CHEMICAL INTERROGATION OF CELL DIVISION IN *STREPTOMYCES*

CHEMICAL INTERROGATION OF SPORULATION AND CELL DIVISION IN *STREPTOMYCES*

By

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ABSTRACT

Cell division is essential for spore formation but not for viability in the filamentous streptomycetes bacteria. Failure to complete cell division instead blocks spore formation, a phenotype that can be visualized by the absence of gray (in *Streptomyces coelicolor*) and green (in *Streptomyces venezuelae*) spore-associated pigmentation. The streptomycetes divisome is however, similar to that of other prokaryotes.

We hypothesized chemical inhibitors of sporulation in model streptomycetes might interfere with cell division in rod shaped bacteria. To test this, we investigated 196 compounds that inhibit sporulation in *Streptomyces coelicolor.* We show that 19 of these compounds cause filamentous growth in *Bacillus subtilis,* consistent with impaired cell division. One of the compounds is a DNA damaging agent and inhibits cell division by activating the SOS response. The remaining 18 act independently of known stress responses and may therefore act on the divisome or on divisome positioning and stability. Three of the compounds (Fil-1, 2 and 3) confer distinct cell division defects on *B. subtilis*. They also block *B. subtilis* sporulation, which is mechanistically unrelated to the sporulation pathway of streptomycetes but which is also dependent on the divisome. We discuss ways in which these differing phenotypes can be used in screens for cell division inhibitors.

In addition to the molecules affecting the divisome, DNA and cell wall damage also affects the process indirectly by temporarily halting the cell division. To further explore the cell division regulation in stressful conditions, we carried the complete transcriptomic

analysis of *S. venezuelae* after the DNA damage. The observed changes in the gene expression as a result of the DNA damage paves the way for identification of the DNA-damage induced cell division inhibitor in streptomycetes.

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ABBREVIATIONS

3-MBA	3-methoxibenzamide
ADP	Adenosine diphosphate
antiSMASH	Antibiotics & Secondary Metabolite Analysis Shell
anti-TB	Anti-tuberculosis
BER	Base excision repair
BrGTP	8-bromoguanosine 5¢-triphosphate
C.F.U	Colony forming unit
DAPI	4', 6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSM	Difco sporulation medium
dTMP	Thymidine monophosphate
DTT	Dithiothreitol
EBNA-1	Nuclear Epstein-Barr virus (EBV) protein
ECT	Electron cryotomography
EM	Electron microscope
FA	Fluorescence anisotropy
FIMO	Find individual motif occurance
FM 4-64	N-(3-triethylammoniumpropyl)-4-(p-diethylaminophenyl-hexatrienyl) pyridinium dibromide
FP	Fluorescence polarization
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
HMMR	Hidden Markov models
HPLC	High-performance liquid chromatography

IS21	Insertion sequence 1
KEGG pathway	Kyoto Encyclopedia of Genes and Genomes – pathway
LB	Luria-Bertani
LDH	Lactate dehydrogenase
MEME	Multiple EM for motif elicitation
MES	2-(N-morpholino)ethanesulfonic acid
MIC	Minimun inhibitory concentration
MMC	Mitomycin C
mRNA	Peptidoglycan
MRSA	Methicillin-resistant Staphylococcus aureus
MYM	Maltose-yeast extract-malt extract
NAD+	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
NMR	Nuclear magnetic resonance
PBP1B	Penicillin-binding protein 1B
PEP	Phosphoenolpyruvic acid
PG	Peptidoglycan
PK	Pyruvate kinase
PSI-BLAST	Position-Specific-Basic Local Alignment Search Tool
PSWM	Position-specific weight matrix
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RecA-Dp	RecA-dependent promoter
RecA-NDp	RecA-independent promoter
RNA	Ribonucleic acid
Rpf	Resuscitation promoting factors
RPM	Revolutions per minute
RSA-tool	Regulatory sequence analysis – tool
SALPs	SsgA-like proteins

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOS	Save Our Soles
ssDNA	Single stranded DNA
UV	Regulatory Sequences Analysis
vHTS	Virtual high throughput screen

CHAPTER 1

INTRODUCTION

1.1 Cell division in unicellular bacteria

Cell division is a process of generating two viable daughter cells from a mother cell. Bacterial cells have evolved a sophisticated system that not only carries out septum synthesis, but also co-ordinates it with other cellular processes such as chromosome replication and cell elongation. The cell division machinery also responds to various environmental signals to regulate this process and ensure that a cell divides only when it is appropriate and safe (Harry *et al.*, 2006,Stragier *et al.*, 1996).

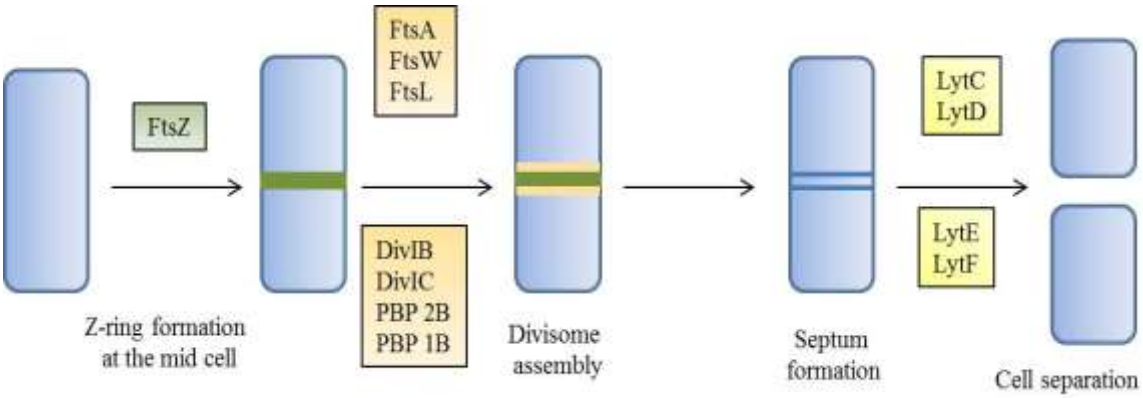
The prokaryotic cell division has been well studied in *Escherichia coli* and *Bacillus subtilis*. It involves the regulated assembly and action of a group of proteins collectively termed the divisome (Gamba *et al.*, 2009). The proteins that make up the divisome are involved in selecting the division site, septum formation and the separation of the two newborn daughter cells (figure 1.1a). The genes encoding most of these proteins were identified as conditional mutants of genetic loci that induce filamentous growth under restrictive conditions and hence they were named *fts* (**f**ilamentous **t**emperature **s**ensitive) (Breakefield *et al.*, 1973).

1.1.1 Divisomal proteins involved in the selection of a division site

In many bacteria, FtsZ is the first protein to assemble at the future division site after chromosome replication and cell elongation. Although mutations in *ftsZ* were discovered as early as 1950 by Y. Hirota, the formation of the characteristic FtsZ ring structure was not demonstrated until 1991 (Bi *et al.*, 1991; Lutkenhaus,1993). Despite limited sequence homology, FtsZ is a structural and functional homolog of a eukaryotic protein tubulin.

Like tubulin, FtsZ polymerizes in GTP-dependent manner resulting in the formation of a ring at the mid-cell called the Z-ring. This ring acts as a scaffold directing the assembly of other proteins of the divisome (Bramhill,1997; Harry *et al.*, 2006).

(a)



(b)

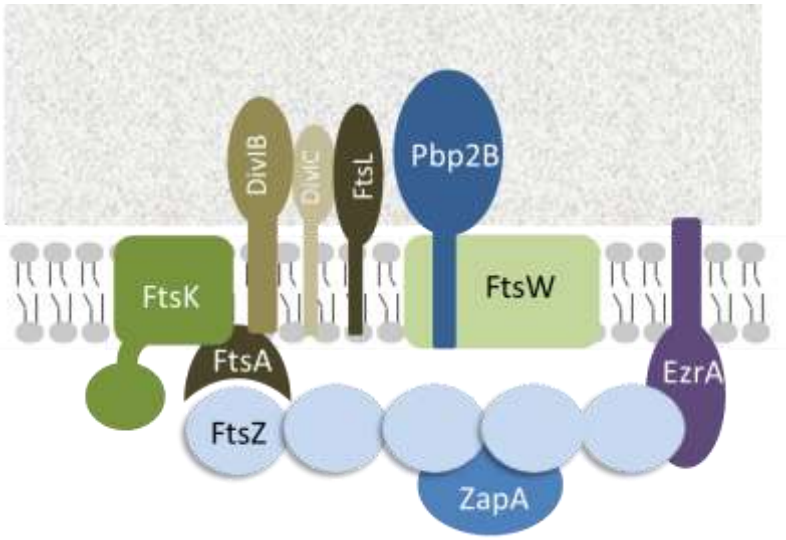


Figure 1.1 Schematic diagram of the *B. subtilis* divisome: (a) Unicellular bacteria divide by the formation of a cell wall in the middle and separation of the cells into two daughter cells (b) The bacterial divisome. A set of around ten proteins called the divisome assembles at the site of septum formation during cell division. Assembly of FtsZ a ring is the first step towards the cell

division, which is assisted by FtsA, ZapA and SepF. The Z-ring formation is followed by the recruitment of the downstream proteins FtsK, DivIB, DivIC, FtsL, FtsW and FtsI.

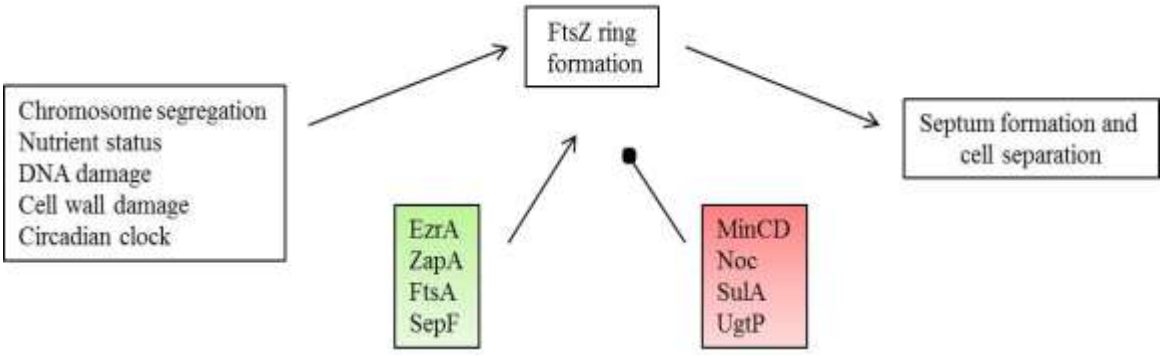


Figure 1.2 Regulation of formation of the FtsZ ring: The ring-like structure formed at the site of septum synthesis is regulated by extracellular and intracellular signals such as chromosome replication, DNA damage cell wall damage, nutrient status etc.

