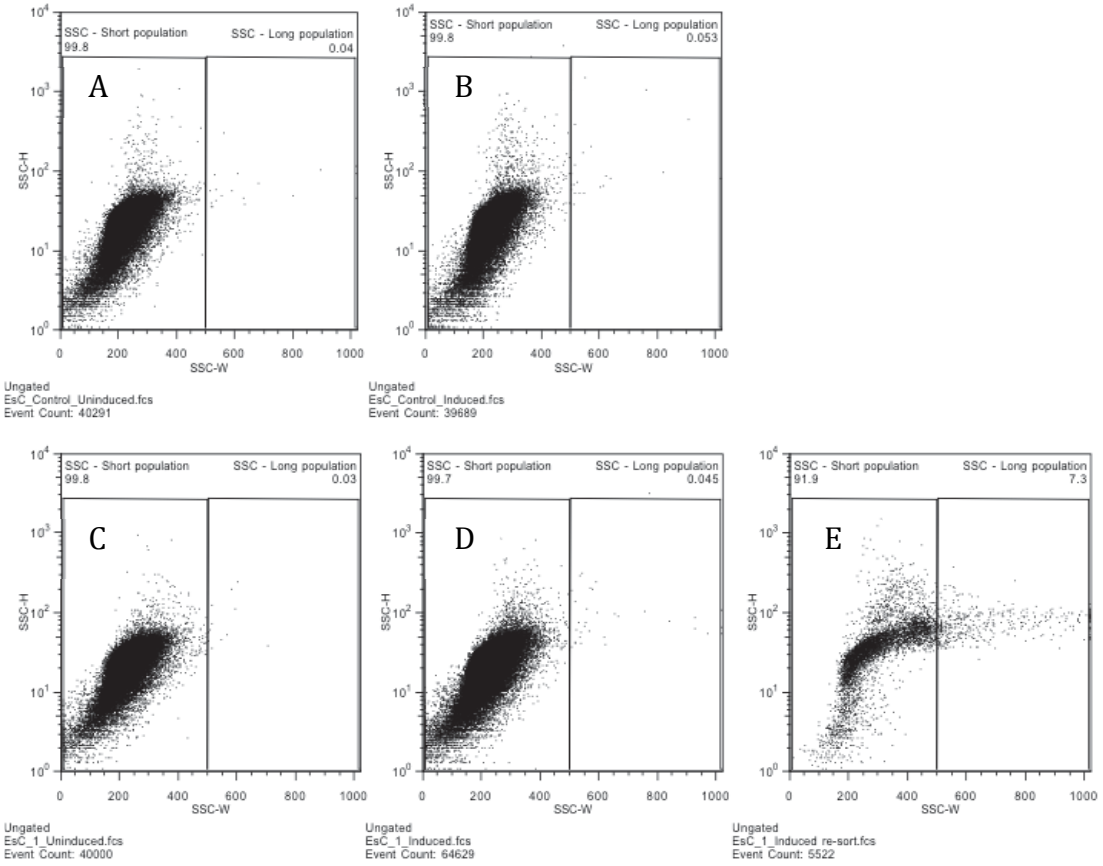

Figure 3.6 - Representative flow cytometry scatter plots taken from the AriaII Cell Sorter. The top row shows plots for the vector control, uninduced (A) and induced (B). The second row shows plots for EsC_1 with uninduced (C), induced (D) and the induced re-sorted population (E). The gates indicate where the population cell lengths sit based upon their side scatter profile.

For each of the induced environmental sub-clone libraries, 10,000 events were collected from the "long" gate in the initial sort. When that population of cells was re-sorted, 1,000 events were collected. Table 3.3 indicates the number of colonies that subsequently grew on agar. These clones were then arrayed into 96-well plates.

Table 3.3 - Number of collected events that grew post screen

	EsC_1	EsC_2	EsC_3	EsC_4
Number of colonies	66	70	78	73

3.2.4. Confirmation of filamentous phenotype in sorted clones using light microscopy

Because sorting cells using flow cytometry does not actually tell us what the individual cell length for each event is, the collected cells need to have the filamentous phenotype confirmed and measured using light microscopy (refer to methods section 2.11), this confirmation is also needed to make sure that the filamentous phenotype that was screened for is reproducible. This involved inducing the sorted sub-clones in the same way as for the flow cytometry screen but in 96-well plates rather than flasks, fixing the cells then adhering them to a glass slide and examining them with phase-contrast light microscopy. A cell was deemed filamentous if the length was greater than 8μm and a population was considered to be filamentous if greater than 25% of the population were filamentous cells. Greater than 8μm was chosen as a filamentous cell length as only a small proportion of the control population (≤5%) of *E. coli* Epi300 were over 8μm. Cell lengths were measured for two sub-clone populations that visually appeared to produce many filamentous cells when the fosmids were induced to a high copy number compared to a vector-only control using microscopy. Cell lengths were not measured for sub-clones that did not appear to have many filamentous cells on first inspection. Figure 3.7 shows the two filamentous sub-clones in comparison to the vector control. These clones were termed, EsC_3-5B

and EsC_4-1G, the first number indicating which of the original clone the sub-clone came from and the second number and digit indicating the well of the 96-well plate the sub-clone occupies.

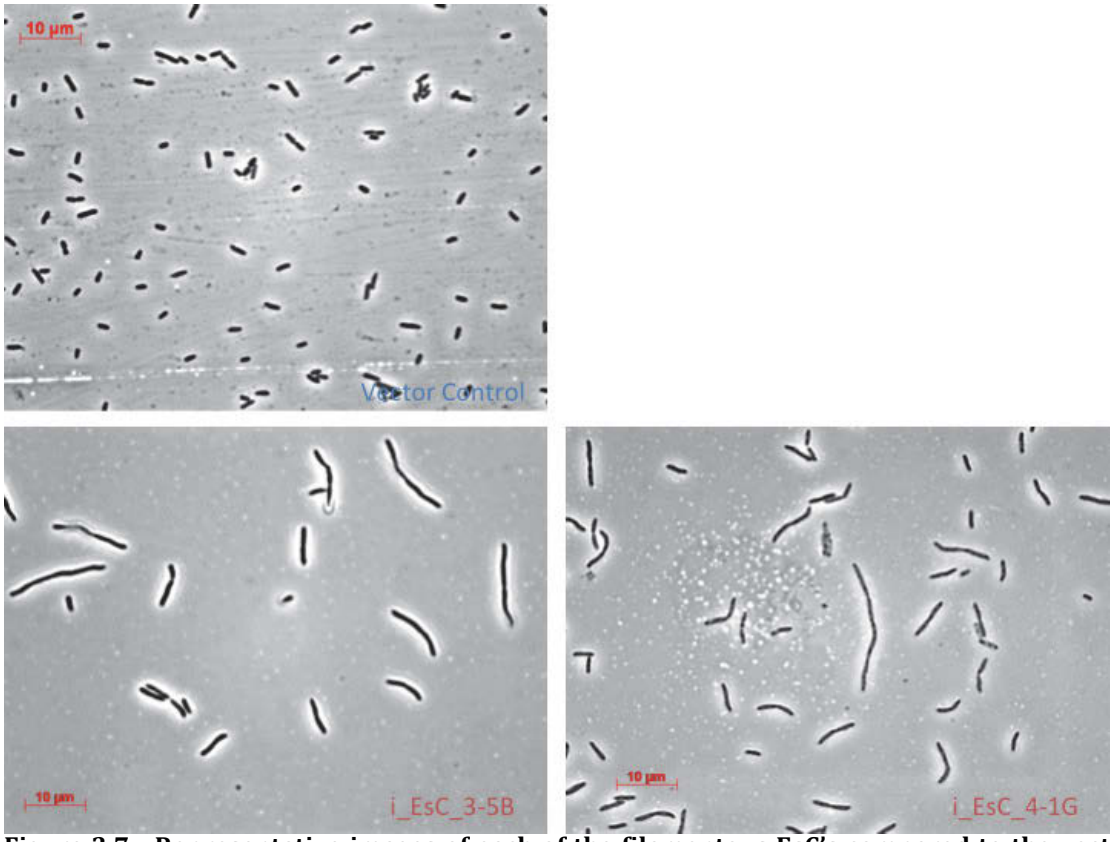

Figure 3.7 - Representative images of each of the filamentous EsC's compared to the vector control. A) Vector control, B) i_EsC_3-5B and C) i_EsC_4-1G. Scale bar is 10µm and the prefix "I" indicates that the clone was induced for overexpression.

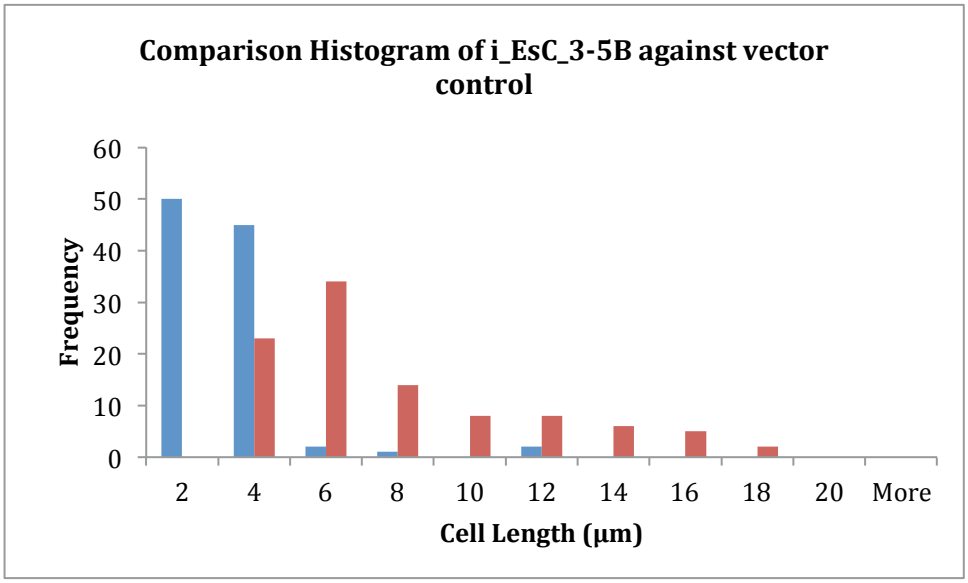

Figure 3.8 - Histogram of cell length distributions of i_EsC_3-5B (red) against the vector control (blue). The prefix "I" indicates that the clone was induced for overexpression.

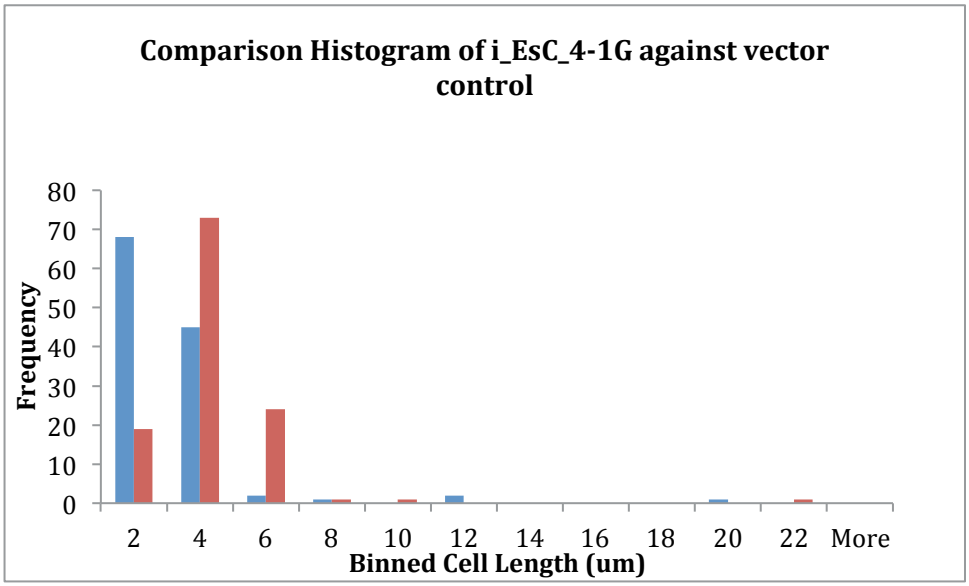

Figure 3.9 - Histogram of cell length distributions of i_EsC_4-1G (red) against the vector control (blue). The prefix "I" indicates that the clone was induced for overexpression.

Table 3.4 – Degree of filamentation of overexpression library subclones. Statistical data corresponds to each of the histograms shown above. Significance was calculated with a Mann Whitney t-test comparing clones to the control with p-values <0.0001

	Vector Control	i_EsC_3-5B	i_EsC_4-1G
Mean cell length	2.39µm	6.82µm	3.35µm
% cells ≥8µm	3%	43%	2.52%
SD of the population	1.46µm	3.66µm	2.12µm
Significantly different	N/A	Yes	Yes

On closer examination and tabulation of the cell lengths of each of the presumptively filamentous cells (Figures 3.7, 3.8 and 3.9), it was found that only 2.5% of cells from the induced populations of clone EsC_4-1G were filamentous, this clone was not investigated further. Sub-clone EsC_3-5B produced the most consistent filamentous cells under inducing conditions, with 43% of the population becoming filamentous with a mean cell length of 6.82µm compared to i_EsC_4-1G, which had 2.5% of the population greater than 8µm and a mean cell length of 3.35µm. Both i_EsC_3 and i_EsC_4 were statistically different from the vector control population according to a Mann-Whitney test but the sub-clone EsC_3-5B was chosen to be further pursued as it had a higher percentage of cells that were filamentous (against the criteria used here).