Since FtsZ is involved in the initiation of cell division, its function is tightly regulated by other proteins at the level of its localization, membrane attachment, GTPase activity and interaction with other divisomal proteins (figure 1.2) (Adams *et al.*, 2009). In *E. coli* FtsA, ZipA, ZapA, ZapB, ZapC, MinCD, Noc are the proteins involved in regulating FtsZ localization, dynamics and membrane attachment (Harry *et al.*, 2006).

FtsA is a homolog of the eukaryotic protein actin and like actin it has ATP hydrolysis activity(Sanchez *et al.*, 1994; Szwedziak *et al.*, 2012). FtsA is involved in tethering FtsZ to the membrane along with ZipA and also helps to recruit the subsequent divisomal proteins (Feucht *et al.*, 2001). FtsA interacts with itself and has been shown to form filaments *in vitro* (Hsin *et al.*, 2013). Since FtsA is essential for growth in most bacteria, it appears to be a very interesting target for discovery of novel antibacterials. Its enzyme

activity as well as polymerization can be measured and quantified *in vitro*, which makes it adaptable for future high throughput screening for inhibitors (Juarez *et al.*, 2012; Szwedziak *et al.*, 2012; Hsin *et al.*, 2013).

The function of ZipA (Z-ring interacting protein A) overlaps with that of FtsA. Though, unlike FtsA, which is a peripheral membrane protein, ZipA has a trans-membrane region that tethers it to the membrane (Pazos *et al.*, 2013). It is conserved in gamma-proteobacteria. In *B. subtilis*, the functional homolog of ZipA is termed SepF (Duman *et al.*, 2013).

1.1.2 Proteins preventing septum formation at the poles: the Min system

In the initial stages of bacterial cell division, cell elongation and chromosomal replication takes place. Septum formation is initiated only after the separation of two newly replicated chromosomes. Bacterial cells have devised mechanisms to prevent damage to the chromosome by the formation of septa. In rod-shaped bacteria, this is carried out by two systems: the Min system and the nucleoid occlusion (Harry *et al.*, 2006; Adams *et al.*, 2009; Lutkenhaus *et al.*, 2012). The Min system, which is named after the mini-cells that are produced in certain cell division mutants, comprised of three proteins: MinC, MinD and MinE. Its role is to prevent the formation of the FtsZ ring at cell poles (Rothfield *et al.*, 2005). MinD is a membrane-localized ATPase that recruits MinC. MinC, a direct inhibitor of FtsZ polymerization, is activated by forming a complex with MinD. The MinC/MinD heterodimer prevents the formation of septa at the cell poles. MinE forms a ring at the mid cell and inhibits the localization of MinCD to that position

by inhibiting local membrane attachment of MinD (Fu *et al.*, 2001; Lutkenhaus,2007a). By preventing the action of MinCD, MinE permits the formation of the Z-ring at the mid cell exclusively (Howard *et al.*, 2005). Interestingly, in *E. coli* MinCD oscillates from one pole to another every 50 minutes (Fu *et al.*, 2001; Hale *et al.*, 2001; Shih *et al.*, 2003; Meacci *et al.*, 2006; Lutkenhaus,2007b). In contrast, the Min proteins in *B. subtilis* are statically localized to their site of action (Jamroskovic *et al.*, 2012).

1.1.3 Proteins preventing the septum formation over the chromosome: the nucleoid occlusion system

The existence of a second regulatory mechanism that positions the site of septum formation relative to the chromosome was suggested long before its relatively recent discovery (Bramkamp *et al.*, 2009; Wu *et al.*, 2011). Two unrelated proteins, both of which are similar to the DNA-binding transcription factors, Noc in *B. subtilis* and SlmA in *E. coli*, inhibit the formation of Z-ring around unsegregated chromosomes (Bernhardt *et al.*, 2005; Wu *et al.*, 2009). Both Noc and SlmA bind to the DNA motifs that are absent near the terminus of the chromosome and inhibit assembly of FtsZ over the chromosome (Wu *et al.*, 2009; Cho *et al.*, 2011; Tonthat *et al.*, 2013).

1.1.4 Regulation of septum formation by external stimuli

A number of environmental and cell stress mechanisms control cell division, in some cases by directly acting on the assembly of FtsZ. For example, SulA is a direct inhibitor of FtsZ polymerization that is activated by DNA or cell wall damage in *E. coli* (Huisman *et al.*, 1984; Jones *et al.*, 1985). It reversibly binds and inhibits FtsZ activity, preventing

the first step in the assembly of the divisome (Jones *et al.*, 1985). SulA and other DNA damage induced cell division inhibitors have been discussed in detail in the following section. In *Streptomyces* , such cell cycle checkpoints have not been identified yet.

Rod shaped bacteria also regulate cell length in response to nutrient availability by regulating the placement of the Z-ring. Recently, Dr. Petra Levin's group has identified cell size regulators, OpgH in *E. coli* and UgtP in *B. subtilis*, which regulate the frequency of Z-ring formation in response to nutrient conditions (Weart *et al.*, 2007; Matsuoka *et al.*, 2011; Hill *et al.*, 2013).

1.2 The assembly of cell division proteins into the divisome

The proper assembly of the cell division proteins into a divisome is critical for the normal cell division event. In *E. coli*, the assembly occurs in a linearly ordered fashion. After the formation of FtsZ ring and its stabilization by FtsA and ZipA, FtsK is recruited to the mid-cell. A complex consisting of FtsQ, FtsB and FtsL is then recruited, followed by FtsW and FtsI and finally FtsN. In *B. subtilis*, it has been shown that before the cell division proteins are recruited to the Z-ring they form bundles by interacting with other divisomal proteins and then assemble into a divisome in two steps. In the first step, FtsZ and regulators of FtsZ assembly: FtsA, SepF, ZapA and EzrA mark the site of the future septum by forming the Z-ring (Goehring *et al.*, 2005; Adams *et al.*, 2009). In the second step, DivIB, DivIC, FtsL and PBP2B are recruited to the Z-ring (Gamba *et al.*, 2009). The assembly of the divisome depends on an extensive network of protein-protein interactions and interactions with the peptidoglycan layer.

1.3 Divisomal proteins involved in the biosynthesis of the new septum

Assembly of the Z-ring is the first and the most critical step in the determination of timing and position of the septum formation (Adams *et al.,* 2009). However, other divisomal proteins play essential roles in the synthesis of septal peptidoglycan and the separation of daughter cells. FtsQ, FtsL and FtsB are involved in the maturation of the divisome (Bramhill,1997; Gonzalez *et al.,* 2009). They are recruited to the divisome by protein-protein interaction, and they recruit the subsequent divisomal proteins. FtsK is involved in translocation of newly replicated chromosome into the daughter cells. Other divisomal proteins FtsW, FtsN, FtsI and PBP1B are involved in the synthesis of a new peptidoglycan layer between two daughter cells (Harry *et al.,* 2006; Lutkenhaus *et al.,* 2012). The precise molecular roles played by many of the remaining proteins are unknown – it is possible that many of them serve primarily to stabilize the structures through protein-protein interactions; however, it is unlikely that this is the whole story.

1.4 Divisomal proteins involved in cell separation

In the final stages of cell division, a part of the new peptidoglycan layer between two daughter cells is degraded resulting in the separation of two cells. The proteins involved in this process have murein hydrolytic activity and are recruited to the septum by protein-protein interactions. Most bacteria possess around 30-60 cell wall hydrolases with overlapping functions, a subset of which is specific to degrading the peptidoglycan layer between two daughter cells (Margolin,2001).

1.5 Cell division during morphogenesis and spore formation

The process of cell division results in the formation of a wall across the cell, separating two chromosomes. There are, however, a number of cases where the cell division is not followed by cell separation, rather results in the formation of a stable cross wall. However, in those cases where the process has been studied, the divisome is still required. There are also examples, were the cell division is not a simple binary event, dividing one cell into two. The best-studied examples of this are the various mechanisms that result in the formation of spores during bacterial development. Bacterial spores are dormant forms of bacterial cells containing a single copy of the chromosome and a thick coat imparting the resistance to a variety of environmental conditions (Errington,2010). The bacterial sporulation process has been extensively studied in *B. subtilis* for the formation of endospores and in *S. coelicolor* for the formation of exospores. In both organisms, the formation of the septum, which occurs in the early steps of sporulation, employs the divisomal proteins discussed above (Errington,1991; Margolis *et al.*, 1991b; McCormick,2009). Therefore, the knowledge gained about the divisomal proteins from the sporulation process can be applied to the vegetative cell division process and *vice versa*.

1.5.1 *Streptomyces coelicolor* development

Streptomycetes is a bacterial group that lives in soil and confers its characteristic earthy smell. The group comprises of GC rich, Gram-positive, filamentous bacteria. Members of this genus are studied mainly because of their ability to produce antibiotics and their

complex life cycle (Hopwood,2007). During its life cycle, *Streptomyces* undergoes various morphological changes (figure 1.3) (Claessen *et al.*, 2006). Under favorable conditions, a free spore of *Streptomyces* germinates and develops into a mass of filamentous vegetative cells called substrate mycelium. Cell division is rare in the substrate hyphae, so these cells are long filaments in which multiple chromosomes share each compartment. Aging of the substrate mycelium initiates the formation of branches that grow away from the soil, called aerial hyphae. In marked contrast to the substrate hyphae, the aerial hyphae undergo synchronous cell division resulting in the formation of uni-genomic spores. As these cells mature they accumulate a grey polyketide pigment in the spore wall *(Flardh,2003b; Flardh et al., 2009)*. This gray pigmentation has been used as a marker of a complete sporulation event. Mutants that result from a blockage in the production of this pigment and interruption of various steps in the sporulation process appear white because of the white fuzzy mass of mycelium on the colony surface. These mutants are thus referred to as white mutants (*whi*).

Mutations that block the formation of the aerial hyphae, an earlier and more profound interruption of the morphogenetic process, are referred to as bald (*bld*) (figure 1.4). These mutants lack the white fuzzy aerial hyphae on the colony surface. Thus, the phenotypic differences in the colony morphology of *S. coelicolor* mutants, blocked at various developmental stages, provide a convenient way to study the development in these bacteria (Chater,1998).

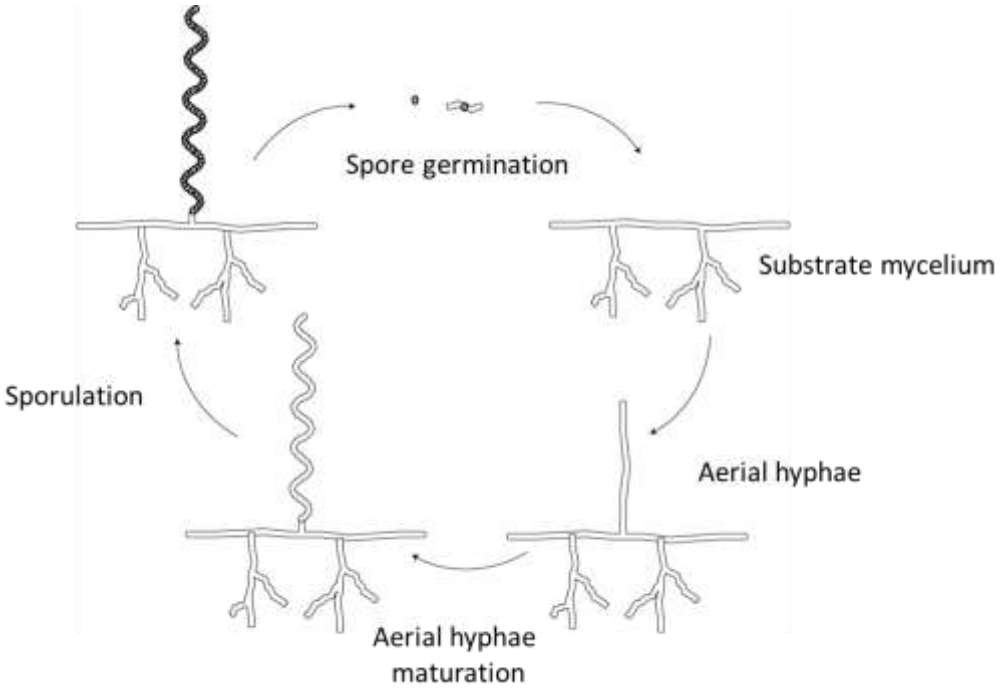


Figure 1.3 Developmental stages of *S. coelicolor*: *Streptomyces* undergoes varies morphological and physiological changes during its life cycle. Favorable conditions allow spore germination, followed by formation of the substrate mycelium which is a mesh of branching hyphae. Prior to spore formation, the substrate mycelium develops aerial growth accompanied by antibiotic production. Aerial hyphae undergo cell division and mature as chains of spores. (figure by Leslie Cuthbertson)

Figure 1.4 Colony morphology of *S. coelicolor* developmental mutants: (A) In a wild-type colony spores mature and produce grey pigments (B) mutants defective in aerial mycelium are termed 'bald mutant' (C) mutants defective in spore formation, forms white colony (from Hoskisson P)

Studies of development in *S. coelicolor* have grouped the sporulation genes into two (Flardh *et al.*, 2009). For example, the early *whi* genes (*whi*A, *whi*B, *whi*G, *whi*H, *whi*I, *whi*J) are involved in the initial stages of sporulation and late *whi* genes (*whiI, whi*E, *whi*L, *whi*M, *whi*O) are required for septum formation and spore maturation (Claessen *et al.*, 2006). In addition to these developmental regulators, cell division genes and the divisome itself, play a vital role in sporulation to occur normally (Flardh,2003a; Willemse *et al.*, 2011a; McCormick *et al.*, 2012).

1.5.2 The role of cell division in sporulation

There are two types of cell division events in *S. coelicolor*. Vegetative cell division occurs in the substrate mycelium and sporulation specific cell division occurs in the aerial hyphae during the spore formation (Flardh *et al.*, 2009). Vegetative cell division in the substrate mycelium is not co-ordinated with chromosome replication and segregation or growth. It is, therefore, manifested as occasional septum formation. As a result, substrate

hyphal cells consist of long compartments, each containing multiple copies of the chromosome. In contrast, the cell division in aerial mycelium is specially organized and synchronized, resulting in the formation of regularly spaced, thicker cell walls dividing off uni-genomic compartments (McCormick, 2009; (McCormick *et al.*, 2012). Each compartment ultimately matures into a spore and accumulates grey pigments. Thus, cell division might not be crucial for growth and survival in *Streptomyces* , but is vital for sporulation.

1.5.3 Cell division in the filamentous streptomycetes is different from that in the rod-shaped bacteria

Most cell division proteins are conserved among bacterial species. However, to match the requirement of a slightly different mode of cell division *Streptomyces* have evolved some unique cell division proteins. These proteins take over the functions served by the well-studied and conserved proteins in other bacteria. One such example is FtsA. Interestingly, even though FtsA is conserved and essential in most bacteria, the streptomycetes lack this protein. Moreover, these filamentous cells do not have proteins similar to ZipA, the Min system or a nucleoid occlusion mechanism. Instead, *Streptomyces* possesses two groups of proteins that uniquely control and coordinate cell division in the vegetative and sporulating cells. The first of these is a CrgA like protein that inhibits FtsZ activity. The second are the SsgA-like proteins that promote FtsZ activity (Traag *et al.*, 2008). SsgA-like proteins were first identified as inhibitors of **s**porulation of *Streptomyces* ***g****riseus* in submerged culture (Kawamoto *et al.*, 1997). The

homologs of SsgA are found only in the morphologically complex actinomycetes and are collectively termed SsgA-like proteins (SALPs). The proteins belonging to this family in *S. coelicolor* are SsgA, B, C, D, E, F and G. These proteins have functions from initial stages of cell division to cell separation (van Wezel *et al.*, 2000; Keijser *et al.*, 2003; Willemse *et al.*, 2011b). SsgA, SsgB and SsgG are involved in determining the site of future septum formation, SsgB has a role in septum growth, SsgD is involved in maturation of the spore wall and SsgE as well as SsgF are involved in autolytic separation of the spores (Traag *et al.*, 2008). The homologs of the small protein involved in cell division inhibition, CrgA, in *Streptomyces* are known as CrgA-like proteins. The molecular role of CrgA-like proteins in the cell remains unknown (Del Sol *et al.*, 2003). Deletion of *crgA* results in early development of aerial hyphae and abnormalities in the spore morphology. In contrast, the overexpression results in an increase in the frequency of Z-ring formation (Del Sol *et al.*, 2006).

Thus, the outcome of cell division in Streptomycete is different from that of unicellular bacteria, but the basic machinery in most part remains similar. As described in table 1.1, homologs of most known cell division proteins are present in *S. coelicolor* (McCormick, 2009). Moreover, cell division is dispensable for colony formation in *Streptomyces.* These features of *Streptomyces* cell division collectively makes *Streptomyces* a good model system to study bacterial cell division.

1.6 Small-molecule inhibitors of cell division

Studies on the bacterial cell division started almost half-a-century ago with the discovery of the first filamentous mutant of *E. coli* that was impaired in cell division (Hirota *et al.* 1968). For several years after that, researchers mainly studied cell division based on the filamentous phenotype of various mutants of *E. coli*. In early 1990s, Erfei Bi and Joseph Lutkenhaus demonstrated the formation of the FtsZ ring during cell division and later elucidated its structure using the electron microscopy (Bi and Lutkenhaus, 1991). These findings constituted a breakthrough of prokaryotic cell division studies and ushered in the cell biological investigation of divisomal proteins.

Table 1.1 Conservation of cell division genes in bacteria

Function	***E. coli***	***B. subtilis***	***S. coelicolor***
Forms a polymeric ring and recruits downstream protein	*ftsZ*	*ftsZ*	*ftsZ*
Tethers FtsZ to the membrane, stabilize the Z rings	*ftsA*	*ftsA*	-
Unknown	*ftsB*	*divIC*	*divIC*
Unknown, similar to ABC transporter	*ftsE*	*ftsE*	*ftsE*
Transpeptidase, introduces cross-links in peptidoglycan	*ftsI*	*ftsI*	*ftsI*
Chromosome partitioning	*ftsK*	*spoIIIE*	*ftsK*
Unknown	*ftsL*	*ftsL*	*ftsL*
Unknown	*ftsN*	-	-
Unknown	*ftsQ*	*divIB*	*ftsQ*
Septal peptidoglycan biosynthesis	*ftsW*	*ftsW*	*ftsW*
Unknown, similar to ABC transporter	*ftsX*	*ftsX*	*ftsX*
Stabilize Z rings at the membrane	*zipA*	*sepF*	-

Promotes Z ring formation by inducing proto-filaments bundling	*zapA*	*zapA*	-
Negatively regulates Z ring formation	-	*ezrA*	-
Inhibitor of FtsZ assembly	*minC*	*minC*	-
Inhibitor of FtsZ assembly	*minD*	*minD*	-
Inhibitor of FtsZ assembly	*minE*	-	-
	-	*divIVA*	*divIVA*
Sporulation-specific cell division in *Streptomyces*	-	-	*ssgA*
”	-	-	*ssgR*
”	-	-	*ssgB*
”	-	-	*ssgC*
”	-	-	*ssgD*
”	-	-	*ssgE*
”	-	-	*ssgF*
”	-	-	*ssgG*
Partitioning of chromosome	*parA*	*Soj*	*parA*
Partitioning of chromosome	*parB*	*spoOJ*	*parB*

In the past 15 years, techniques to study protein localization, genetics, x-ray crystallography and other technologies have played a crucial role in developing a better understanding of the divisome assembly (Adams *et al.*, 2009; Gamba *et al.*, 2009). Despite these technological advances, the specific roles of several components of the core divisome are unknown. Indeed, to date only FtsZ, FtsA, and FtsW have the precise biochemical functions assigned to them (de Boer *et al.*, 1992; RayChaudhuri *et al.*, 1992; Mohammadi *et al.*, 2011; Szwedziak *et al.*, 2012; Hsin *et al.*, 2013). Therefore, the development of novel tools to study cell division could help understand the process better.

There are many examples that use small molecules to study biological processes. A historic example is the use of actin inhibitors to study the dynamic changes in actin during eukaryotic cell division (Peterson *et al.*, 2002). Another classical example of such probe is the use of translational inhibitors such as puromycin, to study the ribosome function (Pestka,1976; Starck *et al.*, 2003). Fluorescently labeled glycopeptide antibiotics, vancomycin and ramoplnin have been used to visualize the site of nascent peptidoglycan biosynthesis (Daniel *et al.*, 2003; Tiyanont *et al.*, 2006; van Oosten *et al.*, 2013). Recently, a small-molecule inhibitor of MreB has been identified, which helped study chromosome segregation and its movement during cell division. A22, the MreB inhibitor allowed researchers to address problems that were very difficult to understand using any existing technique (Dye *et al.*, 2005; Gitai *et al.*, 2005; Dye *et al.*, 2011). Thus, developing such small-molecule tools to study bacterial cell division would significantly expand the current understanding of the process.

In addition to serving as the probes of biological processes, small molecules with a particular target in the divisome could also serve as the lead compounds for the development of anti-bacterial drugs. Most clinically used antibiotics target transcription, translation, cell wall synthesis or folate synthesis (Lock *et al.*, 2008). The bacterial cell division has not yet been exploited clinically for this purpose. Bacterial cell division proteins have several characteristics that make them attractive antibiotic targets. Most of these proteins are essential for growth, which makes them necessary for causing infection, they lack any eukaryotic homologues and because of the presence of a large extracellular domain they are easily accessible to the drug molecules (Lock *et al.*, 2008).

FtsZ is the first among divisomal proteins to initiate the division, and its activity is vital for the process. Moreover, its quantifiable *in vitro* activity allowed FtsZ to emerge as an attractive target in various drug development programs (Lock *et al.*, 2008). Most of these screening endeavors have adopted the existing platforms of high-throughput screening or *in silico* chemical modelling. The screening approaches have relied heavily on the available knowledge in fundamental genetics as well as the biochemical and structural information of the cell division proteins. Various screening strategies used for the identification of small-molecule inhibitors of bacterial cell division have been summarized below.