3.2.5. Characterization of the filamentous phenotype of Clone 3

The insert in the sub-clone EsC3-5B was sequenced and found to contain two complete ORFs (for nucleotide sequences see Appendix section 7.1 and 7.2). A

BLASTx analysis was done on each of the ORFs to identify similar proteins within the NCBI non-redundant protein sequence database. For non-hypothetical proteins, ORF-1 was found to have the closest similarity (85% identity) to a branched-chain amino acid ABC transporter, amino acid-binding protein in Candidatus *Rhodobacter lobularis.* ORF-2 was found to have similarity (50% identity) to an alkyl sulfatase (belonging to the metallo-beta-lactamase group of enzymes [76]) from *Rhodobacterales sp.*. Both of these genera belong within the Alphaproteobacteria class which constituted around 12.5% of the organisms collected and identified using 16S rRNA sequencing from *Ulva australis* in a previous study [77]. Further analysis done on the amino acid sequence for ORF-1 using NCBI's BLASTp (searching a protein database based upon a amino acid sequence query) database showed the presence of a conserved domain belonging to the Periplasmic Binding Protein Type-1 Superfamily (also seen in the BLASTx analysis). This family consists of a diverse range of periplasmic binding proteins that are a part of the amino acid transport systems for bacteria and include ABC (ATPase Binding Cassette)-Type active transport systems used for the uptake of peptides, amino acids or inorganic ions. An example of this is the LIV-system in *E. coli*, which is responsible for the transport of leucine, isoleucine and valine into the bacterial cell [78].

Because the pCC1FOS overexpression vector that was used here is a high copy number of plasmid within the bacterial cell and does not involve the ability to induce expression directly of the cloned genes, a different vector system utilizing the pBAD24 plasmid in host strain DH5α was used for all subsequent experiments. This plasmid allows for induction of the expression of the cloned genes rather than relying on cloned native promoters.

The two ORFs for clone 3-5B were cloned separately and together into the pBAD24 vector to determine whether one or both ORFs were the cause of the filamentous phenotype exhibited by the library sub-clone. As seen in Figure 3.10, only ORF-1 was found to cause filamentation when overexpressed.

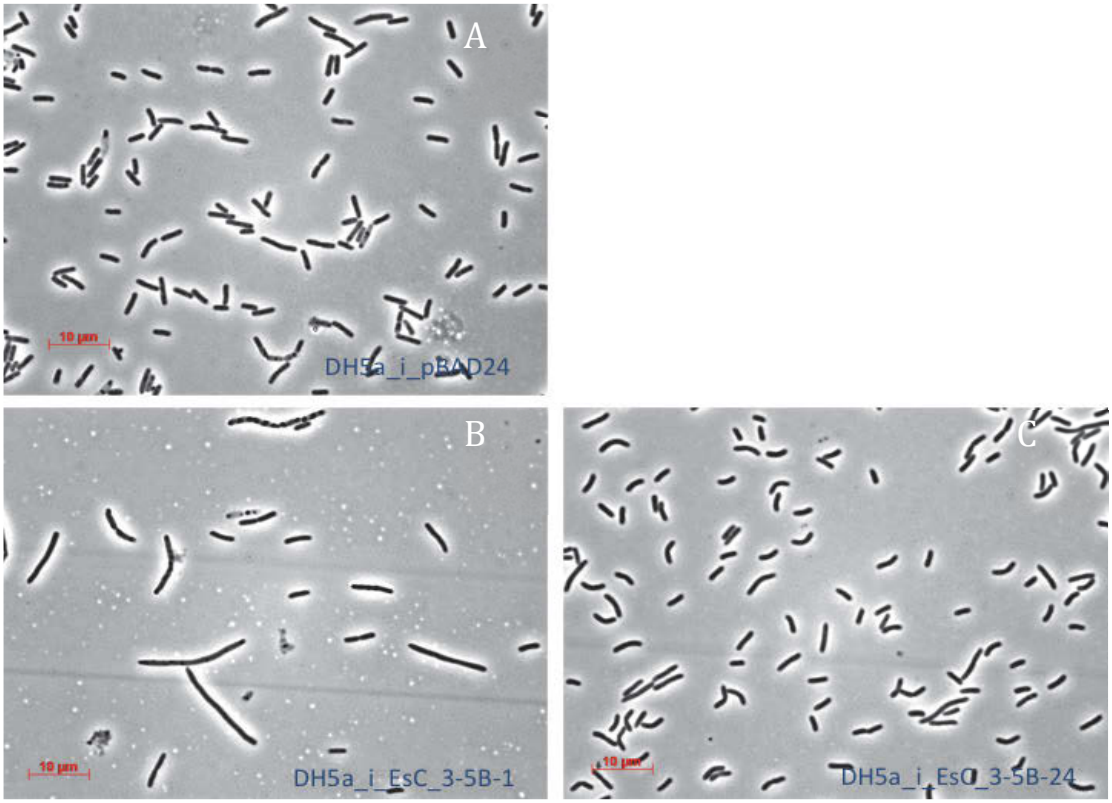

Figure 3.10 - Representative images of each ORF from EsC_3-5B against the vector control (A). ORF-1 (B) shows the observed filamentous phenotype. ORF-2 (C) when induced does not elicit the filamentous phenotype. The prefix "I" indicates that the clone was induced for overexpression.

Figure 3.10 shows that the clone containing ORF 1 gives filamentous cells when overexpressed. This is also shown in Figure 3.11.

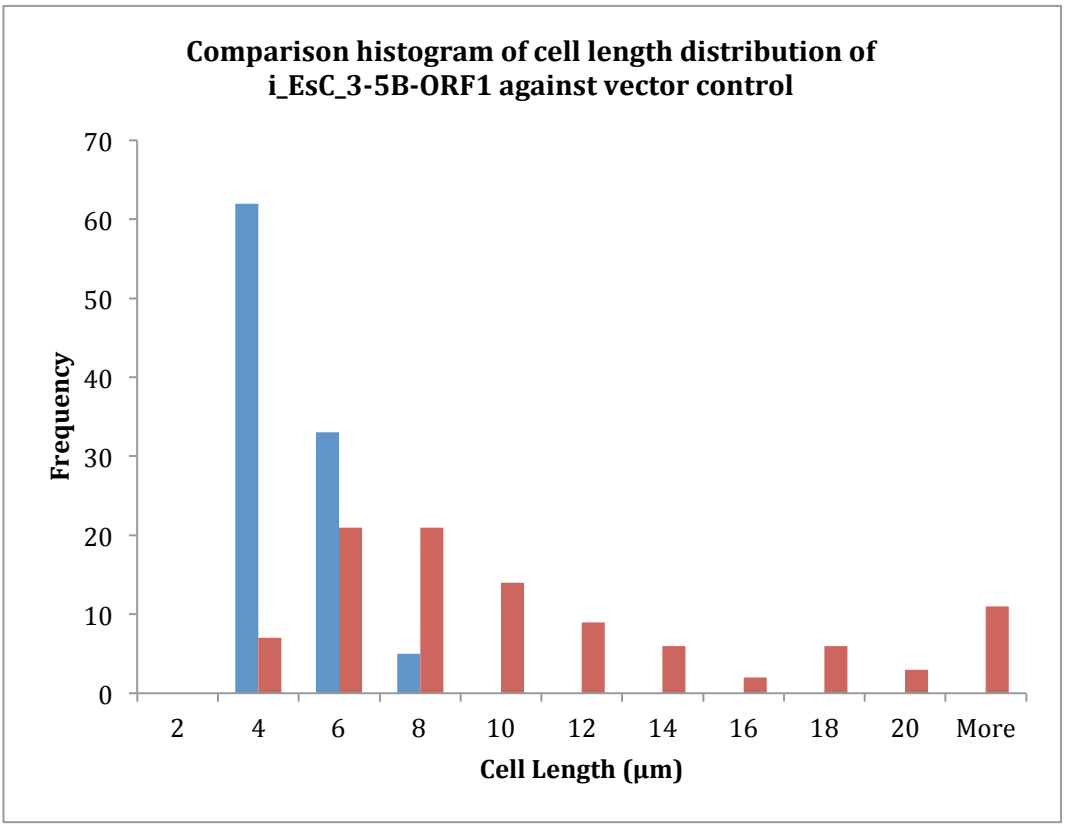

Figure 3.11 - Histogram of cell length distributions of i_EsC_3-5B-ORF1 (red) against the vector control (blue). The prefix "I" indicates that the clone was induced for overexpression.

Figure 3.11 shows a filamentous cell length distribution significantly different (using a Mann-Whitney t-test) from that of the vector control with a percentage of cells greater than 8 µm being 72% compared to the vector control, which was 5%. This data together shows that the filamentation seen in OEC_3 was caused by the overexpression of ORF-1 found in the sub-clone EsC_3-5B. While the data is not directly comparable with the original environmental library or the screen of the sub-clone library due to different expression systems and therefore different levels

of expression, there is an increase of filamentous cells within the population compared to both the control and ORF-2 when induced.

Within the Periplasmic Binding Protein Type-1 Superfamily is the LivK protein, which is the periplasmic component of a leucine specific ABC-type branched-chain amino acid transport system in *E. coli*. The LivK protein in *E. coli* forms a complex with five other proteins as a part of the LIV-transport system and is responsible for the transport of leucine into the bacterial cell [79]. As the protein coded by ORF-1 is similar to and carries the conserved domain present on *E. coli*'s LivK protein, the LivK protein was examined under overexpression conditions to see if the filamentous phenotype seen when ORF-1 is overexpressed is present when *E. coli* has its *livK* gene overexpressed.

To determine whether the *livK* gene of *E. coli* also causes filamentation when overexpressed, *livK* from *E. coli* (MG1655) was amplified and inserted into the pBAD24 vector and expressed using the same conditions as the environmental homologue, the results of which are exemplified in Figure 3.12 below. As can be seen in Figure 3.12 overexpression did not elicit filamentation, which means that there is something about the environmental protein produced by the environmental clone that is unlike that of *E. coli*'s native LivK function. To further examine a possible functional link between LivK and the filamentous phenotype, the phenotype of a *livK* knockout was examined. In bacterial cell division, slight aberrations in regulatory protein levels can cause phenotypic changes [80], and if *livK* plays some regulatory role in cell division then cell division should be inhibited or at least perturbed. A knockout of the *livK* gene in *E. coli* (JW3423-1) was obtained from the Coli Genetic Stock Center (CGSC) from the Keio collection

[81], and the phenotype of this strain observed under normal growth conditions. Again as can be seen in Figure 3.12, there were no cell length abnormalities seen in the knockout clone, indicating that LivK may not be involved in cell division in *E. coli*.

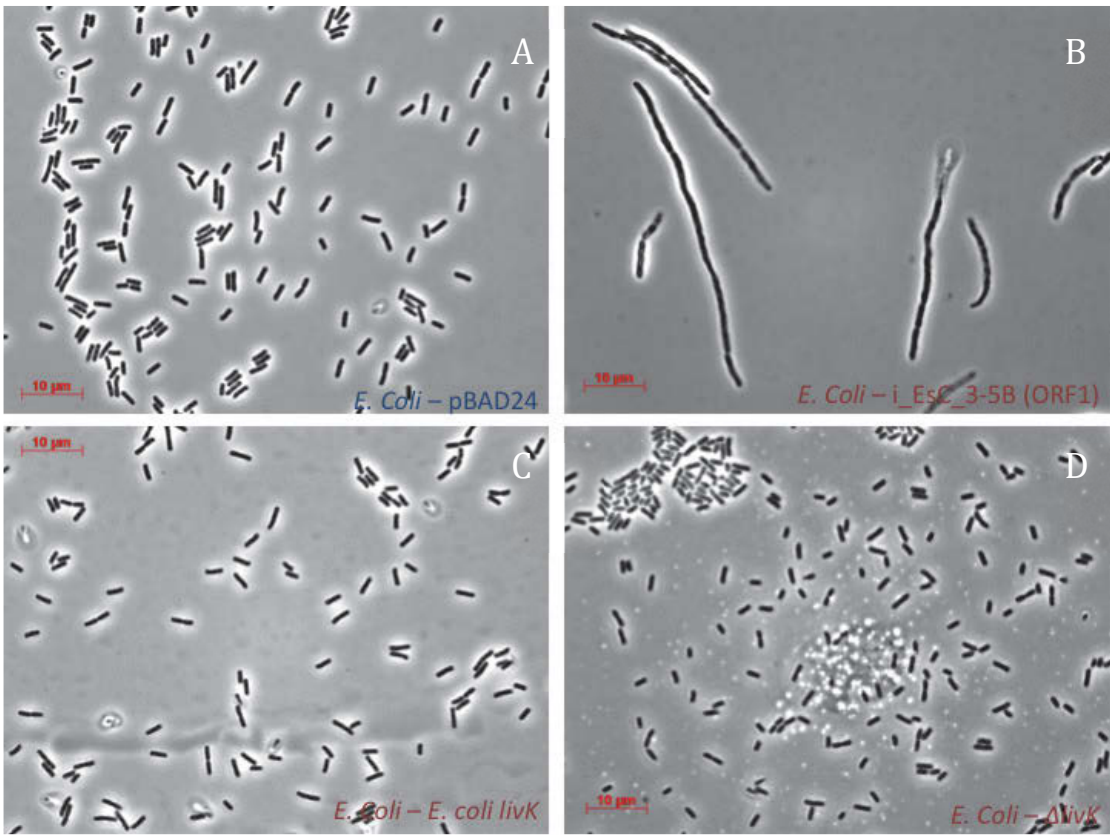

Figure 3.12 - Representative images of each of the *livK* strains with the environmental homologue showing filamentation in B, the *E. coli livK* overexpressed clone with no filamentation in C and the *E. coli livK* deletion also not eliciting filamentation in D. The vector control is in panel A. The prefix "I" indicates that the clone was induced for overexpression.

3.3. Discussion

Environmental bacteria use filamentation to their advantage in a number of situations and in a number of environments, whether it is a mixed microbial community living on a marine algae or a commensal organism living on or inside a human being. The use of strict cell division regulation to either choose to divide or to increase cell length without division based upon the surrounding environment as well as the presence of any stressors confers an advantage against grazing protists or macrophages as well as antibiotics and UV damage.

3.3.1. The environmental library – A proof of concept

The aim of this work was to finish the screen of the environmental library and get to a point where a single or group of genes that elicited a filamentous phenotype when overexpressed could be identified. This would then complete the proof of concept that this screening method using flow cytometry based cell sorting could presumptively identify possible cell division regulatory genes.