1.7 Strategies to identify inhibitors of bacterial cell division

The methodologies used so far, for screening small-molecule inhibitors of bacterial cell division can be broadly grouped in four categories; (1) target based *in vitro* screening, (2) cell based screening followed by the confirmatory assays for the target validation, (3) designing the inhibitor by modification of an already existing molecule and (4) *in silico* virtual screening.

1.7.1 Target based *in vitro* screening approaches

Target based *in vitro* methods are widely used for identifying small-molecule inhibitors of various proteins. However, pre-selection of a suitable target with a quantifiable *in vitro* activity is an essential requirement of this approach. The divisomal protein FtsZ, due to its quantifiable GTPase activity and the ability to polymerize *in vitro*, is a good candidate for this method of screening. Most attempts to screen for the inhibitors of cell division

followed this strategy and identified inhibitors of FtsZ (Wang *et al.*, 2003; Margalit *et al.*, 2004b; Stokes *et al.*, 2005b; Haydon *et al.*, 2008; Eun *et al.*, 2013). Some of those assays and the molecules identified using them are discussed below.

1.7.1.1 Fluorescence-based high-throughput FtsZ polymerization assay

The ability of FtsZ monomers to polymerize *in vitro* has been exploited to screen for cell division inhibitors. A fluorescence-based polymerization assay was developed by Trusca and Bramhill, which relies on the use of fluorescent FtsZ monomers. In the screen that the authors performed, the fluorescent monomers were induced to polymerize by the addition of GTP in the presence and absence of the molecules. Polymers were then separated from the monomers by centrifugation. The degree of polymerization is calculated based on the fluorescence from the pelleted fraction of the protein (Trusca *et al.*, 2002). The fluorescent FtsZ required for the assay was prepared by fusing fluorescein at T65C position of the FtsZ monomer from *E. coli*. Addition of the tag did not interfere with the ability of the protein to polymerize *in vitro*. The polymerization assay is easily adaptable to 96-well plate format and requires small quantities of purified protein. Since the fluorescence can be measured quantitatively, the assay also allows detection of the molecules that affect the FtsZ activity by inducing polymerization or bundling of the proto-filaments. Unfortunately, small-molecule libraries contain many auto-fluorescent compounds that can interfere with the measurements and generate false positive hits. This is a major drawback of the technique and to overcome this barrier the authors have

introduced a quenching counter screen. The counter screen identifies and discounts the auto-fluorescent compounds among the hits.

Table 1.2 Strategies used for the identification of known bacterial cell division inhibitors

Screening method	Molecules	MIC Gram-negative	MIC Gram-positive	Target (mode of action)	Assay performed	library screened	Tubulin inhibition	Reference
GTPase assay								
(pyruvate kinase couples assay)	Zantrins (Z1-Z5)	5-40 µM	0.3-10 µM	FtsZ inhibition	FtsZ polymerization by sedimentation and EM assay, GTPase assay by malachite green and using radioactive GTP.	DiverSet E, ChemBridge and Diversity Set, National Cancer Institute.	not tested	(Margalit *et al.*, 2004a)
Fluorescence based FtsZ polymerization assay	Viriditoxin	>64 µg/ml	2-16 µg/ml	FtsZ inhibition	GTPase assay by using radioactive GTP	>100,000 microbial and plant extracts	not tested	(Trusca *et al.*, 2002); (Wang *et al.*, 2003)
Fluorescence anisotrophy based competition assay	UCM05	>32 µg/ml	4	Induced FtsZ polymer formation	Fluorescence anisotropy, EM, light scattering, sedimentation assay and *in silico* docking			(Schaffner-Barbero *et al.*, 2010; Ruiz-Avila *et al.*, 2013)
NMR-based fragment screening approach	Compound 4	not tested	not tested	FisZ-ZipA interaction, binds to ZipA interaction pocket	NMR, X-ray crystallography	selected commercially available molecules	not tested	(Tsao *et al.*, 2006)

Molecules designed to bind the target protein	Carboxybiphenylindole	0.1-2 µg/ml	2-16 µg/ml	FtsZ-ZipA interaction	NMR to test binding to the protein	Molecules synthesized	not tested	(Sutherland *et al.*, 2003; Jennings *et al.*, 2004b)
***Bacillus* sporulation (sigmaF induction)**	PC58538, PC170942	>256 µg/ml	8-64 µg/ml	FtsZ	*B. subtilis* filamentation, GTPase activity by malachite green dye, FtsZ polymerization by sedimentation assay, generation of resistant mutants	Synthetic compounds	not tested	(Stokes *et al.*, 2005a)
***Streptomyces* sporulation (visual phenotypic screen)**	Fil-1, Fil-2, Fil-3	not active	*B. subtilis*	Not known, non-FtsZ	*Streptomyces* sporulation, *B. subtilis* filamentation, FtsZ polymerization and GTPase assay NADPH based			
Modification of already existing weak inhibitor	3-MBA derivatives -		*S. aureus* MRSA	FtsZ inhibition	FtsZ polymerization by sedimentation, light scattering		No	(Stokes *et al.*, 2013)
	PC190723	Not active			GTPase assay by Malachite green assay			(Haydon *et al.*, 2008)
	Compound 1	not tested						(Haydon *et al.*, 2010)
	Compound 2	not tested						(Czaplewski *et al.*, 2009)
								(Sutherland *et al.*, 2003)

Developed from tubulin inhibitors	2-Alkoxycarbonylaminopyridines	not tested		FtsZ	Polymerization of FtsZ and tubulin by light scattering	Synthetic compounds	Yes	
	SRI-7614		19 µM		GTPase by monitoring radioactive phosphate	Southern Research Institute (SRI)		(White *et al.*, 2002)
	SRI-3072		0.28 µM			tubulin inhibitors		
Developed from GTP analogs	BrGTP	not tested	not tested	FtsZ	EM, GTPase (HPLC and fluorescence based)	Molecule synthesized	No	(Lappchen *et al.*, 2005)
Bacterial filamentation	534F6 derivatives	<10 µM		not known	GTPase assay (does not inhibit FtsZ GTPase activity)		not tested	(Mukherjee *et al.*, 2007)
	trans-cinnamic acid		not tested	FtsZ	*E. coli* filamentation, FtsZ polymerization	Selected phytochemicals	not tested	(Rastogi *et al.*, 2008)
Anucleate cell blue assay	A189				GTPase activity, *E. coli* filamentation			(Ito *et al.*, 2006)
Virtual screen	Quinuclidine 12	49 µM	25 µM	FtsZ inhibition			no effect	(Chan *et al.*, 2013a)
Classic bacterial kill assay	Psammaplysin F	Resistant	50 µM	Inhibition of chromosome portioning	MIC, microscopy	Natural product library	not tested	(Ramsey *et al.*, 2013)

Another limitation of the assay is that the molecules inducing non-specific protein polymerization increases the false positive rate, however, this can be addressed in the followup experiments (Trusca *et al.*, 2002).

This technique has been successfully used leading to the discovery of viriditoxin. The molecule is the only known bacterial cell division inhibitor of fungal origin. Wang and colleagues screened >100,000 natural product extracts of microbial and plant origin. A candidate extract from *Aspergillus sp.* was positive for the assay. The pure molecule was then isolated from a mixture of eight molecules in the extract and was identified as viriditoxin. The inhibition of FtsZ activity was further confirmed by *in vivo* fluorescent microscopy and GTPase assay using radiolabelled GTP (Wang *et al.*, 2003).

1.7.1.2 Pyruvate kinase and lactate dehydrogenase coupled GTPase assay

There are several enzymes that yield products (or intermediates) that are not detectable following the existing techniques. Coupled enzyme assays offer a rescue for detecting the activity of those enzymes. In such an assay, the undetectable product of one enzyme serves as a substrate for a subsequent enzymatic reaction that can be quantified. For example, pyruvate kinase and lactate dehydrogenase (PK/LDH) coupled enzyme assays have been classically used to measure the activity of enzymatic reactions that produces ADP or GDP (by enzymes having ATPase or GTPase activity respectively). In the cell, the enzyme pyruvate kinase is involved in glycolysis. It transfers a phosphate from phosphoenolpyruvate to ADP resulting in the formation of ATP and pyruvate. Lactate dehydrogenase uses this pyruvate and converts it into lactate. During this process lactate dehydrogenase utilizes NADH and oxidizes it to NAD^+. NADH absorbs light at 340 nM and therefore decrease in

the absorbance at 340 nM provides an indirect readout for the activity of the test enzyme (Pon *et al.*, 1967). Although indirect, coupled enzyme assays provide an opportunity to track the activity of an enzyme in real time. One of the drawbacks of the high-throughput screen designed based on such assays is that the activity of the downstream enzyme may also be affected by the molecules being screened leading to high false positive rate.

$$\text{GTP} + \text{H}_2\text{0} \xrightarrow{\text{FtsZ}} \text{GDP} + \text{P}_\text{i}$$

$$\text{GDP} + \text{PEP} \xrightarrow{\text{PK}} \text{GTP} + \text{Pyruvate}$$

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{NAD}^+ + \text{Lactate}$$

The coupled enzyme assays have been used to detect the inhibition of cell division protein FtsZ. Since, the end product of the enzyme reaction of FtsZ, GDP, can be utilized by pyruvate kinase its activity can be conveniently coupled to PK/LDH system. Margalit and collogues have developed a high-throughput assay and screened a chemical library of 18,320 compounds to identify the group of FtsZ inhibitors named Zantrins (Margalit *et al.*, 2004b).

1.7.1.3 Fragment-based screening approach

Fragment-based discovery is a rational approach of drug development. Here, instead of using the molecules, their precursors, constituents or fragments of very small sizes (<150 Da) are screened against the target protein. Any activity obtained therein serves as a lead. Selected lead compounds are then enhanced chemically, in a series of steps, to achieve improved activity.

The fragment-based screen is suitable for those targets where the information on the three-dimensional structure of the protein is available. The idea is to identify the precursor molecules that can occupy and block the active site of the target protein. Such precursors can be further modified and developed into a complete molecule to increase specificity or *in vivo* efficacy. The identification of the precursor fragment can be done using one of the two approaches; computer-aided drug design or *in vitro* drug design (Kumar *et al.*, 2012).

In the field of bacterial cell division, the fragment based screening has been successfully used to screen the inhibitors of protein-protein interactions. Tsao *et al* discovered an inhibitor of *E. coli* cell division affecting interaction of FtsZ and ZipA through this approach (Tsao *et al.*, 2006). ZipA is a membrane-anchored protein that interacts with the cell division protein FtsZ. ZipA-FtsZ interaction is essential for *E. coli* cell division (Liu *et al.*, 1999; Hale *et al.*, 2000; Mosyak *et al.*, 2000; Moy *et al.*, 2000). The authors screened 825 synthetic compounds, having molecular weight around 200 Da, for their binding ability to C-terminal of the FtsZ interacting pocket of ZipA. The authors identified seven lead compounds one of which was chemically modified into 14 analogs. The analogs were further analyzed by NMR and X-ray crystallography. Using the same approach this group has designed several other inhibitors of ZipA/FtsZ interaction (Sutherland *et al.*, 2003; Jennings *et al.*, 2004a; Jennings *et al.*, 2004b; Tsao *et al.*, 2006).

One of the drawbacks of this approach is; if used *in vitro* it requires a sophisticated technique to detect the interaction of the molecules with the protein. Various techniques such as NMR spectroscopy, isothermal colorimetry, surface plasmon resonance, X-ray diffraction have been used for the purpose (Schulz *et al.*, 2009; Wyss *et al.*, 2012). If

performed *in silico*, this approach requires extensive knowledge of computational modeling for the analysis and interpretation of the data. Moreover, since the screen is searching for weakly interacting molecules, the compounds have to be screened at higher concentrations and needs to have greater solubility.

1.7.1.4 Fluorescence polarization/anisotropy (FP/FA)

Fluorescence polarization is a widely used method for detecting protein-protein, ligand-receptor, drug-protein and enzyme-substrate interactions. It has also been employed to study cell division as well as to screen for inhibitors of FtsZ and ZipA/FtsZ interaction (Kenny *et al.*, 2003; Reija *et al.*, 2011; Ruiz-Avila *et al.*, 2013)**.**

The principles of fluorescence polarization were first theorized in 1926 (Perrin,1926). They were based on the observation that when light polarized in a particular plane is projected on a fluorophore in the solution; it emits light polarized in the same plane

as long as the molecule remains stationary. However, molecules in the solutions do not remain stationary and are constantly moving due to the Brownian motion. When a molecule rotates or tumbles, the emitted light is polarized in a different plane. This fluorescence polarization can be quantified by measuring light emitted in a plane parallel and perpendicular to the excited light and changes upon binding of the fluorophore to a protein (Reija *et al.*, 2011). This forms the basis of application of FP/FA techniques in detecting the interactions between a small-molecule and a protein or between two proteins.

Kenny *et al*., in 2003 adapted this principle for the high-throughput screening of inhibitors of ZipA/FtsZ interaction (Kenny *et al.*, 2003). Using the phage display technique, they first

identified a 12 amino acid peptide named PD1 that binds to ZipA with higher affinity than FtsZ. Subsequently, one of the fluorescently labeled PD1, instead of FtsZ protein, was used for a high-throughput screen of 250,000 small molecules. Using fluorescence polarization as the detection method, 29 molecules that competitively inhibit the interaction of PD1 to ZipA were identified. These candidate molecules showed potential as inhibitors of FtsZ-ZipA interaction and could be further developed as antibacterials targeting cell division (Kenny *et al.*, 2003).

Claudia Schaffner-Barbero and colleagues used the same principle of light polarization to detect the inhibition of protein-ligand interaction, FtsZ-GTP in this case, instead of protein-protein interaction as described earlier (Schaffner-Barbero *et al.*, 2010). They developed a method to screen small-molecule inhibitors of FtsZ based on the competitive FP/FA analysis. In this study, a fluorescently labeled GTP (mant-GTP) was used as the fluorophore and its displacement by the small-molecule inhibitor binding to the GTP binding site of FtsZ was observed by measuring the change in the fluorescence polarization (figure 1.5a and b). In a recent screen, the same group identified a synthetic molecule UCM05 (figure 1.5c) and its two analogs UCM44 and UCM53 as the competitive inhibitors of *B. subtilis* FtsZ. The activity was verified using light scattering, sedimentation assay and electron microscopy techniques (Ruiz-Avila *et al.*, 2013).

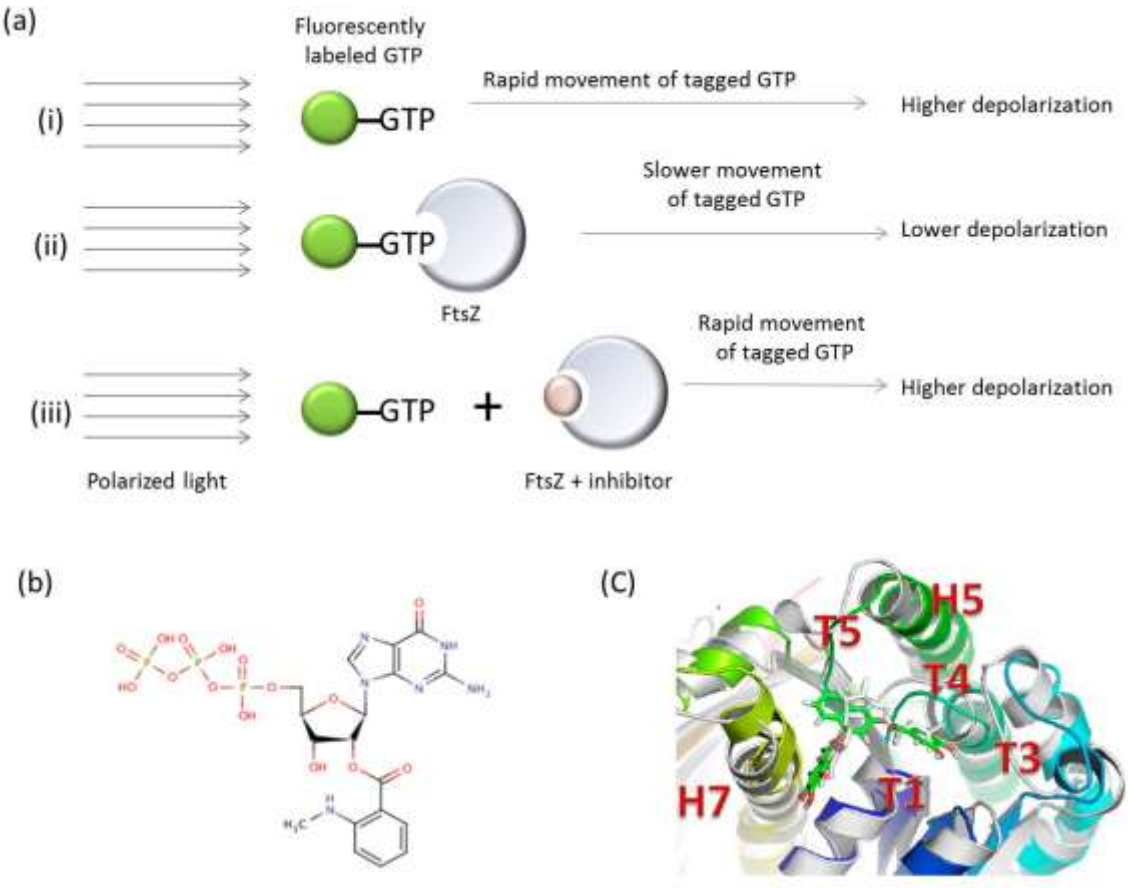


Figure 1.5 Use of fluorescence polarization technique to screen inhibitors of FtsZ: (a) Principle of an FtsZ inhibitor screen based on competitive fluorescent polarization. (i) When fluorophore is excited with plane polarized light due to the rapid movement in the solution the emitted light is not polarized in the same plane (ii) when the fluorophore is bound to a bulkier protein (FtsZ in this case), the rate of movement in the solution is reduced and as a result depolarization of the emitted light is reduced. (iii) A competitive small-molecule inhibitor of FtsZ displaces the fluorophore that in turn increases its rate of movement and increases depolarization of light. (b) Structure of mant-GTP, the fluorophore used for the detection of interaction of small-molecule inhibitor with FtsZ by Laura B. Ruiz-Avila *et al*. (c) Representative snapshots of the simulation for the first binding mode of UCM05, the inhibitor identified through FP/FA method, to FtsZ (Laura B. Ruiz-Avila *et al*., 2013)

1.7.2 Modification of known molecules (drug repositioning)

Modification of known molecules with a validated mode of action is another approach for developing new drugs. In this approach, the lead molecules obtained from a target based screen are chemically modified to increase potency, target specificity and affinity to improve the pharmacokinetics of the molecule. This approach significantly shrinks the drug development pipeline and bypasses the requirement for the initial discovery of the weak inhibitors; thereby provides a significant advantage. This strategy has been applied to bacterial cell division inhibition by modifying known weak inhibitors of FtsZ, the analogs of GTP and inhibitors of the eukaryotic FtsZ homolog, tubulin. Selected cell division inhibitors discovered this way are discussed here.

1.7.2.1 Modification of the known weak inhibitors - the curious case of 3-MBA

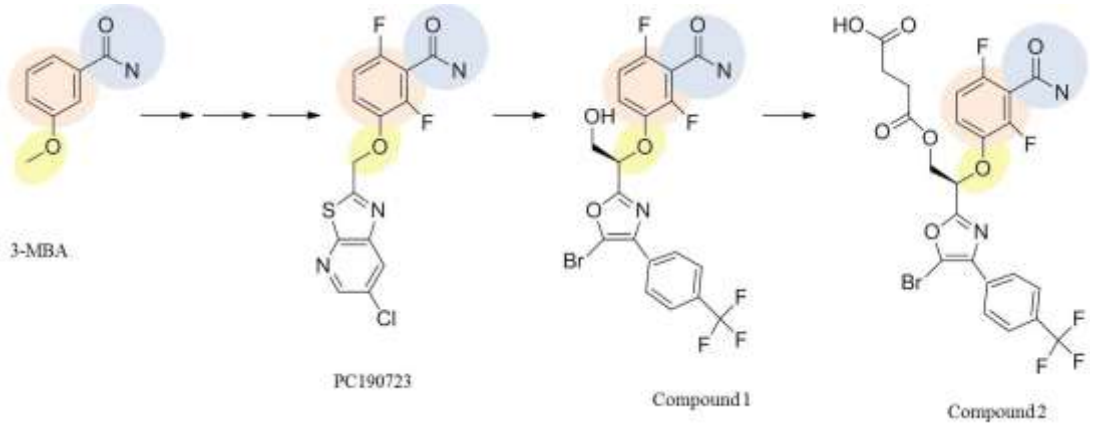


Figure 1.6 Evolution of more potent compound from the weaker inhibitor of FtsZ: 3-methoxybenzamide a weak inhibitor of FtsZ was modified by a series of chemical modification which at each step lead to a compound with higher potency or better

efficacy than the preceding molecule. The most recent compound originated from 3-MBA has lower MIC, higher *in vivo* efficacy in the mouse model and specifically targets FtsZ. At each stage of modification the target specificity is ascertained by *in vitro* and *in vivo* experiments.

Chemical modification of a weak inhibitor with the known target has been a very successful technique for the development of novel cell division inhibitors. PC190723 is a very well-known example and the first cell division inhibitor with *in vivo* efficacy, which was developed by the modification of a weaker FtsZ inhibitor 3-methoxibenzamide (3-MBA) (Haydon *et al.*, 2008).