A library of environmental DNA collected from a biofilm on the marine algae *Ulva australis* was screened previously for filamentation in *E. coli,* four environmental clones (OEC_1-4) had a filamentous phenotype when screened and each OEC contained upward of 20 genes. Environmental sub-clone libraries were created from each of the four original clones to narrow down the number of possible genes causing the filamentous phenotype. The only sub-clone to be reproducibly filamentous came from OEC_3. The sub-clone contained two full ORFs and ORF-1 was found to be the one causing filamentation.

ORF-1 contained the Periplasmic Binding Protein Type-1 Superfamily conserved domain and we found that the overexpression of this ORF caused a reproducibly filamentous phenotype. It is unlikely that this phenotype result is from a *livK* type function as seen in E. coli where it is the periplasmic binding component of the branched-chain amino-acid transport system specific for leucine, since when *E. coli livK* was overexpressed no filamentation occurred and when the *livK* gene was deleted in *E. coli* there was again no filamentation or change of phenotype observed.

What this may mean is that the *E. coli livK* gene and its translated protein have no frontline role during the cell division process in *E. coli*. What this could mean for the overexpressed environmental clone is that it is somehow inhibiting bacterial cell division by perturbing the cell division process in some way and not actually acting as a negative regulator of bacterial cell division (i.e. interfering with the assembly of the cell division machinery or septation of the divided daughter cells) or by interfering with *E. coli*'s own LivK protein and its ability to bind and transport leucine into the cell. There is also the possibility that the environmental LivK and *E. coli* LivK have differing functions.

3.3.2. Flow cytometry as a screening method for cell division inhibition

Through the course of this work the use of flow cytometry based cell sorting has been shown to be a viable method that can be utilized to screen for cell division inhibitors and regulators. The use of flow cytometry based cell sorting to search for novel bacterial cell division regulators by the change in their cellular

phenotype was successful and a single gene of interest that caused a filamentous phenotype when overexpressed was found.

The use of a first sort for the filamentous population then a re-sort of those collected events greatly enriched the entire filamentous population of events; those as a result of induced expression of cloned genes and those that are filamentous for reasons not to do with overexpression of the cloned genes. When all the collected events were enriched by culturing in the lab, induced and then screened, there was a great number of false positives constituting those clones where filamentation was not due to the overexpression of environmental genes. This has been documented before, where filamentation was seen in a non-inducing population of a vector control in *E. coli* DH5α directly after cell sorting and fixation [63]. This may have been due to induction of the SOS response in these cells due to DNA damage or some other cellular stressor during the procedure [82].

Even though the induced environmental *livK*-like gene does not have any annotated function in cell division, the screening method was able to isolate a single clone where cell division was perturbed and filamentation occurred from a large library of hundreds of clones, thus proving that the screening method works.

3.3.3. The leucine binding protein livK

The identified putative gene causing the filamentous phenotype from the environmental clone contained a Periplasmic Binding Protein Type-1 Superfamily conserved domain and had similarity to branched-chain amino acid transporters, namely the leucine specific periplasmic binding protein LivK. The LivK protein in *E.*

coli forms a complex with five other proteins and is responsible for the transport of leucine specifically into the bacterial cell. The LivK protein is a part of the LS-transport system [79] and is well conserved across bacteria as a transport mechanism.

As yet there is no published role for this protein in bacterial cell division in any organism, but the overexpression of the environmental clone that contained the LivK conserved domain did elicit a reproducibly filamentous phenotype. While the environmental protein could be having a negative regulatory effect on cell division in *E. coli* different to the function of *E. coli*'s own LivK protein, it is also possible that the filamentous phenotype was an artifact of the overexpression of the environmental gene in *E. coli*. This has been noted before where the Tn5 transposase (Tnp) was found to cause filamentation and be lethal to *E. coli* when overexpressed as it was interacting with an essential factor that has a role in cell division or chromosome segregation. The overexpression of Tnp titrated the essential cell division factor below the threshold needed for cell division to occur [83]. This could be the case for LivK protein of *R. lobularis* expressed *in E. coli*, where the expressed LivK is interfering with endogenous LivK and interrupting the function of the protein, which in turn is inhibiting cell division *in E. coli*. As cell division is a tightly regulated process in bacteria, interference of cell division proteins by genes under inducible expression can cause an inhibition to cell division and filamentation.

3.3.4. Future work on this area

For future work in this area, it would be good to look at other gene knockouts in the LIV-system to see if the environmental clone is having an effect on one of those proteins which may be the cause of cell division inhibition. Further to this, overexpression experiments could also be done where other LIV-system genes are investigated. This may identify one of the other proteins in the system as a cell division component.

Another avenue of investigation may be using a defined medium with little to no leucine and see if there is a filamentous phenotype present in either the WT *E. coli* strain, the overexpressed *E. coli* clone or the *E. coli* deletion mutant. This may show that a decrease in the levels of intracellular leucine could be the cause of the blockage of cell division as the LIV-transport system, while being specific for leucine, is not the only means of leucine transport into the cell.

Another tactic could be to create a GFP reporter conjugated to Clone3_ORF-1 to visualize the placement of the protein within the cell and to see if the protein moves to the division sites of the *E. coli* cells (i.e. mid-cell). This may give insight as to what part of the cell division process is being blocked with regards to the environmental clone with the LivK-like conserved domain.

The main conclusion of this chapter is that method of using flow-cytometry based cell sorting to screen for cell division inhibition phenotypes works in that we can identify a single gene responsible for a filamenting phenotype. We successfully identified a putative gene from an environmental bacterium that perturbs cell division causing filamentation in *E. coli*. This proof-of-concept result means that we can use this screening process to look at cell division regulation and attempt to

identify novel cell division genes in other bacteria including the more clinically relevant bacterial genus *Mycobacterium sp.* whose species include *Mycobacterium tuberculosis* the causative organism for tuberculosis (TB).

Chapter 4

Screening *Mycobacterium bovis* DNA for cell division regulators

4. Screening *Mycobacterium bovis* DNA for cell division regulators

4.1. Introduction

Mycobacterium tuberculosis (*Mtb*), the causative bacterium of the tuberculosis disease, is a clinically relevant pathogen with a long and proven track record for lethality. The human pathogen has become drug resistant and the level of resistance is increasing from multi-drug resistant (MDR) (resistant to isoniazid and rifampicin) to extremely drug resistant (XDR) (same resistance as MDR but also fluoroquinolones and second line injectable drugs) [84]. The rise of drug resistance to this pathogen makes clear the need for new drugs to be developed but for this to occur, novel targets for these drugs must be investigated [63].

As mentioned previously in this body of work, filamentation is a useful and important mechanism of survival for bacteria. *M. tuberculosis* grown within THP-1 derived macrophages (a model for what happens during infection) were observed to be filamentous and deficient in mid-cell Z-rings, the primary scaffold for the bacterial divisome [48]. This has led some to speculate that filamentation may be involved in the persistence of Mycobacteria during infection [62] and allow the organism to survive intracellularly within the granuloma that forms as a result of infection but this has not been extensively studied.

The protein Ssd, which promotes filamentation in Mycobacteria, is also associated with induction of the dormancy regulon [51], indicating that *M. tuberculosis* may be able to arrest its own cell division process to enhance its survivability within the human macrophage. The mechanism for this tight regulatory control over cell division is not known in the bacterium and many of the

cell division regulators, proteins and systems that are known in the model organisms *Escherichia coli* and *Bacillus subtilis*, like the min-system have no homologue at least sequence-wise in *Mycobacteria sp*. [6].

Previous studies of cell division in *Mycobacterium sp.* have used various methods including quantitative real-time PCR (QRT PCR) to look at expression level changes of genes under stress [85, 86], electron microscopy to visualise the division and septation of the bacterium [87] as well as single gene deletions [58]. In this chapter, regulators of cell division in *Mycobacterium bovis* BCG were investigated using flow cytometry-based cell sorting, which was employed in Chapter 3 of this thesis and shown to work well with an environmental library of bacterial DNA (see chapter 3), as well as a library of *E. coli* DNA in other work [63].

The use of shotgun genomic overexpression libraries has been shown to be effective in investigating novel cell division regulators in bacteria [63]. The overexpression of a regulatory gene or component of the division machinery can perturb the cell division process and lead to filamentation (cell division inhibition) of the host bacterium. Isolation of these filamentous clones and sequencing the portion of the genome they contain, along with further characterising experiments, can identify novel cell division genes or regulators in the organism the library was derived from as well as indicating novel targets for the development of antimicrobial agents [63].

Mycobacterium bovis (BCG) can be used as a model organism for investigating the cell biology of *Mycobacterium tuberculosis* [35, 88]. The genomes of the two organisms are similar and well conserved, with *M. bovis* BCG being 99.95% identical on a nucleotide level to *M. tuberculosis* in comparative genome

studies [89]. For this work *Mycobacterium bovis* (BCG), rather than *Mycobacterium tuberculosis* DNA was used, as it was deemed safer and more amenable to the PC2 facilities available at this initial stage of screening.

Escherichia coli was to be used as the host organism for this body of work. *E. coli* has been used previously to express and investigate cell division regulatory proteins in *M. tuberculosis* [90]. The investigators expressed a eukaryotic-type Ser/Thr protein kinase, PknA from *M. tuberculosis* and found that the mycobacterial protein phosphorylated both *E. coli* and *M. tuberculosis* FtsZ when co-expressed, resulting in a filamentous phenotype in the *E. coli* host. This indicates that at least some of the cell division processes of bacteria are highly conserved across taxa, provides proof that Mycobacterial cell division genes can be successfully expressed in an *E. coli* host and arrest division and further proves that cell division genes of Mycobacteria can be identified by their expression in an *E. coli* host. *E. coli* also has the benefit of being easier to work with experimentally as it has a faster generation time and the molecular methods used for *E. coli* are well described in the literature [66]. If filamentous *E. coli* clones are found then the filamentation effect of the cloned gene(s) can then be tested in a mycobacterial species. The use of flow cytometry as a screening mechanism has been shown to be effective both through the work of Catherine Burke [63] and in this work (Chapter 3), it was therefore chosen as the screening method for this work.

The aims for the work carried out in this chapter was to build a shotgun genomic expression library of *Mycobacterium bovis* BCG DNA then screen the overexpression library hosted in *E. coli* for filamentous cells using flow cytometry-based cell sorting. The collected filamentous clones were then to be confirmed

using light microscopy then characterised through sequencing and bioinformatics. This then allows for initially testing their role in filamentation using molecular and other microscopy methods.

Unfortunately when the cells were screened using microscopy, there were no filamentous clones seen. The aim was then changed to build the *M. bovis* BCG DNA library in *Mycobacterium smegmatis* using the same screening methods described above. For this to be done a new vector system was used and so another aim of this work was to modify and develop an expression vector able to be used in *Mycobacterium sp.* so that it could be used to screen a library of *M. bovis* BCG DNA. Based upon the advice of our collaborators Jamie Triccas and Warwick Britton and our own investigation, the TET-On/Off expression system was chosen [91]. This system utilizes the specific binding of the constitutively expressed TetR protein to the operator *tetO*. Whilst TetR is bound to the operator there is no expression of the cloned genes downstream of the binding site. When anhydrous-tetracycline (ATc) is added to the culturing media, TetR, which has a higher affinity for tetracycline relieves repression of the operator *tetO* and binds to ATc allowing for the expression of cloned downstream genes, which in this case is a 2-5 kb fragment of the *M. bovis* BCG library.

4.2. Results

4.2.1. Constructing the Mycobacterium bovis BCG DNA library in the expression vector pBAD24 to be hosted in E. coli

For construction of the BCG DNA library in *E. coli* using the pBAD24 vector, the gDNA from BCG (isolated by Rachel Pinto in Jamie Triccas' lab at the University of Sydney) was partially digested with the 4-base cutter *FatI*. These restricted fragments of DNA could then be ligated to the vector backbone that was restricted with *NcoI* (compatible sticky ends). It is worth noting here that the *FatI* palindromic recognition sequence is CATG. This allows restriction of the DNA at the possible start codon of a gene, so that when it is cloned into the expression vector there is a possibility that we are transcribing from the start of the gene and not truncating the gene that is inserted.

To obtain 2-5Kb fragments of BCG gDNA for cloning, the digest time with the *FatI* enzyme had to be optimized. Approximately 1µg of BCG gDNA was placed in a reaction with 0.2 U of the FatI enzyme and 5 µl aliquots were removed and heat inactivated at 5-minute intervals for 50 minutes. Figure 4.1 shows the results of this experiment (refer to methods section 2.5.3).

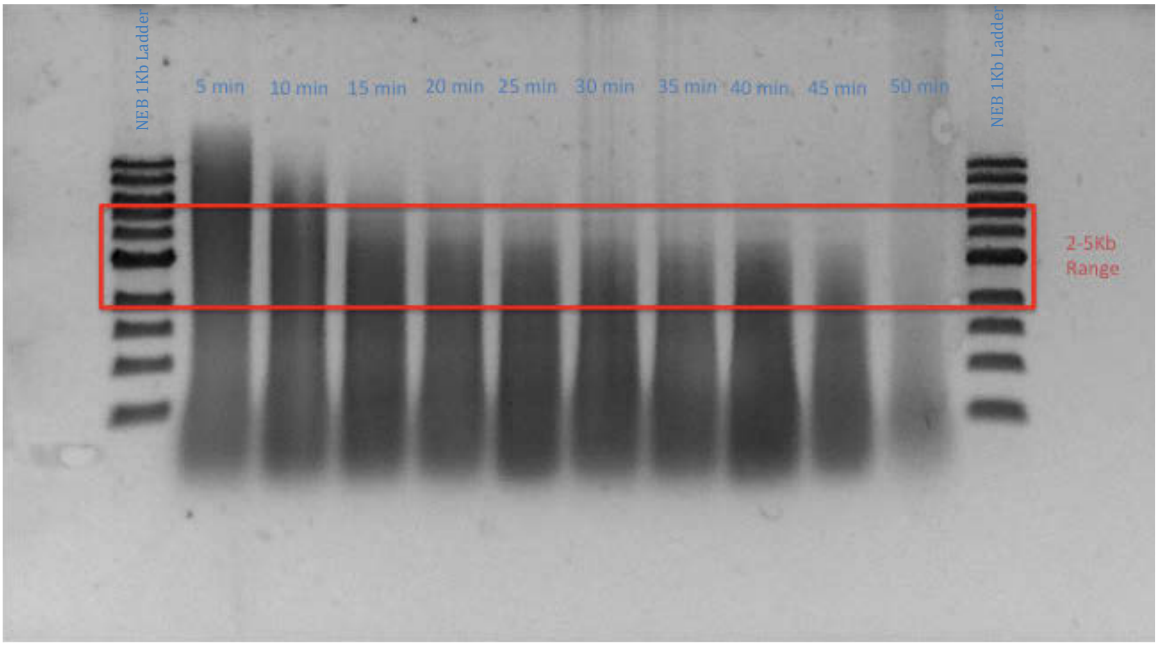

Figure 4.1 - GelRed stained 0.6% agarose gel imaged with UV transillumination showing the time-point digests of BCG gDNA. The aliquot's time-point is given at the top and the red box indicates the 2-5Kb size range of linear DNA according to the NEB 1Kb ladders in lanes 1 and 12.

From Figure 4.1 above the optimal digest time of BCG gDNA using the *FatI* restriction enzyme at 55°C was estimated to be approximately 7 minutes. Using this information the digest reaction was scaled up and the products separated by agarose gel electrophoresis (refer to method 2.5.9). The 2-5Kb DNA fragments were excised from the gel and purified using a gel purification kit as per the manufacturers instructions (Qiagen) but at the end of the process no DNA was detected using the Nanorop (refer to method 2.5.9). The method was repeated with the same result.

An alternative method was then tried called the freeze-squeeze method, which involved snap freezing excised slices of low-melt agarose containing DNA in liquid nitrogen, centrifuging at high speed then heating to 65°C, which was then

repeated twice more to elute DNA that was then cleaned using ethanol precipitation. Unfortunately this method did not yield 2-5 Kb fragments of DNA.

The next method tried was the GELase enzyme kit (Epicenter) (refer to method 2.5.10), which involved incubation of the GELase enzyme with the gel slice containing the DNA of interest, then purifying using ethanol precipitation. This method did yield DNA after the extraction and purification process and can be seen below, faintly, in Figure 4.3 (lane 4).

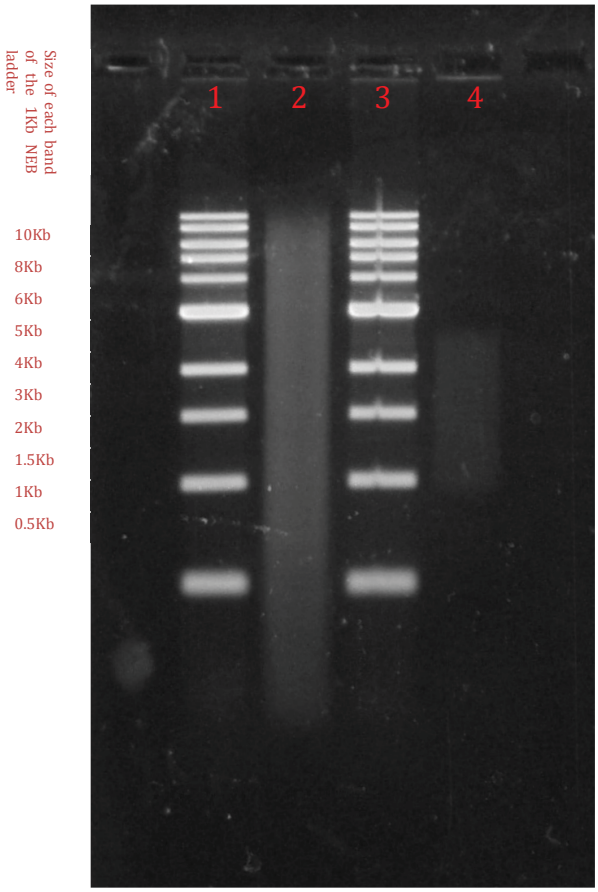

Figure 4.2 - 0.6% agarose gel showing in Lane 2 the FatI digested *E. coli* DNA and in Lane 4 the extracted and purified *E. coli* DNA after using the GELase protocol. The ladders in Lanes 1 and 3 are NEB 1 Kb ladders.

With the method for size selection and purification of the digested *M. bovis* BCG gDNA working, the method was scaled up and performed, ready to be ligated to the NcoI digested pBAD24 vector.

The ligated vector and BCG DNA insert was then transformed via electroporation into *E. coli* (DH5α) and tested for efficiency of transformation by spread plating an appropriate dilution of the transformed cells onto ampicillin (100μg/mL) LB plates. After 17 hours, the colonies that grew on the plates (18 colonies) underwent colony PCR (refer method 2.5.8). Unfortunately there were no positive clones for the insert of the BCG DNA. The negative control plate (no plasmid) had no colonies and the positive control plate (vector only) had approximately $2x10^5$ cfu/mL. The transformations were performed twice more with the same result. It was theorized that the lack of colonies resulting from the ligated vector/insert DNA might be attributed to the restriction/modification (R/M) systems of *E. coli* DH5α digesting the foreign *M. bovis* BCG DNA [92] as the *E. coli* DH5α cells were competent enough to be able to take up plasmid DNA, indicated by the positive control.

To move forward we used the *E. coli* strain lacking in R/M systems ER1793 [92, 93]. We made the strain electrocompetent (refer to method 2.7) and performed the transformation of the *M. bovis* BCG DNA in pBAD24 again. The use of the new strain relieved the problem of transformation efficiency, yielding approximately 47% of clones containing an insert of BCG DNA, with an average size of approximately 2 kb. With this efficiency in mind, and using the same calculation as for determining the number of clones needed for full coverage of the approximately 4.27Mbp genome, we would need approximately 6,605 clones.

When the transformations were performed for the construction of the library, we collected conservatively 60,505 clones meaning we covered the entire *M. bovis* BCG genome 9.2 times. The clones were pooled together and stored as single use aliquots at -80°C.

4.2.2. Screening the Mycobacterium bovis BCG DNA library in E. coli using the expression vector pBAD24 and flow cytometry based cell sorting

The library was screened under arabinose-induction for filamentous *E. coli* cells caused by the expression of the cloned *M. bovis* DNA. As described in Chapter 3, the library was screened using the BD Aria II at the Centenary Institute for Life Sciences in Sydney. Prior to the screen the library, as well as a vector only (pBAD24) control, were grown to mid-exponential phase then diluted down to an OD_{600} of 0.04 and induced with either 0.02% arabinose or 0.2% arabinose and incubated for 3 hours or approximately 4 generations (refer to methods section 2.2.4). Two levels of induction were used with a 10-fold separation to see if different levels of expression elicited different levels of filamentation within the population.

The library was screened as described previously (refer to method 2.10) except that only the 0.2% arabinose induced culture went through a purity re-sort as this was the culture expected to have the highest degree of filamentous cells due to the higher level of gene expression. Table 4.1 below shows the percentages of filamentous events detected via flow cytometry within the area of the scatter plot where filamentous cells were expected to be present (the long gate).

Table 4.1 - Summary of values from the flow cytometry screen of the BCG DNA library hosted in *E. coli*

	Vector Control 0.02%		Vector Control 0.2%	
	Total number of events analysed	Percentage of events in "long" gate	Total number of events analysed	Percentage of events in "long" gate
Initial sort	100,000	0.40%	100,000	0.30%
Purity re-sort	N/A	N/A	N/A	N/A

	*E. coli*_BCG Library 0.02%		*E. coli*_BCG Library 0.2%	
	Total number of events analysed	Percentage of events in "long" gate	Total number of events analysed	Percentage of events in "long" gate
Initial sort	100,000	0.40%	100,000	0.40%
Purity re-sort	N/A	N/A	1,565	5%

Table 4.1 shows the percentage of events in the “long” gate that were derived from the flow-cytometry dot plots. The top panel shows the values for the vector controls at 0.02% and 0.2% arabinose induction and from this data we can see that there is a difference of 0.1% between the two levels of induction. The populations for the *E. coli*_BCG Library have the same percentage of 0.4% for both 0.02% and 0.2% arabinose induction for the initial sort and collection of events in the “Long” gate. However, when the collected events from the 0.2% arabinose induced *E. coli*_BCG Library were screened again, the proportion of cells within the “Long” gate increased to 5%. These events from the re-sort were collected for further growth and screening.

The events that were re-sorted for purity and collected were plated onto LB plates containing ampicillin and incubated at 37°C overnight. 384 colonies grew on the plate (24% of the number of collected events) and these were manually arrayed out into 96-well plates where each colony/clone was allocated an

individual well. Each of these clones was then further tested for filamentation under inducing conditions (refer to methods sections 2.2.4 and 2.11.3), and screened via microscopy. None of the 384 clones isolated from the original screen elicited a filamentous phenotype. Figure 4.5 below shows example images of the negative control (pBAD24 vector only, top left panel) and three of the screened clones collected via flow and visualized for phenotype confirmation (top left and bottom panels). Figure 4.5 shows visually that there is no difference between the screened library clones and the negative control.

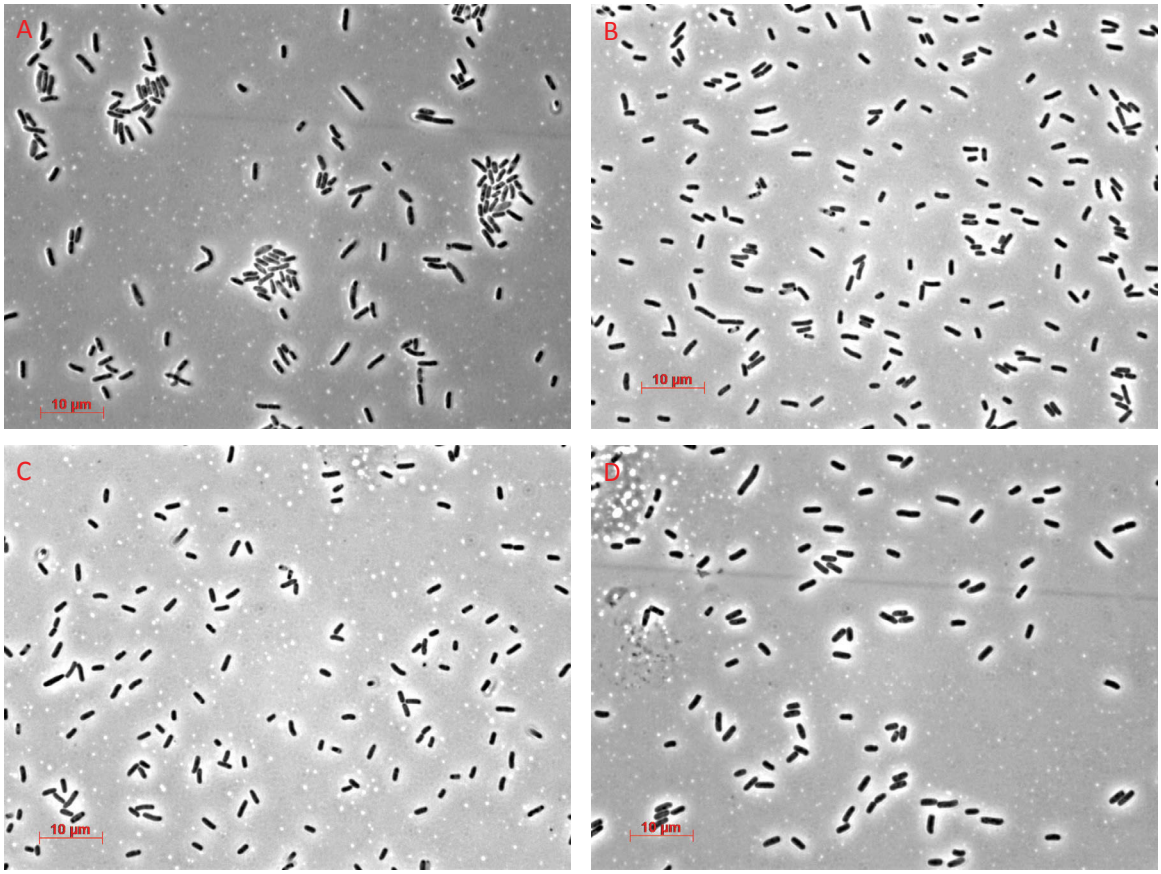

Figure 4.3 - Representative images of screened and collected clones from the *M. bovis* BCG library hosted in *E. coli*. All clones and the control were induced with 0.2% arabinose. A) pBAD24 vector (negative) control. B-D) Representative images of flow cytometry collected clone, further screened using wide-field microscopy. Scale bar is 10 µm.

Despite repeated efforts including growing the library in M9 minimal medium where expression under the arabinose inducible promoter on the pBAD24 vector has been reported to be higher than in rich medium [65], no filamentous clones were isolated from the screen of the BCG DNA library hosted in *E. coli*. As such, an alternative approach was formulated. To overcome possible issues related to the difficulties of expressing mycobacterial DNA in a heterologous host, the approach of creating a library of *M. bovis* DNA to be hosted and screened in the faster growing mycobacterial species *Mycobacterium smegmatis*, was explored.

4.2.3. Determining the appropriateness of M. smegmatis as a host species for the M. bovis DNA library

For the purposes of the planned screen, it was important to determine whether *M. smegmatis* could indeed form filamentous cells when cell division was perturbed, and that these cells could be identified and isolated using the flow cytometry screening protocol. To accomplish this, the *M. tuberculosis ftsZ* gene was overexpressed in *M. smegmatis,* as overexpression of the *ftsZ* gene in bacteria, including mycobacteria, typically leads to a blockage of cell division and eventual bacterial filamentation [94]. The *M. smegmatis* strains mc^{2}155_pJFR11 and mc^{2}155_pJFR14 (obtained from Jamie Triccas' lab at the University of Sydney) were used for this purpose. mc^{2}155_pJFR11 containing an episomal copy of the *M. tuberculosis ftsZ* gene and mc^{2}155_pJFR14 containing a gene coding for Green Fluorescent Protein (GFP) (to be used as a negative control for filamentation) [95].