3-MBA was initially identified as an inhibitor of ADP-ribosyltransferase that served as the precursor of PC190723 (Romano *et al.*, 1988). It had been observed previously that 3-MBA inhibits cell division and induces filamentation in *B. subtilis*. In 1999, Ohashi, Y. *et al.* discovered that certain mutations in *ftsZ* could overcome the lethal effects of 3-MBA (Ohashi *et al.*, 1999). Later Czaplewski and co-workers carried out an extensive structural activity relationship (SAR) study of 3-MBA to synthesize 43 compounds with improved antibacterial activity (Czaplewski *et al.*, 2009). Several of these compounds induced filamentation in *B. subtilis* and cell enlargement (ballooning) in *S. aureus* indicative of cell division inhibition. Haydon *et al.* synthesized 500 analogues of 3-MBA including the potent cell division inhibitor PC190723 (Haydon *et al.*, 2008). The molecule showed lower MICs against Gram-positive bacteria compared to the parent molecule, 3-MBA. The target specificity of PC190723 was demonstrated by the generation of resistant mutants and mapping the point mutation in the *ftsZ* gene. The target specificity and the first ever reported *in vivo* efficacy in a mouse model of infection for a cell-division inhibitor proved it to be a strong candidate for clinical trials against methicillin-resistant *Staphylococcous aureus*

(MRSA). The same group then went on to develop another series of chemical modifications. Two derivatives of 3-MBA from this series named, compound 1 and compound 2 have been recently developed showing superior potency than PC190723 against various strains of *Staphylococcus* species (Stokes *et al.*, 2013). Compound 2 has been further modified by SAR studies and two more compounds with better *in vivo* pharmacokinetics as well as lower antibacterial activity have been developed (Stokes *et al.*, 2014). Thus, 3-MBA served a very good starting point for the development of new FtsZ inhibitors because, in spite of being a weak inhibitor of bacterial growth, it was able to penetrate the cell membrane and had a known target with defined mode of action.

Another example of the use of this strategy to develop FtsZ inhibitor is the development of an anti-malarial precursor 2-alkoxycarbonylaminopyridine as the inhibitor of *Mycobacterium tuberculosis* FtsZ (Mathew *et al.*, 2011).

This approach provides a a major advantage in being cost effective and less time-consuming compared to the starting a screen with a huge set of unknown molecules. Moreover, since the target is already deciphered there are fewer chances of generating false positives and lesser efforts are required for target validation.

1.7.2.2 GTP analogues

Two studies published in 1992 demonstrated the ability of *E. coli* FtsZ to bind and hydrolyze GTP (de Boer *et al.*, 1992; RayChaudhuri *et al.*, 1992). These were the first evidence showing a non-regulatory role for FtsZ in bacterial cell division. It was later shown that GTP binding (but not hydrolysis) is essential for the polymerization of FtsZ (Mukherjee *et al.*, 1994). Early work to study the effects of non-hydrolysable GTP analogs on the FtsZ

GTPase activity and polymerization was specifically aimed at studying the protein's function in detail and not at developing the inhibitors of cell division (Scheffers *et al.*, 2000; Mingorance *et al.*, 2005).

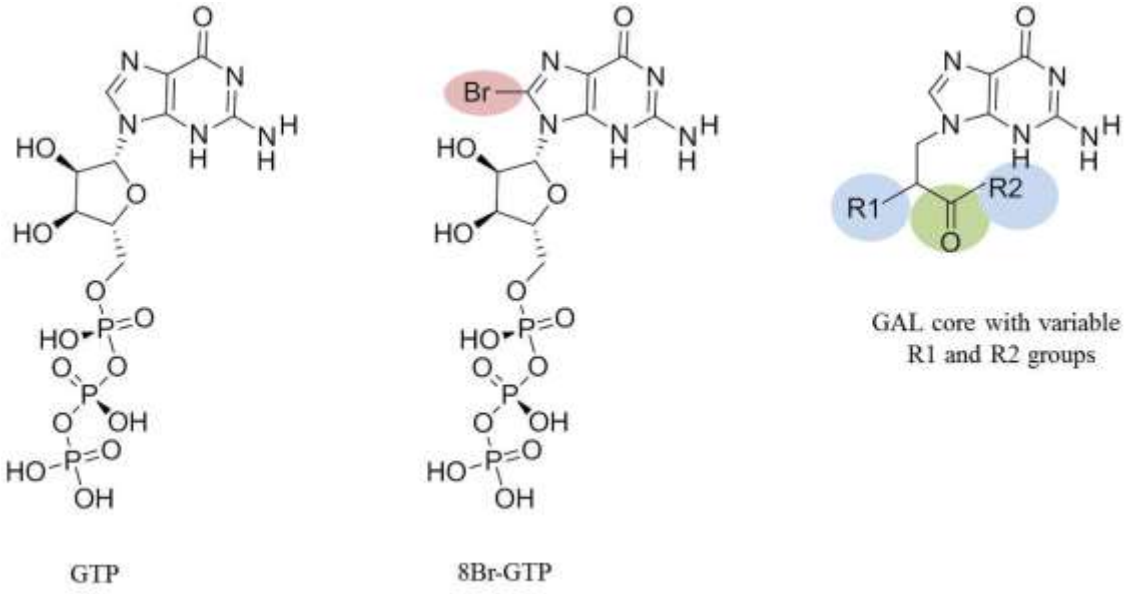


Figure 1.7 GTP analogs as FtsZ inhibitors: GTP analogs have been synthesized by chemical modification of GTP making it un-hydrolysable by FtsZ and keeping GTP's FtsZ binding site constant. These molecules when specifically bind to FtsZ form a very good starting point for discovery of novel FtsZ inhibitors.

Later, when the importance of the bacterial cell division as an antibiotic target was realized, the GTP analogs were channelized to the development of FtsZ inhibitors. In one of the early works Lappchen *et al*. showed the inhibition of FtsZ activity by a GTP analog, 8-bromoguanosine 5'-triphosphate (BrGTP), which had no inhibitory effect on tubulin

polymerization (Lappchen *et al.*, 2008). Using solid phase chemistry and keeping the core FtsZ binding structure constant, several novel GTP analogs with potent anti-staphylococcal activities have been designed (Lappchen *et al.*, 2005). GTP analogs have thus proved to be a very good starting point for the development of antibiotics targeting FtsZ. Such molecules are relatively easy to develop and test since the core structure and its interaction with the protein is well understood. However, the presence of several essential GTP-binding proteins in the cell might pose a challenge in developing a molecule specifically targeting FtsZ.

1.7.2.3 Tubulin inhibitors

Tubulin, a eukaryotic protein belonging to the family of small globular protein, is studied extensively for the formation of cytoskeleton filaments, microtubules and for its role in eukaryotic cell division (Jacobs,1975; Mazia,1975). There are six families of tubulin of which alpha-tubulin and beta-tubulin are well studied for their role in microtubule formation (Fujiwara *et al.*, 1975; Inoue *et al.*, 1975). The existence of a prokaryote homolog of tubulin was unknown until early 1990s when several groups showed the presence of a glycine-rich sequence motif, (GGGTGTG) in FtsZ involved in GTP binding. A similar motif was known to be present in tubulin (A/G)GGTG(S/A)G, and until then was thought to be unique to the tubulin family of proteins (Erickson,1997). These were the early evidence indicating FtsZ could be a prokaryote homolog of tubulin. Although, there is little (<20%) sequence homology between FtsZ and tubulin, the extensive structural characterization, sequence homology and studies of GTPase activity of FtsZ confirmed it to be the structural and functional homolog of tubulin (Erickson,1997).

Tubulin had been discovered long before its prokaryote homolog FtsZ and the small-molecule inhibitors of tubulin have been identified since the late 50s (Wilson,1975a; Wilson,1975b). These tubulin inhibitors have received much attention because of their importance as cancer drugs and their role as probes of eukaryotic cell division (Wilson,1975a; Wilson,1975b). Since, FtsZ is a structural and functional homolog of tubulin, these inhibitors form very good starting point for the development of FtsZ inhibitors which after chemical modifications can be made specific to FtsZ. In fact, it was found that the most acclaimed FtsZ inhibitor PC190723 binds to the taxol binding site of tubulin without affecting its activity, which substantiates the potential of tubulin inhibitor to be developed as FtsZ inhibitors (Haydon *et al.*, 2008; Haydon *et al.*, 2010).

Qing Huang *et al*. took this approach to discover drugs against *M. tuberculosis* targeting the cell division protein FtsZ using a group of plant-based diterpene tubulin inhibitors, called taxane as the starting point (Huang *et al.*, 2006). Amongst 17 taxans tested, selected molecules with lower MIC and cytotoxicity were followed up for chemical modification. This led to the development of a novel anti-tuberculosis agent targeting FtsZ without any cytotoxicity to the eukaryotic cells. Lucile White *et al*. in a similar study screened >200 known tubulin inhibitors and found two potent FtsZ inhibitors that could be developed further as anti-microbial agents (White *et al.*, 2002).

1.7.3 Cell based screening approaches

Although in theory, *in vitro* screening approaches apparently yield drug-like molecules, in reality, most of these molecules get dropped out from the drug discovery pipeline due to

poor *in vivo* efficacy. In contrast, even though target identification of molecules identified by an *in vivo* screen is the most challenging step, a potent drug-like molecule with multiple targets or a well-validated target could prove to be very useful lead compound. The simplest *in vivo* screen is based on standard kill assay where the molecules are screened for the inhibition of growth of the chosen organism followed by target identification of the most useful hit. However, several screens have taken advantage of the distinct phenotypic changes imparted by the inhibition of the target protein or a reporter system to screen for the pathway-specific inhibitors. Some of these screens leading to the identification of the bacterial cell division inhibitors are discussed below.

1.7.3.1 *Bacillus subtilis* sporulation as an assay for cell division inhibition

B. subtilis is a Gram-positive rod-shaped bacterium that, when starved for nutrients, form endospores. This involves the formation of an asymmetrically positioned septum that divides the cell into a smaller forespore and a larger mother cell. In the subsequent events of sporulation, the forespore thus formed is engulfed by the mother cell which eventually lyses. The miniaturized cell develops a thick coat and matures into a spore (Errington,1991; Margolis *et al.*, 1991b). Although the outcome is different from vegetative septation, the same cell division machinery is employed during sporulation (Errington,1991). Therefore, a small molecule inhibitor of the cell division would also result in the inhibition of sporulation.

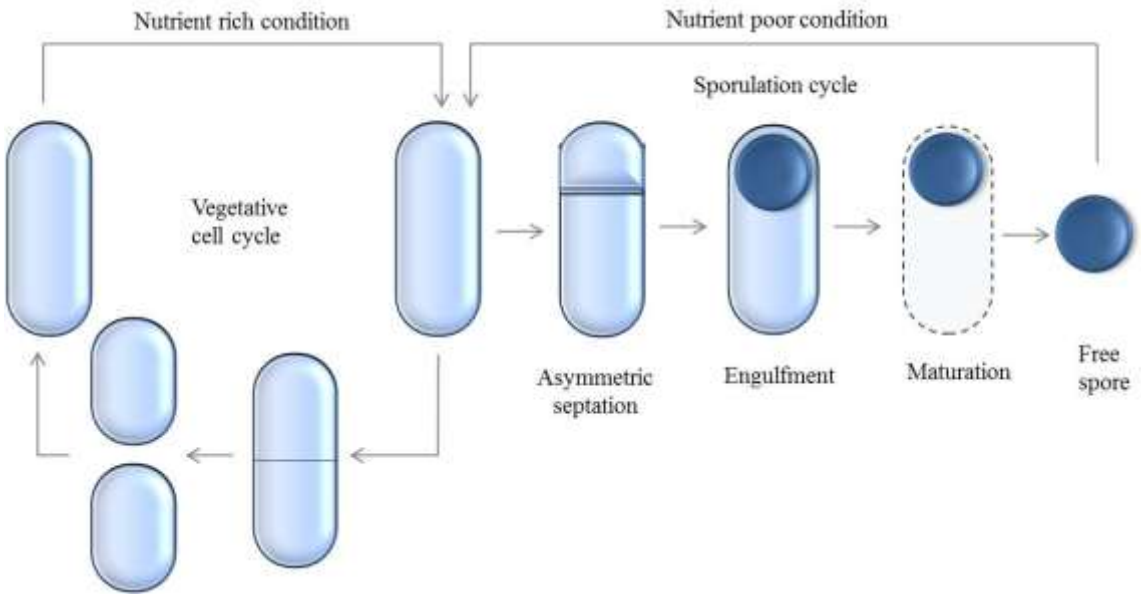


Figure 1.8 Endospore formation in *B. subtilis*: Endospore formation in *B. subtilis* is initiated by nutrient limitation and leads to the formation of an asymmetric septum. In the subsequent steps of sporulation, the smaller compartment formed by the asymmetrically positioned septum ultimately develops into a mature endospore. The larger compartment that played the role of a mother cell lyses to release the endospore. The cell division machinery involved in formation of the vegetative septum is also responsible for the formation of the asymmetric septum during sporulation.

Unlike eukaryotic system, vegetative cell division in bacteria does not have a checkpoint system. However, *B. subtilis* sporulation is heavily regulated at the transcriptional level, specifically, by alternate sigma factors that direct the activity of RNA polymerase to stage-specific sporulation genes in a manner that depends on preceding developmental steps. One such sigma factor, SigmaF is activated only after the formation of the asymmetrically positioned septum is complete (Margolis *et al.*, 1991a; Stragier *et al.*, 1996). Taking advantage of this, Stockes *et al*. developed a high throughput screen to identify the inhibitor of the septum formation by monitoring expression levels of a SigmaF controlled gene. They fused the promoter of the sporulation gene *spoIIQ,* one of the SigmaF controlled gene, to a

gus reporter and used this construct to analyze the changes in expression levels by the addition of molecules *(Stokes et al., 2005a)*. This is the first cell-based high-throughput screen for the inhibitors of bacterial cell division based on the expression level of a gene. Stockes *et al*. identified several potent cell division inhibitors that were in the confirmatory assay found to be targeting FtsZ of Gram-positive bacteria.

1.7.3.2 Bacterial filamentation assay

Another phenotypic change of cell division inhibition in the rod-shaped bacteria is bacterial cell filamentation. In the absence of cell division when the rod-shaped bacteria like *E. coli* and *B. subtilis* continue to grow, they form long tubular cells which may or may not contain septa. This microscopic phenotypic change serves as a clear indication of the inhibition of cell division. It is often used as the confirmatory assay but could be adapted to screen cell division inhibitors *in vivo*. Mukherjee *et al*. screened and identified a compound named 534F6 through observing such phenotypic changes in *E. coli (Mukherjee et al., 2007)*

Although every single cell division inhibitor identified so far has been tested for its ability to filament rod-shaped bacteria for the target validation, there are relatively fewer reports relying on this simple assay for screening such inhibitors. This is due to the difficulty involved in adapting this assay for higher throughput screening. However, recent advances in high-content microscopy and programs to analyze and interpret a large number of images may well facilitate screens for cell division inhibitors using filamentation based phenotypic assays.

1.7.3.3 Anucleate cell blue assay

The anucleate cell blue assay was originally designed as a screening platform for defects in chromosome partitioning in *E. coli* and serendipitously resulted in the identification of an FtsZ inhibitor. The assay used an *E. coli* strain containing a plasmid that carries two genes: *repA*, responsible for the amplification of the plasmid and *lacZ*, responsible for the production of blue color. Both these genes are under the control of a repressible promoter. The repressor was encoded by chromosomal DNA. In the absence of proper chromosomal partitioning, the cells lacking the chromosome but containing the plasmid would lose the repression resulting in the increased plasmid copy number by expression of *repA* as well as the production of blue color by *lacZ* expression (Wachi *et al.*, 1999). Using this reporter system, Oyamada Y. *et al*. screened 95,000 compounds and identified around 50 molecules that affected chromosome partitioning (Oyamada *et al.*, 2006). Ito *et al*. later tested some of these compounds for the inhibition of FtsZ activity and the filamentous phenotype in *E. coli*. One of the molecules named A189 was found to inhibit cell division via affecting the GTPase activity of FtsZ (Ito *et al.*, 2006).

1.7.4 Virtual screening

The technique of *in silico* docking involves the computational modeling of compounds into various regions of the protein of interest. The *in silico* docking approach has been updated to allow structure-based virtual screening of small molecule inhibitors of a protein (Simmons *et al.*, 2010). A prerequisite for this screening strategy is the determination of a target protein with a validated crystal structure. In the first step, the target protein is screened against a database of molecules for their ability to bind. This step is performed entirely *in silico,* which is followed by the target confirmation *in vitro* and *in vivo*. The virtual high-

throughput screening has been made possible because of the advancement in software support to carry out such screening. Some example of the programs that have been used for such screening include AutoDock, Glide (Schrödinger), GOLD (The Cambridge Crystallographic Data Centre, Cambridge, UK), DOCK and eHiTS (SimBioSys Inc., Toronto, Canada) (Simmons *et al.*, 2010).

Since the crystal structure of several bacterial cell division proteins; FtsZ, FtsA, ZipA, ZapA, FtsK, FtsQ, FtsI, FtsN and AmiC has been determined, virtual *in silico* screening may be an attractive strategy for the development of cell division inhibitors (Lowe *et al.*, 1998; Cordell *et al.*, 2001a; Cordell *et al.*, 2001b). Structure of the central divisomal protein FtsZ had been determined in the late 90s and using this structural information Chang F.Y. *et al*. carried out the vHTS (virtual High-Throughput Screens) of a natural product library to identify a pyrimidine-quinuclidine scaffold as the potential FtsZ inhibitor (Chan *et al.*, 2013b). The molecule was further refined for potency by virtual SAR studies. This resulted in an inhibitor of FtsZ inhibitor with potent antimicrobial activity against Gram-positive and Gram-negative bacteria. Though it has been shown that the molecule does not inhibit tubulin activity, its specificity for FtsZ needs to be determined at the molecular level (Chan *et al.*, 2013b). Nevertheless, this example proves the potential of a virtual screen for the identification of novel cell division inhibitors.

1.8 Regulation of cell division in stressful conditions

For a living cell, DNA serves as a blueprint of all the cellular functions and messages. Most organisms at some point get exposed to agents that damage their DNA. Since DNA is, from the evolutionary point of view, the most important cellular component, cells have developed

mechanisms to tightly limit and quickly repair the damage caused to it. Both eukaryotic and prokaryotic cells sense the presence of damaged DNA and change the transcription of a number of genes that facilitates the survival of cells in such adverse conditions. This transcriptional response of the cell to the DNA damage is called the SOS response (Radman,1975; d'Ari,1985b; Peterson *et al.*, 1988).

1.8.1 RecA-dependent SOS-response

The prokaryotic SOS response has been extensively studied in *E. coli*. The two most important regulators of this response are RecA and LexA. In the absence of DNA damage, LexA binds the promoters of the SOS genes in stretches of DNA called the "SOS box", inhibiting their expression. In the event of DNA damage, ssDNA is generated either as a result of the damage to DNA or because of the stalled replication fork. In the first series of events, RecA immediately binds to ssDNA and forms a nucleoprotein complex that activates its co-protease activity (figure 1.9). The RecA-ssDNA complex then activates autocatalytic activity of LexA resulting in the cleavage and release of LexA bound to the SOS box. This enables RNA polymerase to transcribe the SOS genes (Janion,2008).

LexA is not the only protein cleaved by the activated RecA. The C-terminal of LexA shares homology with the C-terminal of two other proteins in *E. coli*: UmuD and the lambda phage repressor. UmuD, in the absence of DNA damage represses the expression of *umuDC* operon, which codes for the error-prone DNA polymerase pol V. In the situation where the damage to the DNA cannot be repaired, DNA polymerase V can resume the replication, albeit with a high mutation rate (d'Ari,1985a; Elespuru,1987; Janion,2008).

Interestingly, the lysogenic phage residing within the genome of *E. coli* has devised a way to lyse and escape the bacterial cells under stressful conditions. As mentioned earlier the repressor of the phage λ, cI, has homology to the repressor LexA in its C-terminal. The RecA-ssDNA complex can cleave the repressor which results in the expression of the genes responsible for entering and carrying out the lytic phage cycle. This results in replication of the phage and lysis of the host to release the phages.

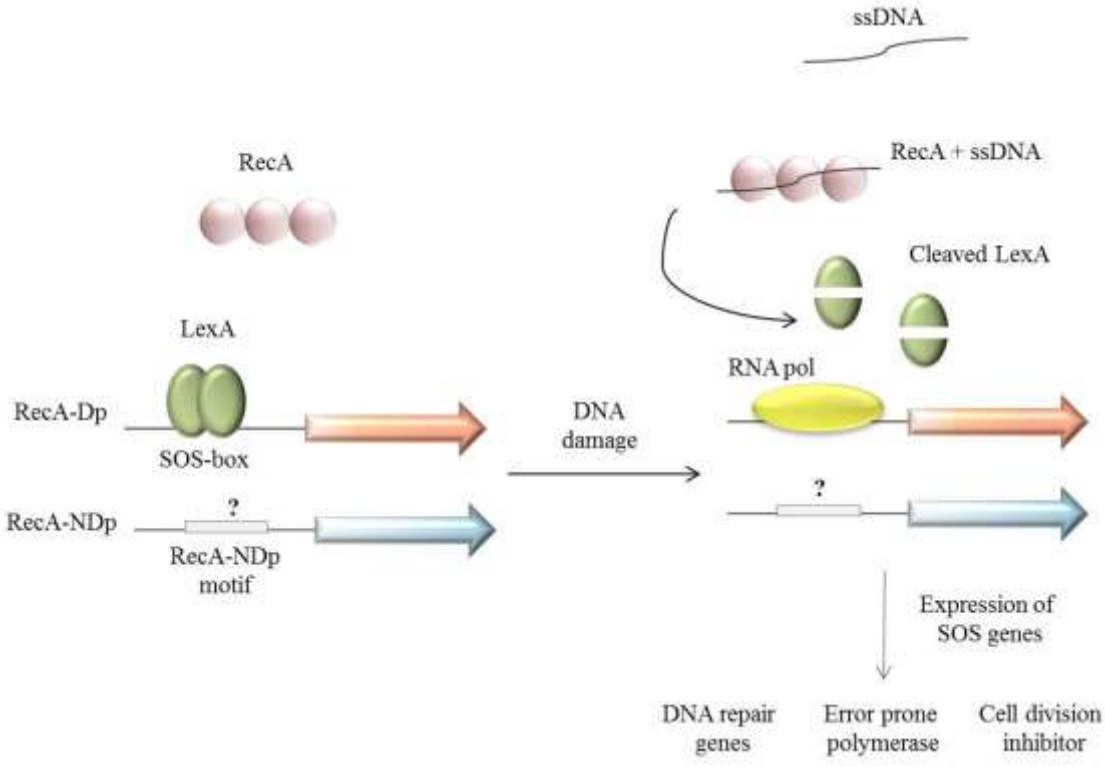


Figure 1.9 The bacterial SOS response: Bacterial SOS response which is induced by DNA damage, replication block or in some cases cell wall damage results in expression of genes that are regulated by the single-stranded DNA binding protein RecA or independently of RecA. The SOS genes induced by this stress response are involved in repairing the damage, resisting the damage and temporarily halting cell division.