Before the cells could be induced with acetamide to overexpress the cloned *ftsZ*, a growth curve of *M. smegmatis* WT was constructed (Figure 4.6) (refer to methods section 2.4) so that we could calculate the mid-exponential growth point to induce overexpression of *ftsZ*.

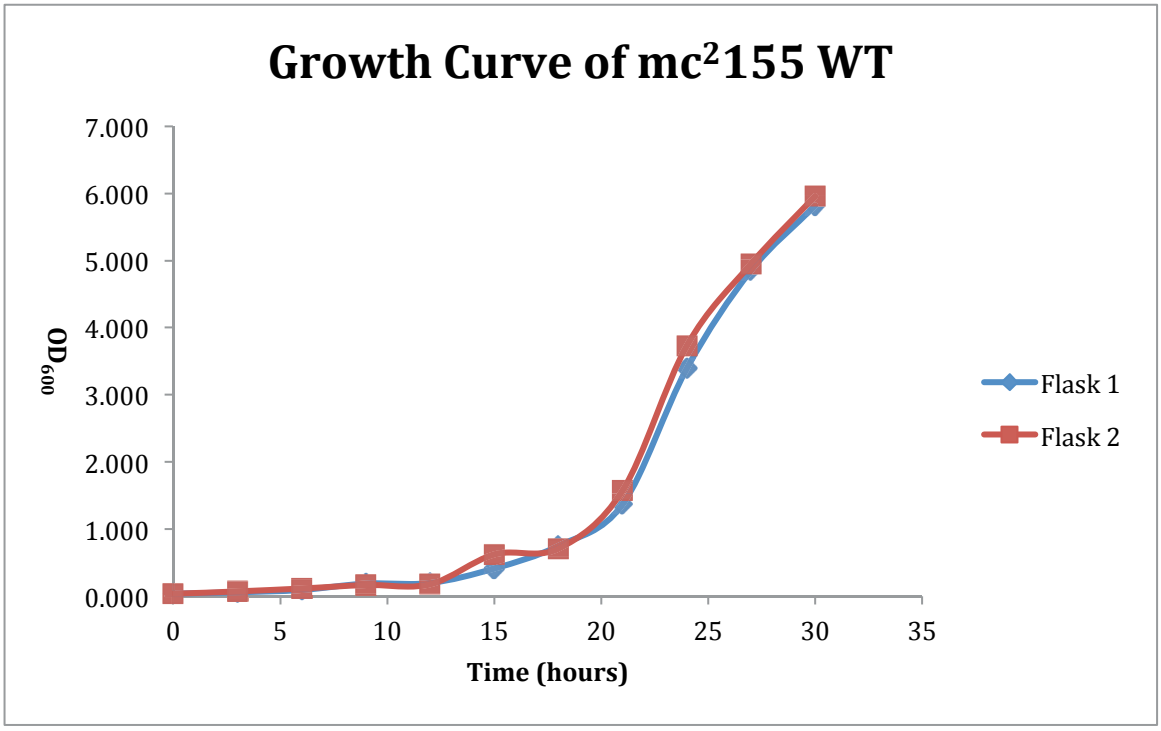

Figure 4.4 - Growth curve of WT *M. smegmatis* mc2155. Optical Density readings at 600 nm were taken every 3 hours over a period of 30 hours. The experiment was run in duplicate and the generation time was calculated from the graph to be approximately 2.5 hours

Once the generation time had been established to be 2.5 hours, the *ftsZ* and WT strains were induced at the mid-exponential point with 0.5% acetamide for approximately 5 generations (refer to method 2.3.1) and screened via flow cytometry (refer to method 2.10). Table 2 below indicates the proportions of filamentous cells detected in populations of WT *M. smegmatis*, and *ftsZ*-overexpressing *M. smegmatis*.

Table 4.2 - Percentage data of the proportion of "long" cells within a population of WT *M. smegmatis* and *M. smegmatis* overexpressing *ftsZ*.

	WT MSMEG 0.5% acetamide		MSMEG_*ftsZ* 0.5% acetamide	
	Total number of events analysed	Percentage of events in "long" gate	Total number of events analysed	Percentage of events in "long" gate
Initial sort	100,000	1.20%	100,000	1.80%
Purity re-sort	N/A	N/A	5,888	12.0%

Table 4.2 above illustrates that *M. smegmatis* containing and overexpressing *ftsZ* has a slight increase of 0.6% in the proportion of events in the "Long" gate over that of the WT *M. smegmatis* control, which can then be increased from 1.8% to 12% via a purity re-sort, which is excellent as the flow cytometry-based screening method is able to be used for an expression library hosted in *M. smegmatis*. The filamentous cells were also visualized and measured using wide-field phase-contrast microscopy to confirm their filamentous phenotype, indicated in Figure 4.7.

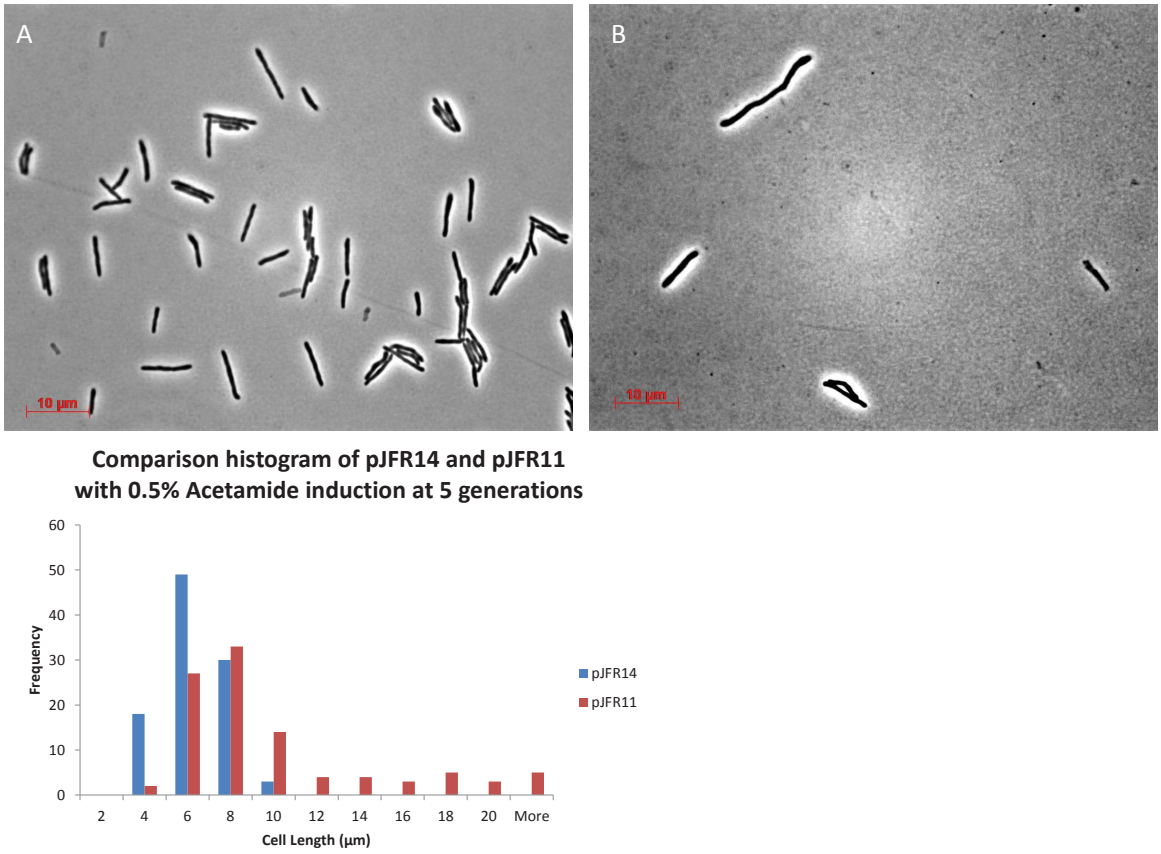

Figure 4.5 - Representative images of *M. smegmatis* mc2155 containing either pJFR14 (vector control) (A) or pJFR11 (*ftsZ*) (B). The graph below the images (C) is a cell length frequency histogram comparing pJFR14 and pJFR11.

Figure 4.7 above shows that the acetamide-induced population of *M. smegmatis* overexpressing *M. tuberculosis ftsZ* from the pJFR11 plasmid (B) does form filamentous cells, differing from the vector control (pJFR14) (B), which is highlighted in the frequency histogram. The mean cell length for the pJFR14 population was 5.5µm with 3% of the population having a cell length greater than or equal to 10µm, the mean cell length of the pJFR11 population on the other hand was 9.2µm with 38% of the population having a cell length greater than or equal to 10µm. The filamentous range for the *M. smegmatis* cells was extended to greater than or equal to 10µm from the value of 8µm for *E. coli*, as the mean cell length of the control population (pJFR14) was 5.5µm and only 3% of the population had a

cell length between 8 and 10 μm with no cells having a greater value. This confirms that *M. smegmatis* can have a filamentous phenotype and that this is a suitable host strain for the library of *M. bovis* BCG DNA with the intent of screening for filamentous cells.

4.2.4. Modification and testing of the new mycobacterial expression vector utilizing the TET-On/Off repressor system

A suitable vector and expression system for investigating mycobacterial cell division genes and regulators in *M. smegmatis* needed to be identified. Based upon the advice of our collaborators and through a search of the literature, the TET-On/Off expression system was chosen [91, 96-98]. The pSE100 plasmid containing the *tetO* operator (kindly provided by the Ehrt lab (Cornell) was designed for cloning of individual genes from Mycobacteria, and does not contain a ribosome binding side (RBS). Because of the nature of the genomic library to be created (a shotgun library of random fragments) an optimized RBS is necessary on the plasmid to ensure for the expression of cloned genes and through a search of the literature one was found [99, 100]. Additionally, there was not a compatible restriction site within the multi-cloning site (MCS) for creation of a shotgun library. As such a new plasmid was created from the pSE100 backbone, which incorporated both a RBS and a compatible restriction site (SphI) for cloning of the partially digested genomic DNA (digested with NlaIII) (Figure 4.8).

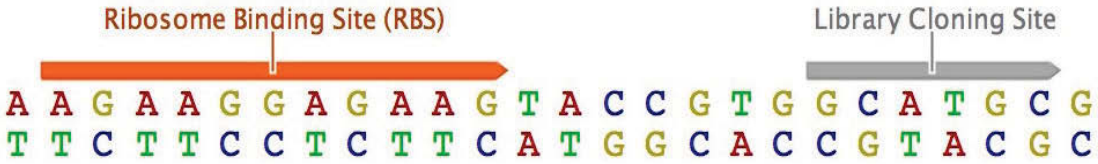

Figure 4.6 - Sequence of the RBS and SphI cloning site that was cloned onto the pSE100 plasmid. The orange bar indicates the RBS and the grey bar indicates the cloning site for the *M. bovis* BCG DNA fragment.

Figure 4.8 above indicates the DNA sequence that was added into the pSE100 plasmid (refer to method 2.6). The orange bar indicates the position of the RBS within the fragment and the grey bar indicates the included *SphI* restriction site compatible with the *M. bovis* BCG DNA library restricted with *NlaIII*. The RBS insert of DNA was directionally cloned into the pSE100 vector between the *SphI* site and the *BamHI* site then transformed into *E. coli* DH5α; clones were recovered and sent to AGRF for sequencing to determine the correct insertion of the DNA sequence. On confirming the correct sequence had been inserted, the modified pSE100 was named pSAM1.

4.2.5. Checking the expression of the pSAM1 vector with the TET-ON system in *M. smegmatis*

The modified vector was tested to confirm that the cloned genes could be expressed, and secondly, that *M. smegmatis* filaments when *M. bovis* BCG *ftsZ* is expressed from pSAM1 in these cells. Thirdly, does the absence of Anhydrous Tetracycline (ATc) in the growth media effectively repress the expression of cloned genes on pSAM1.

To test whether cloned genes are actually expressed when cloned into pSAM1, a **m**onomeric **s**uper-**f**olding **G**reen **F**luorescent **P**rotein (**msfGFP**) [67] was

amplified from pDHL1029 (lab stock) and cloned directionally into the SphI site on pSAM1 (the same site as for the library) then transformed into *M. smegmatis*. Using fluorescence microscopy, the *M. smegmatis* clone containing the msfGFP plasmid was examined in both non-inducing and inducing conditions (refer to method 2.3.1 and 2.11.3) (0ng/mL ATc and 50ng/mL ATc respectively) (Figure 4.9).

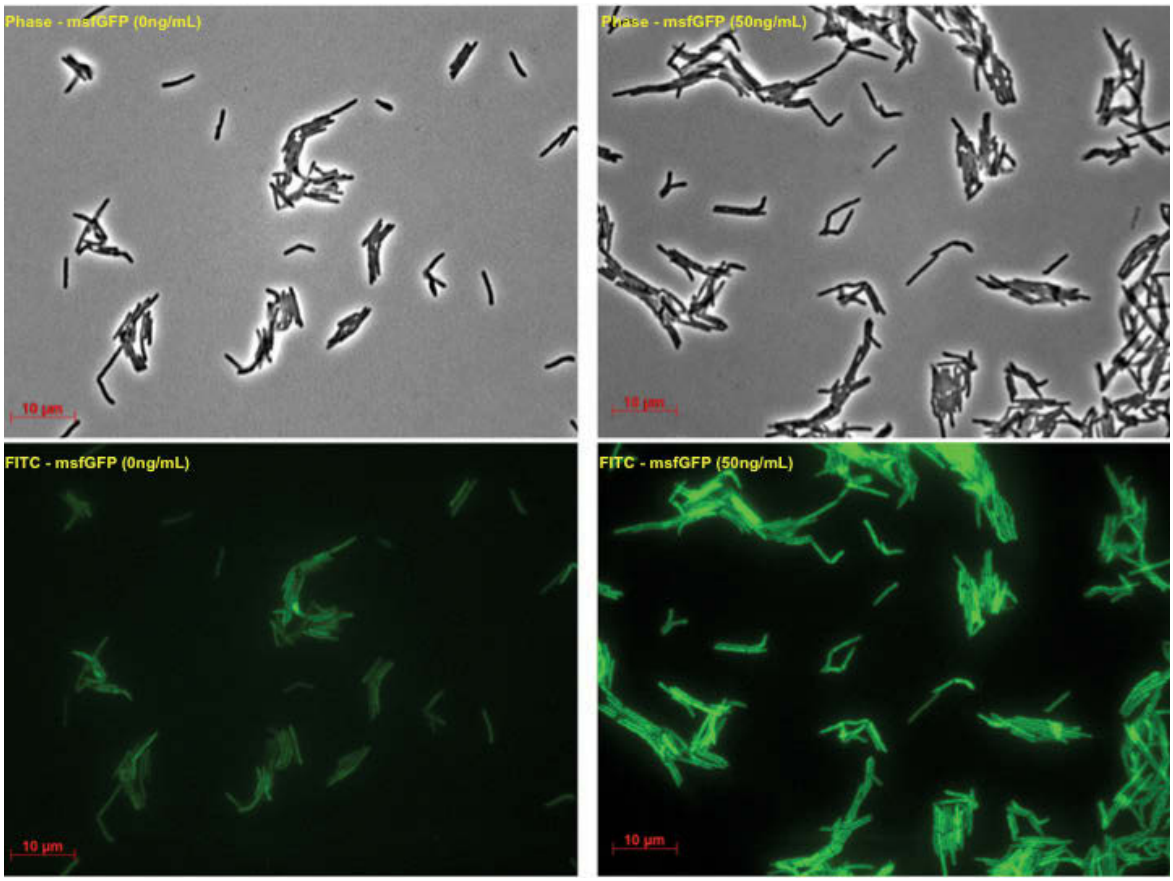

Figure 4.7 - Microscopic images of *M. smegmatis* cells expressing msfGFP at 0ng/mL ATc and 50ng/mL ATc induction. Phase contrast images are above the images of the same field of view for the FITC channel. Scale bar is 10µm.

Figure 4.9 above shows the difference in expression between zero induction and high induction of pSAM1_msfGFP (0 ng/mL and 50 ng/mL ATc respectively). There is a distinct difference in the brightness between the msfGFP expressing *M. smegmatis* cells when there is no inducer and when the inducer is present. With

this information it was determined that the expression system does work although there is some leaky expression of cloned genes when no inducer is added to the growth media. The next step was to determine if the expression system is able to elicit a filamentous phenotype when a cell division related gene is overexpressed.

4.2.6. Expression of the *M. bovis* BCG ftsZ from pSAM1 elicits filamentation in *M. smegmatis*

To determine whether overexpression of *ftsZ* in pSAM1 elicits a filamentous phenotype, the principle cell division gene was amplified from the *M. bovis* BCG chromosome and cloned into pSAM1 (refer to methods section 2.5). An experiment was performed in which cultures of *M. smegmatis* hosting either pSAM1 (vector control) or BCG_*ftsZ* were grown in the presence of ATc inducer and under non-inducing conditions (refer to method 2.3.1). The cells were fixed and examined using phase-contrast microscopy and the cell lengths of the different populations were measured (refer to method 2.11.3). Figure 4.10 below shows the populations of *M. smegmatis* with pSAM1 and BCG_*ftsZ* both induced with 50 ng/mL of ATc.

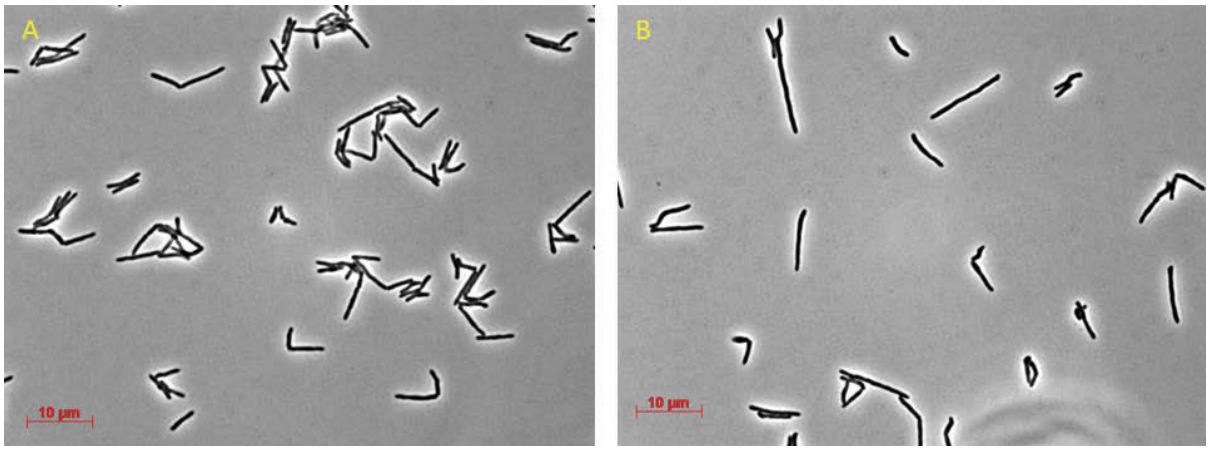

Figure 4.8 - Comparison images of *M. smegmatis* with pSAM1 (A) and BCG_*ftsZ* (B) with 50ng/mL ATc induction

Figure 4.10 shows visually a slight difference between populations of cells, with the cells containing pSAM1 (vector control) only, not eliciting filamentation when induced with 50 ng/mL ATc and the cells containing the BCG_*ftsZ* construct eliciting filamentation when induced with 50 ng/mL ATc. Further to this the cell lengths of these populations were tabulated and cell length histograms were constructed to show the differences between the two cell types under inducing and non-inducing conditions (Figures 4.11, 4.12 and Table 4.3).

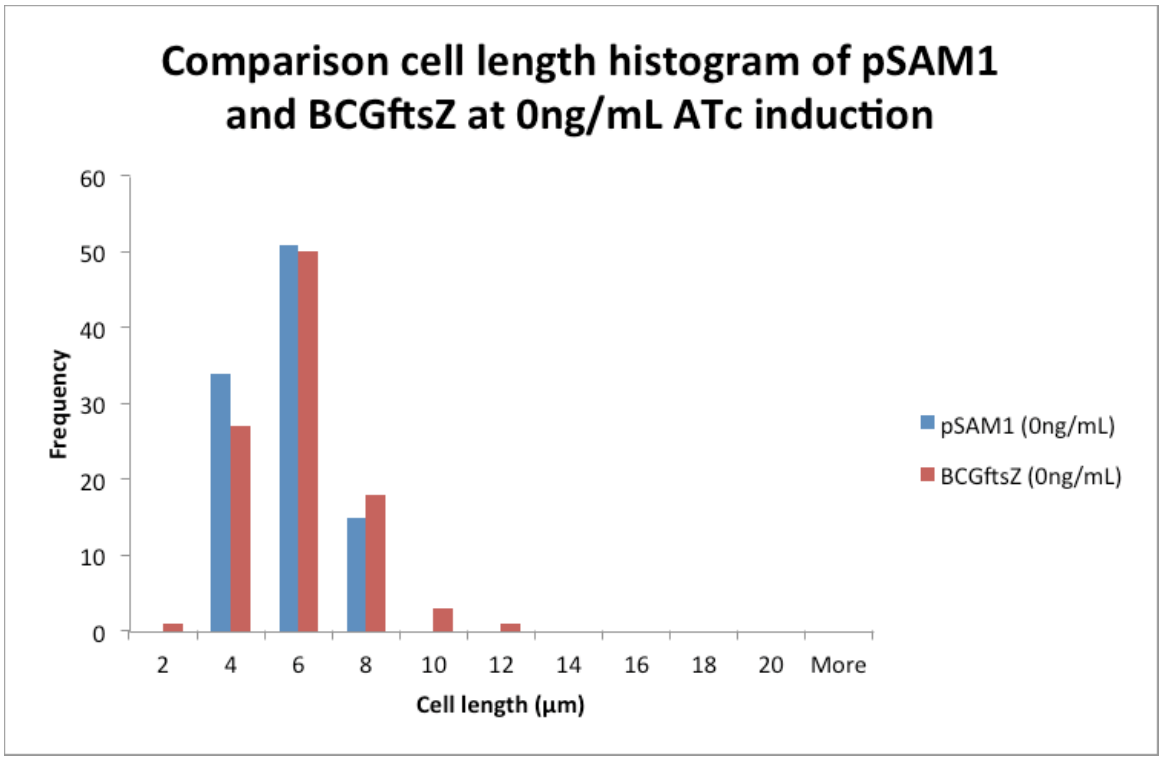

Figure 4.9 - Frequency histogram showing the difference in cell length distribution between vector controls when induced with 0 ng/mL ATC or left uninduced.

Figure 4.11 above indicates that while there is a slight difference between the cell lengths for *M. smegmatis* MSTR1 containing the vector control or BCG_*ftsZ* when left uninduced, this difference was not significant when investigated using a Mann-Whitney test. This data indicates that the small amount of leaked expression

seen in the experiments using the msfGFP would not affect the experiments using BCG_*ftsZ;* nor would it affect the library to be built and screened for filamentous cells.

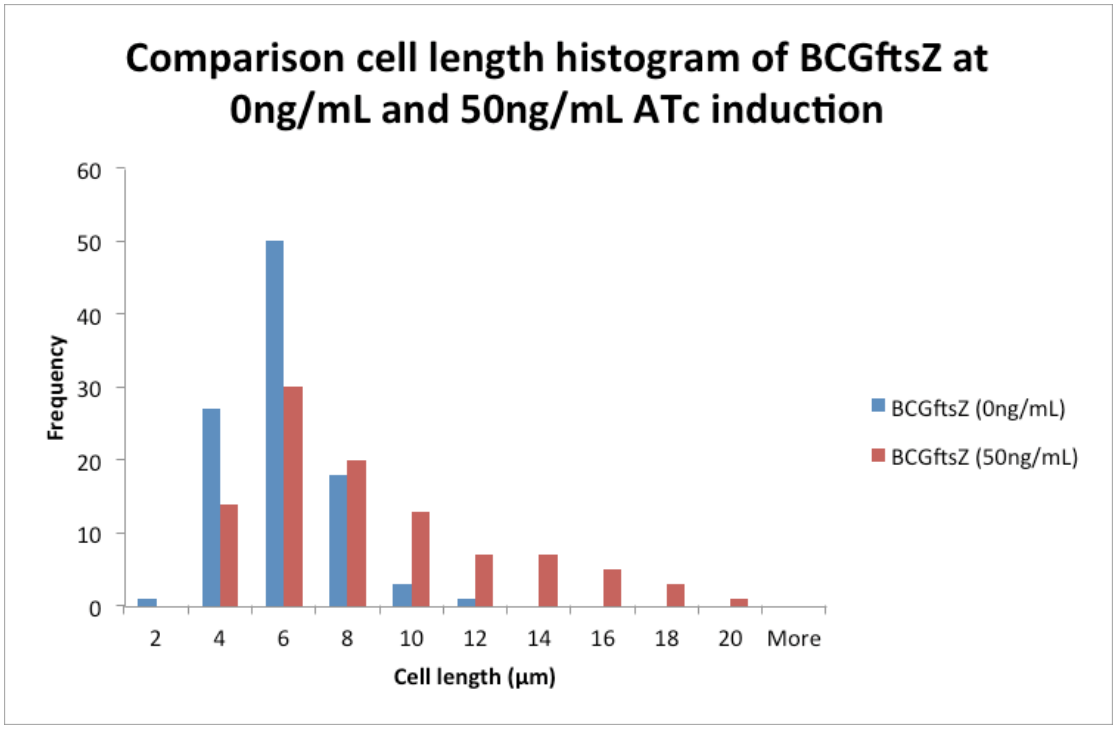

Figure 4.10 - Frequency histogram showing the differences in cell length distributions between *M. smegmatis* MSTR1 cells expressing *M. bovis* BCG_*ftsZ* with 50ng/mL induction with ATc and no induction.

Figure 4.12 illustrates that there is a difference between populations of cells that are induced to express BCG_*ftsZ* and those that are left uninduced. The difference between the induced and non-induced populations of BCG_*ftsZ* was checked for significance using a Mann Whitney test and found to be significantly different with an exact p-value of <0.0001.

Cell lengths were also measured for the vector control in inducing and non-inducing conditions and statistical analysis was also performed on those data sets in the same way as above. This is shown in Table 4.3 below.

Table 4.3 - Basic statistics for pSAM1 (vector control) and BCG_*ftsZ* in *M. smegmatis* MSTR1 when uninduced and induced with 50ng/mL ATc.

	pSAM1		BCG_*ftsZ*	
	0ng/mL	50ng/mL	0ng/mL	50ng/mL
Number of cells	100	100	100	100
Mean cell length (µm)	4.693	4.614	5.016	7.572
Std. Deviation	1.311	1.349	1.553	3.836
Std. Error of Mean	0.1311	0.1349	0.1553	0.3836
Percentage of cells ≥10µm	0%	1%	4%	36%

Table 4.3 highlights the percentage of cells for each population either grown in inducing or non-inducing conditions. What can be seen is that there is a 35% increase in the number of cells that are filamentous (≥ 10 µm) for the BCG-_*ftsZ* population when compared to the pSAM1 population grown in inducing conditions. What can also be seen is that there is no significant difference between the non-induced populations for both pSAM1 (vector control) and BCG_*ftsZ* as well as the induced population for pSAM1.

Further statistical analysis was done using the Mann-Whitney test on each population of cells and it was found that there was no significant difference between both induced and non-induced populations of *M. smegmatis* MSTR1 containing pSAM1 and the non-induced population of cells containing *M. bovis* BCG_*ftsZ*.

What this data shows is that under ATc induction of *ftsZ* gene expression, using 50 ng/mL ATc, elicits filamentation of cells in a significant proportion of the population. This indicates that the modified vector system can be used as expression of cloned cell division genes or regulators would yield a filamentous phenotype that could then be screened using flow cytometry based cell sorting.

4.2.7. Building a *M. bovis* BCG chrDNA library in *M. smegmatis*

A shotgun library of gDNA from *M. bovis* BCG was cloned into pSAM1 using DNA received from collaborators (Jamie Triccas and Rachel Pinto at the University of Sydney) (refer to methods section 2.5). Initial attempts at library construction in *E. coli* yielded only 320 cfu/mL. 32 colonies were checked for the presence of an insert in the plasmid using colony PCR, however no inserts were detected.

A second attempt at library construction yielded a $2.6x10^3$ cfu/mL for the *M. bovis* BCG DNA library in *E. coli* ER1793. The controls were as expected and 32 colonies were randomly picked from the library plates for colony PCR to test the insertion but again there were no clones that contained the correct insert.

The next steps were to determine what has occurred that has impacted the ligation of digested *M. bovis* BCG DNA to the restricted pSAM1 vector. A series of experiments were done to determine if the pSAM1 expression vector was at fault, i.e. whether digested DNA fragments could be ligated to the *SphI* restricted pSAM1 vector. *E. coli* DH5α chrDNA was partially digested and ligated to the pSAM1 vector in the same way as for the *M. bovis* BCG DNA (refer to methods section 2.5) but without the gel extraction step. When colony PCR was done on 24 recovered clones,

15 of the clones had insert present indicating that the problem did not lie with the ligation of insert DNA to the vector.

The *M. bovis* BCG chrDNA was checked to see if there was a problem with the DNA preparation that was received. Gel electrophoresis (refer to method 2.5.9) indicated that the DNA had degraded (Figure 4.13).

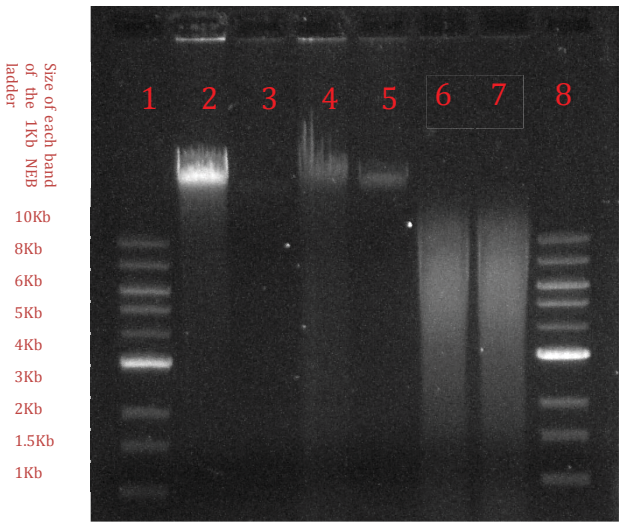

Figure 4.11 - UV transillumination image of different *M. bovis* BCG chrDNA preparations received. Lanes 1 and 8 each contain 500ng of NEB 1kb DNA ladder. Lane 2 is *E. coli* DH5α chrDNA. Lanes 3-7 are different *M. bovis* BCG chrDNA preparations that were received. Note the degradation of the chrDNA in lanes 6 and 7.

Figure 4.13 above indicates the degradation of the *M. bovis* BCG chrDNA. The cause of the degradation is undetermined, as the method for preparation had not changed since the initial preparations. Fortunately there was enough of the earlier chrDNA preparations which when added together would be enough to build a single library.

The pooled *M. bovis* BCG chrDNA was partially digested with the restriction endonuclease *NlaIII* (refer to method 2.5.3), size selected for 2-5kb fragments (Figure 4.14 below) (refer to method 2.5.10) and ligated to *SphI* restricted pSAM1

(refer to method 2.5.3 and 2.5.5). The ligation reaction was transformed into *E. coli* ER1793 via electroporation and plated onto appropriate media along with positive (ER1793 with vector only) and negative (ER1793 only) controls (refer to method 2.7).

Figure 4.14 shows the partial digestion of the *M. bovis* BCG chrDNA (Lane 3) compared to the chrDNA prior to digestion (Lane 2), as can be seen from the image and especially lane 3, the chrDNA has been partially digested and fortunately does not look degraded like the other previous chrDNA preparations (Figure 4.13, Lanes 6 and 7).

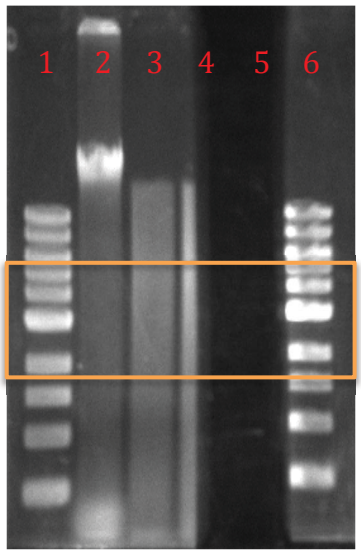

Figure 4.12 - UV transillumination image of the partial digest of *M. bovis* BCG chrDNA. The ladders (lanes 1 and 6) are NEB 1kb DNA ladders. The blacked out section (lanes 4 and 5) are the gel lanes of DNA to be size selected. Lane 2 is an aliquot of *M. bovis* BCG chrDNA and lane 3 represents the digested chrDNA. The orange box represents the 2-5kb region of DNA fragments.

Unfortunately again there were no colonies present on the plate neither for the M. bovis BCG DNA library nor on the plate for the negative control but there were too many to count on the positive control plate even at $1x10^3$ dilution. The

transformations were attempted twice more with the same negative result for library clones.

4.3. Discussion

The original aim for this body of work was to build a shotgun genomic library of 2-5kb DNA fragments from *M. bovis* BCG contained within the arabinose inducible pBAD24 vector in *E. coli* covering the entire genome of the organism. This library was then screened for clones eliciting filamentation when the cloned genes were induced to a high expression level. After screening the library no clones were found that could reproduce a filamentous phenotype after collection from the flow cytometer.

During this work there was also a change in the host organism from *E. coli* DH5α to *E. coli* ER1793. DH5α is the traditional cloning strain for *E. coli* but we were unable to generate clones although other studies had been able to clone Mycobacterial DNA into this strain [56]. A review of the literature indicates that this kind of expression library has not been constructed before using mycobacterial DNA and when libraries have been built, it was done through the use of mycobacterial fosmids with large inserts [101] or packaged into phage [102]. This being the case, we changed the host *E. coli* strain to ER1793, a strain that lacks restriction/modification systems based on literature [92] and on the advice of colleagues; using this strain we were able to collect the clones necessary for a complete library. It is unknown why library clones in *E. coli* DH5α were unable to be generated but because clones were generated using *E. coli* ER1793 a strain lacking restriction/modification systems, there is a possibility that the *M. bovis* BCG DNA hosted in *E. coli* DH5α was being digested possibly based upon the methylation patterns of that DNA [101].

We were able to build the *M. bovis* BCG DNA library in *E. coli* ER1793 but when that library was screened using both flow cytometry and microscopy there were no reproducibly filamentous clones. This could be due to the heterologous expression of cloned genes and differences in codon usage between *M. bovis* BCG (has a G-C content of 65.6% [103]) and *E. coli* (has a G-C content of 50% [104]) as it has been documented that the differences in codon usage between the G-C rich *Mycobacterium avium* and A-T rich *Lactobacillus salivarius* species, which can hamper protein synthesis [105]. As this may have been the case and repeated attempts to generate filamentous clones in *E. coli* using *M. bovis* BCG DNA in pBAD24 failed, the aim was modified so that a library of *M. bovis* BCG DNA was to be screened in *M. smegmatis*, an organism more closely related to the organism we wished to investigate. This may in fact be a better experiment because codon usage would be less of a problem and as the project aims to investigate cell division regulators and genes in *Mycobacterium sp.* the use of *M. smegmatis* as a host strain for the library would generate more robust results.

As the library was to be hosted in *M. smegmatis*, it was not a simple matter of shuttling the pBAD24 *M. bovis* BCG library into the new *M. smegmatis* host organism as the pBAD24 vector system does not have an origin of replication compatible with Mycobacteria [65]. This being the case, a new vector system was needed and through investigation and recommendations from collaborators the TET-ON/OFF system was chosen to be the vector system for the library [96]. The modified vector pSAM1 was able to express the principle cell division gene *ftsZ* and with a high level of ATc induction did elicit a filamentous phenotype consistent with what is seen in other organisms during overexpression of *ftsZ* [106]. The *M. bovis* BCG DNA library was then built using the pSAM1 vector but unfortunately

this could not be done due to reasons including poor DNA preparation and time constraints.

As unfortunate as that is, the vector system that has been modified to express single genes can be used as a fluorescent reporter construct utilizing the cloned msfGFP for use within mycobacterial species, an area that is lacking in reliable fluorescent reporters which can be utilized with techniques such as fluorescence microscopy and flow cytometry. As the vector carries an origin of replication for both *E. coli* and *Mycobacterium sp.* constructs can be built in the faster growing *E. coli* and shuttled over to mycobacterial species. To move forward in an attempt to create a shotgun DNA library of the *M. bovis* BCG genome, it is imperative that good quality template gDNA is used, as this would increase the efficiency of the process and while this approach is feasible currently there are also other methods that are worth looking into which may in the end lead to the same result. A Method such as random inducible controlled gene expression (RICE) that has been used in mycobacteria to identify genes involved in adherence, entry and survival within human macrophages [107]. Or, perhaps using a different mycobacterial plasmid vector like pMycoFos, which the authors state has copy number control and inducible gene expression but has also allowed the cloning and expression of an ethane monooxygenase gene cluster that had previously been unable to be done due to heterologous gene expression [101].

Chapter 5

General Discussion

5. General Discussion

5.1. Introduction

Mycobacterium tuberculosis the causative organism of tuberculosis (TB) has been afflicting humanity for centuries and will continue to do so until infection with *M. tuberculosis* can be eradicated. With a rise of multi-drug resistant strains of *M. tuberculosis* [108], the emergence of extremely-drug resistant [109, 110] and the reporting of totally-drug resistant strains of *M. tuberculosis* [8, 15] new strategies are needed to effectively combat this human pathogen. One method to control infection is to stop the essential process of cell division through the use of a targeted drug to bacterial cell division [9, 18, 19]. The inhibition of bacterial cell division in rod-shaped cells leads to a filamentous phenotype, where the bacterial cell continues to elongate without dividing into two identical daughter cells [51, 62, 83, 85, 106]. Without relief of the cell division block the bacterial cell will lyse and in the case of *M. tuberculosis* clear the infection. To utilize this method against *M. tuberculosis* new cell division targets need to be investigated for the development of the next generation of antibiotics and as little is known about cell division in *Mycobacterium sp.* when compared to model bacteria like *Escherichia coli* and *Bacillus subtilis*, investigation in this area can give new insight into how the essential process of cell division is carried out.

Filamentation is seen when bacterial cell division has been inhibited but cell growth continues and has been identified in various bacterial species such as *Escherichia coli* [69] and *Mycobacterium tuberculosis* [48]. The phenotype has been seen in situations where bacteria have come under some form of stress including DNA damage [73], grazing protist evasion in marine environments [71] and human

host evasion [111]. The filamentous phenotype allows bacteria to survive under these conditions and then when the stress has passed, divide and grow as normal.

This work was started as a means to screen for and discover novel and hopefully well conserved regulators or genes responsible for bacterial cell division as a first step in a process hopefully ending in a new antimicrobial. As cell division is essential and the genes that it requires relatively well conserved across bacteria, a viable a target could have a broad-spectrum effect and be a step forward in combating the rising tide of antibiotic resistance [9, 19]. This study was also a means to investigate cell division of the clinically relevant *Mycobacterium sp.*. As discussed in the introduction of this work, in comparison to the model bacterial organisms *Escherichia coli* and *Bacillus subtilis*, not as much is known about bacterial cell division in Mycobacteria even though it is a clinically relevant genus that warrants further examination [84].

The aims of this work were to create and screen a sub-clone shotgun expression library of a previously screened library of environmental DNA isolated from the surface of the marine algae *Ulva australis*. The screening method utilized flow cytometry based cell sorting to discriminate between populations of *E. coli* cells that were filamentous as a result of expression of cloned genes, these filamentous cells were then collected and the genes responsible for the filamentous phenotype elucidated through wide-field microscopy, sequencing and bioinformatics. This was done to complete a series of experiments that were proving a concept that the flow cytometry based method for screening filamentous cells could lead to the identification of novel cell division inhibitors.

Once the concept was proven to be viable, the second aim of this work was to apply the flow cytometry based screening method to the clinically relevant genus *Mycobacterium sp.* through the construction and screen of an expression library of *Mycobacterium bovis* BCG gDNA. Clones shown to have a reproducibly filamentous phenotype would then be investigated as in the first aim above to isolate novel cell division genes or regulators in mycobacteria with the hope of identifying novel targets antibacterial drug development.

5.2. Using flow cytometry based cell sorting and microscopy as a screening method for bacterial cell division inhibition

Flow cytometry based cell sorting has been shown to be effective in screening for bacterial cells that have a filamentous phenotype as a result of cell division inhibition both in this work and in Burke *et al.* 2013 [63]. The method relies upon the differences in side-scatter profiles between filamentous cells and the normal wild-type (WT) phenotype and is very good at capturing a small proportion of filamentous cells from a larger population. A limitation of this method is that cells that are filamentous at the time of the screen and are therefore collected may be filamentous for reasons other than the expression of the cloned genes of the library. Filamentation may be elicited through the induction of the SOS response as a result of DNA damage [73] or little nutrient availability [112] or other as yet unknown reasons and not because we have overexpressed a cloned cell division gene or regulator. It is also worth noting that strains of *E. coli* have varying levels of filamentation when compared to other strains of *E. coli*, for example the BL21 strain of *E. coli* has a higher proportion of filamentous cells

within the population when compared to the DH5α strain of *E. coli* (Burke, personal communication). As the flow cytometry method cannot distinguish between these differing types of filamentation, it is necessary to confirm the filamentous phenotype and to make sure that the phenotype is reproducible and not transient as reproducibility would indicate that the filamentation seen is as a result of overexpression of the cloned genes from, as the case is here, the DNA library. To confirm the filamentous phenotype we used light microscopy in this study, which was also used by Burke *et al.* 2013 [63]. The problem with using this method is the time consuming nature of the screen where each of the collected filamentous clones from the flow cytometry screen are visualized individually. This would not be such an issue if there were a higher proportion of filamentous clones as a result of the cloned genes being expressed but as there is a low hit rate from the expression library and the flow cytometry screen is collecting all of the filamentous cells within the population regardless of the cause of the phenotype the confirmatory screen is time consuming using the wide-field microscopy method.

One method that could overcome the time consuming nature of the confirmatory screen is using a high-content analyzer (HCA) for a high throughput microscopy screen. This method could be adapted to be the confirmatory screen of clones collected from the flow cytometry screen and as the microscopy process is quick and relatively well automated, the screen could be performed in a fraction of the time it took to do the screen manually. Image analysis software has come such a long way recently as well, to such an extent where batch analysis of images can be done, which can analyze different parameters including cell shape, size and length automatically. With these two technologies, microscopy screening for cell

division inhibition phenotypes can be rapid and quicken the pace that data can be generated.

With the cost of DNA sequencing declining rapidly and the power of bioinformatic tools increasing, there is another method that is proving to be useful as a confirmatory screen for filamentous clones and is being used by Daniel Mediati in Dr. Iain Duggin's lab at The University of Technology, Sydney (UTS). Performing large-scale plasmid DNA preparations of collected clones from a flow cytometry screen of filamentous clones and then sequencing the cloned genes (Mediati, personal communication). This method benefits from having a complete reference genome of the organism the library came from to where the sequencing reads from the plasmids can be aligned. If you have an area of the reference genome with a large number of library DNA alignments, then that would indicate a gene or region of the genome where the overexpression of those gene(s) results in bacterial filamentation and therefore may be a regulator or at least important for bacterial cell division. There can also be an internal control with this method as known cell division genes like *ftsZ* when overexpressed cause cell division inhibition and filamentation [113] and if this gene has a high number of reads aligning to the reference genome then it shows that filamentous clones are being found. Another method to accomplish this would be to align the plasmid sequences against one another then searching nucleotide databases for similar genes. The use of next-generation sequencing as a part of a high-throughput screen has been used in conjunction with phage display libraries and biopanning [114-117], where a library of phage particles with random surface peptide sequences are exposed to a target (in the case of Ngubane *et al*. 2013 it was *M. tuberculosis*). The bound phages are then eluted and enriched through infection of a host bacterium. High-

throughput sequencing is then used to sequence and analyze the millions of DNA inserts and the corresponding peptides with the higher number of sequencing reads corresponding to and identifying the peptides with the best binding efficiency [114]. Though a few limitations with this method outlined by Rentero Rebollo *et al*. were the lack of open-access computational tools to analyze all of the sequences generated with the high-throughput screening method and sequencing errors misleading computational algorithms designed to identify consensus sequences and sequence similarities [117]. These were overcome through the development of their own analysis software that as well as being able to analyze high-throughput sequencing reads could also filter out the sequencing errors leading to a more robust data set [117].

Transposon **D**irected **I**nsertion **S**equencing (TraDIS) [118] would also be a viable method for the detection and identification of cell division genes and regulators within a bacterial genome. TraDIS could, as an example, be applied to the four original filamentous clones screened by Dr. Catherine Burke. Sub-clone libraries could be constructed using random transposon insertions that would knockout each of the genes within the large 40 kb clone. These transposon libraries could then be screened for clones that have reverted to the WT cell length phenotype. If a clone exhibiting the WT cell length was identified the DNA sequence surrounding the transposon insertion site would then be sequenced and a possible causative gene for the filamentous phenotype seen in the original environmental clone elucidated.

If the initial screen done in Chapter 3 of this thesis were to be done today, it would be good to use the HCA method as a confirmatory screen for filamentous

bacteria as it would be quicker than what was done at the time. The use of the sequencing method however could not be done with the environmental libraries, as there was no reference genome of an organism to work with due to the fact we do not know what organism or group of organisms the DNA came from. Metagenomic sequencing could be done to generate a reference meta-genome but it is expensive to do. If the *M. bovis* BCG DNA library had been created then the sequencing method would have been used as a confirmatory screen as we have a reference genome.

5.3. Shotgun DNA expression libraries as an investigative tool for discovering novel cell division regulators and genes

In this work shotgun DNA expression libraries were constructed and screened for genes that inhibit cell division when overexpressed. This is a method that Burke *et al* 2013 had used to great effect in screening for cell division genes and regulators in *B. subtilis* and *E. coli* [63]. We were able to isolate a gene of interest within the environmental library (Chapter 3) to complete the proof of concept for the following work to be done on *M. bovis* BCG. While the work done on *M. bovis* BCG was unable to bear fruit, the method of construction for the libraries is sound, just not with *M. bovis* BCG.

The initial shotgun genomic DNA library of *M. bovis* BCG DNA constructed using the pBAD24 vector system and hosted in *E. coli* did work in so far as the library was built and screened but no filamentous clones within the library were found using the confirmatory microscope screen. In the discussion of Chapter 4 differences in codon usage between high G+C content genomes [105, 119] and

others was hypothesized as the reason for the inability to generate filamentous cells with the library in *E. coli*. *M. tuberculosis* has for example a G+C content of 65.6% [120] to *E. coli*'s G+C content of 50.8% [121]. Codon usage also differs between organisms with different growth rates and translational efficiency with the relative number of tRNAs at different points of the growth cycle having an effect on the efficiency of translating expressed genes [122]. It has also been shown that mRNA that consists of preferred codons for the organism is translated faster than mRNA consisting of rarer codons [123]. When this information is applied to what we were doing and while it is true that overexpression of *M. tuberculosis ftsZ* in *E. coli* will inhibit cell division and form filamentous cells [94], in the case of the library screen we were hoping to overexpress all the genes within the *M. bovis* BCG genome, including those that are not expressed at high rates and which are more likely to contain rarer codons and genes that are not as well conserved as *ftsZ*.

Another possible reason filamentous clones were unable to be generated through the expression of cloned *M. bovis* BCG DNA hosted in *E. coli* may be that the translated proteins from the expressed library were insoluble as it has been reported that only a third of proteins from *M. tuberculosis* are soluble in *E. coli* [124]. One way to overcome this shortfall in soluble protein expression would be to construct the library to be expressed in the faster growing species *M. smegmatis*, which is becoming the workhorse for the expression and production of recombinant proteins for mycobacteria.

5.4. Future work

Cell division can prove to be a valuable target for antimicrobial development as it is essential for survival [19]. Conversely bacterial filamentation as a part of cell division inhibition is also important for survivability of an organism in varying environmental niches including that of a human host [48, 111]. If filamentation can be prevented in organisms like Uropathogenic *Escherichia coli* (UPEC) and *Mycobacterium tuberculosis* then that may lead to a new form of treatment for infection. Rather than relying on antibiotics to eliminate infection themselves, target the survival mechanisms that bacteria employ and then let the host immune system eliminate the infection.

One area of work that would be interesting to look in to is cell division inhibition and filamentation of *M. tuberculosis* within human macrophages. This could be done with high power microscopy to visualize fluorescent protein expressing cells within the cell and then measure their cell lengths. If the population is found to be filamentous then further experiments could be done to investigate the cause, for example using transcriptomics to investigate what genes are either up-regulated or down-regulated. Another method could be to use random transposon mutagenesis to build a knockout library of *M. tuberculosis* mutants, and then screen that library for clones that do not elicit filamentation within a human macrophage.

Bacterial filamentation and cell division are important areas of investigation for clinically relevant bacteria. With the information that can be gleaned from these investigations, the next generation of antimicrobials can be invented and halt a currently losing battle.

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6. References

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Appendix

7. Appendix

7.1. Nucleotide sequence of ORF1 from environmental library sub-clone EsC_3-5B

CTGCTGCAAGGCCATCGCCAAGCTTTCCTGAACGACCCTTTACAGCTGTGGGGTCTCAA
AAAAATCGTTGATCGAATGCTCTACCTGTGTACTGTATACAGGATACAGAACGACTCCG
GCAAACGGGGCTTTCAAAACTGGGAGAATGATATGACTTTCAAACATAGTATTGTGCT
GGGTGTTACGGCCTCATTGCTCGCAAGCACGGCGCTTGCAGATGTCAAAATCGGCTTTA
TCGGAAGCCTGAGTTCGGACACCGGCCTTAGCACCCTGCGCGGCGCAGAGATCGCCATT
GAAGAGATTAACGCCGCCGGTGGTATGAGTGGCGAGAAGCTTGTGCTTGTCAGCGCTG
ACACAGGTGAGGACGTCACCGAGGGTGTCAAAGCCTACGAATATCTGGCCGAGACCGAG
GAGGTTGATTTCATCATCTCCGGGTCCATCGACGATGTGAGCCTAGGCTGGCTGCCGCG
TATGCAGGAGTTCCAGATCCCTACGCTGGACACATGGACATCCTACATCGGTATCATCG
ACATGGTCATCGAAGACCCCGAAACCATGGCGCCTTACTTTATGAACATCGCCTCGGAC
GAGGCGCTGGCCACGCTTTACATCAACTTCGGTGCAGACGTGCTGAAAAAGGAGATGGG
TTGGGACAGCGTCGTCATTCTGGCCGAGGACACTGCCTTTGGTGAAGCAATCACCGGCC
TTGTCACCGAAGCGTTGGGCCCCGTTGCAGGGATCGAGACCAAGGAAATCATCACCTAT
GACGTAGGCACGGTCGACTTTGCGCCGCTGTTCTCCAAGGCGCAGGCCTCGGGAGCGGA
TTTTATCTATCAGGTCAGTTCGGTGAATTCGCAGGTGATTTCCTCGCAATATGTGAAGT
TGCAGGTGCCTATGGCGATGACCGGTGTGAACGTCTCAGCGCTGGGTCTTGAGTACTGG
GAAGACACTGGCGGTGCCGGTGGCGGTATTTCCACCCTGTCGCCCATTCCGTCGGTCGG
CTTTGAGCTTGACCCTGAAAGCCAGAAGTTCGTCGACACCTATCAGGCCAAGTATGACA
GCCGCCCCAAGATGCCCCACTTTAACGGGTTCAACGCCTACTGGGGCCTAAAGCAGGCG
GTTGCCGCCGCCGAGGCAGCAGGCGGCTTCGGTGACGCGAAAGCATGGTCCGCCGAGAT
GAAAAAGCAGGACATCACGCTGAAGAAAGACGGCAAGATGTGGTTGCGTTACGCGTTC
TGGGGCGATGACGAGGTCGAGGAAGTCACCGGCCGGACCTATCCGCACAACGCTCGTTT
CGACCTGACGGCGCCCTACGACGACGCGCAGCCGTCCATGGTTGTTATCCAGTGGAAGG
AAGATGGCGAAGTTGGTGTGGTCTATCCACCCGAATACGCCACGACCAAGTTCACAGTG
CCAAGCTGGGTCAAGTAA

7.2. Nucleotide sequence of ORF2 from environmental library sub-clone EsC_3-5B

TTGTTATCCAGTGGAAGGAAGATGGCGAAGTTGGTGTGGTCTATCCACCCGAATACGCC
ACGACCAAGTTCACAGTGCCAAGCTGGGTCAAGTAACCCCAAGCGGCAGGGCGGTTGCG
CCCTGCCGCATTCTTCCTCTCCATTTGGCAGGACAGACAAGATGTCTCCACATCCGGCG
CTGGTCGCCCATTCGGATACCTTTGCAAAGTCGGTCCATCAATTGACGCCAGAAGTCAG
GGGCTTTGTCGGAAATGCCGCCTCCAACGTCTATGTGATCGAAGGCGCAGACAGCTTTG
CTGTGATCGACACAACGGAATCCACAGGTGCTGCAACCAACATTCTTGCGGCATTGCGC
GAACAGACCGCCAAGCCGGTCACGCAGATCCTCTATACCCACAGTCACCGCGACCACAT
CTCGGGGGCGTCCGTTTTTGCGGAGGGGCAGAACCCGCAGGTGATTGCGTGGCACGGTT
TTGCCTCCGACATTGTGGGCGAAGTTGCTGGCCCGTCAAGGGCGATCCTTGCGCGTACC
CGCAAACAGTTCGGTTTCGGCCTGAGCTTTCCCGACGAGCGGGTCAATCTGGGCCTTGG
TCCAGGCGATCGCCCCACCGAAGGCATGGGCGCAGGGCATATGACGCCCACAACGTTTG
TTGATAGCACGCCCTGCACATTCACGCTGGCAGGCCATGAGGTCTCCCTGATCCACGCA
CCCGGTGAGACCGCCGATCATATGATGGTCTGGATGGAAGACAGCGGTCTGCTGATATC
GGGTGACAATTACTATCACGCGTTTCCAAATCTCTATCCCATTCGCGGATCCGCCTATC
GGGACTTCAACGCATGGGCGGACAGCCTTGAGCTGATCTTGCAGATTGATCCGGCCATC
CTCGCCCCCGGTCATTCGCAACCGCTGACCGACCGCGCCACCATTCGTGCGCGGGTAGGG
GATTACCGCGACCTCATCCGTTTTATCATCGACACCACAGCGGCGGGGCTGAACGCGGG
CCAAACGCCAGACCAGATCGTTGCTGAGGCGCGTCTGCCCAAGGCGCTTGCGGACAAGC
CTTGGCTGGGTGAGTTCTATGGCCGGCTTGATTGGTCGATCCGCGCCTATGCTGCGGGT
ACTGTCGGGTGGTTTGATGGCAATCCCACCAACTTGGCGCGGTTATCCCCGGCGGATGA
GGCCGCCCGCTTTGTCGCTCTGGCTGGTGGCGCGGATCAGGTTCTGGACGCCGCATGTG
CATCGGATGATGCGCAGTGGACGCTGGAACTTTGTGACCGCCTCATCACGGAGGAAAGC
CACGCCACAGCCGCTTTGGCGCTTAAGGCGAAAGCCATGTGCGCACTGGCGGACCAACA
GATCAACGCCACCGCGCGGAACTATTACCTTGTCTCCGCCAAGGACATCGAGGACGCAC
AGCCATGA