1.8.2 RecA-independent detection of the DNA damage

When there is damage to the DNA, most bacteria use the classic DNA damage response described above, which involves the induction of SOS genes by RecA and LexA regulated mechanism. However, using the microarray technique, Rand L. *et al*. found that the majority of the DNA damage-induced genes in *Mycobacterium* are expressed independently of RecA (Rand *et al.*, 2003). The presence of a conserved motif was also shown by computational analysis of the upstream regions of around 50 SOS genes from *Mycobacterium*. The similar motif is also shown to be present in some other Actinomycetes, including *Streptomyces* (Rand *et al.*, 2003). Although, the exact mechanism of regulation of such response is not yet characterized, involvement of an activator protein has been suggested (Rand *et al.*, 2003). The promoter motif regulating the RecA-independent SOS genes in actinomycetes is discussed in detail in chapter 3.

1.8.3 Repairing DNA damage

Before the invention of transcriptomic techniques very common today, the identification of SOS genes relied on the expression analysis by random transcriptional fusion of reporter genes. The genes identified in such a way were initially termed as din for DNA **d**amage-**in**ducible. Induction of the DNA damage response in *E. coli* leads to the expression of around 40-50 genes (Love *et al.*, 1985). These genes are involved in repairing the damage, protecting the DNA from further damage, inhibiting the cell cycle and regulating the SOS response (Sedgwick,1986; Elespuru,1987; Yasbin *et al.*, 1991; Janion,2008).

The DNA damage is repaired either by the base excision repair (BER), which removes the damaged base and replaces it by the correct one or by recombination, which removes the stretch of damaged DNA and synthesizes a new one by homologous recombination. If the damage is severe and cannot be repaired by these mechanisms, an error-prone polymerase (pol IV/V) bypasses the damage and resumes the replication (Janion,2008).

Besides the induction of DNA repair genes, the SOS response also inhibits cell division for two reasons: (i) to maximize resource allocation for the repair of damaged DNA and (ii) to avoid propagation of the damaged genome. SulA, YneA, Rv2719c, DivS and SidA have been identified as the DNA damage induced cell division inhibitors in *E. coli*, *B. subtilis, M. tuberculosis, Corneybacterium glutamicum* and *Caulobacter cresentus* respectively (Huisman *et al.*, 1984; Chauhan *et al.*, 2006; Ogino *et al.*, 2008; Modell *et al.*, 2011a; Vadrevu *et al.*, 2011). However, these inhibitors are not related to each other and their homologs are not present in *Streptomyces.*

1.8.4 Shutting down the damage response

After the DNA damage is repaired, the SOS response needs to be shut down. For a long time it was believed to occur passively by decreased expression of *lexA* and *recA* after the damage repair. However, recently Galkin *et al*. have found that a DNA damage induced protein, DinI binds and inhibits RecA resulting in the active inhibition of the SOS response (Galkin *et al.*, 2011). Inhibition of the RecA activity by RecX is also a proposed mechanism for the active inhibition of SOS response (Long *et al.*, 2009).

1.8.5 DNA damage induced cell division inhibition

In addition to the expression of genes that are involved in repairing the DNA damage, the SOS response also expresses genes that are involved in inhibiting cell division to avoid propagation of a damaged chromosome.

In *E. coli*, the gene responsible for inhibiting cell division as a part of the SOS response is named *sulA* (Huisman et al., 1984; Jones et al., 1985; Love et al., 1985). SulA binds and inhibits polymerization of the cell division protein FtsZ. FtsZ is one of the earliest proteins to determine the timing and site of cell division. Inhibition of FtsZ by *sulA* expression imparts filamentous phenotype to *E. coli*. After the damage is repaired, the SOS response machinery shuts down and transcription of genes resumes to the normal levels. However, some of the SulA protein still lingers in the cell which is sufficient to inhibit cell division. A site specific ATP-dependent protease, Lon degrades the residual SulA and cells begin to divide normally (Higashitani *et al.*, 1997).

After the discovery of the DNA damage checkpoint protein SulA in *E. coli*, there was a lag until the discovery of similar proteins in other organisms. This was because SulA served as the prototype of bacterial SOS induced cell division inhibitors. However, *sulA* is conserved only in gamma-proteobacteria.

A structurally and functionally distinct protein YneA was identified in *B. subtilis* as the SOS response induced cell division inhibitor (Kawai *et al.*, 2003). YneA is a secreted protein containing the peptidoglycan binding domain LysM. It is expressed upon DNA damage from an SOS-box containing promoter and appears to inhibit cell division independently of FtsZ (Kawai *et al.*, 2003).

Another LysM domain containing protein, ChiZ was later identified as the DNA damage induced cell division inhibitor in *M. tuberculosis (Vadrevu et al., 2011)*. *chiZ* is expressed divergently from *lexA* and its expression is regulated by RecA-dependent and RecA-independent mechanisms. ChiZ in addition to the peptidoglycan binding domain also contains peptidoglycan hydrolase domain and the hydrolase domain is sufficient for the cell division inhibitory activity of ChiZ. This indicates that the presence of the LysM domain in the cell division inhibitors may be only for directing the protein to the correct destination (Chauhan *et al.*, 2006). In the bacterial two-hybrid assay, ChiZ was found to interact with FtsI and FtsQ making it the first non-FtsZ cell division inhibitor to be discovered. Later based on the presence of the protein domain and the gene's position on the chromosome such inhibitors have been identified in *Corneybacterium* species as well (Ogino *et al.*, 2008).

Recently, Modell *et al*. identified a small protein SidA as the DNA damage induced cell division inhibitor in alpha-proteobacteria, *C. cresentus*. SidA does not contain a LysM domain or a peptidoglycan hydrolase domain. It directly binds FtsW and inhibits constriction of the cytokinetic ring (Modell *et al.*, 2011b). Modell *et al*. argue that a stress-induced cell division inhibitor acting at the later stages of divisome assembly provides a distinct advantage of quick resumption of the functional divisome when the stress is relieved.

It is also apparent from the DNA damage induced cell division inhibitors identified later that SulA, which was considered a paradigm of such inhibitors, is actually an outlier (Modell *et al.*, 2011b).

1.9 *Streptomyces* as a model to study bacterial cell division

Cell division is a cardinal process of bacterial cell biology and is equally important for the therapeutic purpose. As discussed earlier, in the genus *Streptomyces* , cell division is tightly linked to the developmental cycle. Dispensability of cell division for the colony formation in these bacteria, along with the phenotypic changes associated with it; provide a unique opportunity to study bacterial cell division. Although, the mode of cell division in *Streptomyces* is different from the unicellular bacteria, the cell division machinery is identical, which allows a broader application of the knowledge gained from these bacteria.

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CHAPTER 2

STREPTOMYCES – A SCREENING TOOL FOR BACTERIAL CELL DIVISION INHIBITORS

(A version of this chapter has been published: *Jani et al.*, 2014. Journal of Biomolecular Screening)

2.1 Abstract

Cell division is essential for spore formation, but not for viability in the filamentous streptomycetes bacteria. The failure to complete cell division, therefore, confers a developmental phenotype in which colonies fail to produce the gray, spore-associated pigment. This phenotype can be scored by visual examination of the colonies. While this is an unusual mode of cell division, the streptomycetes divisome is very similar to that of other prokaryotes. We, therefore, hypothesized that screening for chemical inhibitors of sporulation in model streptomycetes will be a good way to identify compounds that interfere with divisome function. To test this, we investigated the effect of 196 previously identified compounds that inhibit sporulation in *Streptomyces coelicolor*. We show that 22 of these compounds cause filamentous growth in *Bacillus subtilis* consistent with impaired cell division and 3 of them decreased the cell size. One of the compounds is a DNA damaging agent and therefore inhibits cell division by activating the SOS response. The remaining 21 act independently of known stress responses and may therefore act on the divisome itself. Three of these compounds, which we name Fil-1, 2 and 3, confer distinct cell division defects on *B. subtilis* as determined by microscopic examination as well as inhibit the formation of endospores. Consistent with the essentiality of cell division in most prokaryotes, all three compounds have antibacterial activity against *B. subtilis.*

2.2 Introduction

Cell division, the cellular event as a result of which two daughter cells are generated from a single parent cell, is essential for viability in most organisms. Bacterial cells express a set of proteins, referred to as the divisome, which brings about the biosynthesis of new cell wall and membrane at the site of division. The divisome works in conjunction with proteins that convey spatial information to ensure accurately positioned septum biosynthesis and cytokinesis. This machinery responds to environmental signals and stresses to ensure that cell division does not occur when conditions are unfavorable. For example, damage to genomic DNA or the cell wall can lead to the arrest of cell division, allowing repair proteins to serve their purpose (Harry *et al.*, 2006; Adams *et al.*, 2009).

The divisome of *B. subtilis* consists of FtsZ, FtsA, FtsW, PBP1, PBP2B, EzrA, DivIB, FtsL, DivIC, EzrA, SepF and other proteins that regulate the divisome assembly and placement (Thomaides *et al.*, 2001; Gamba *et al.*, 2009). The divisomal proteins have been described genetically and in a few cases, biochemically. Enzymatic activities ascribed to it so far include the GTPase activity of FtsZ, the ATPase activity of FtsA and flippase activity for lipid-linked cell wall precursors associated with FtsW (de Boer *et al.*, 1992; Feucht *et al.*, 2001; Mohammadi *et al.*, 2011).

The biochemical functions of the other divisomal proteins are less well understood and the development of new tools for studying this apparatus, including chemical probes, could be very significant. Given the essentiality of the divisome for pathogenic bacteria, such probes might also serve as lead compounds for the development of new therapeutic

agents. Efforts to develop such lead compounds for bacterial cell division are, therefore, an active area of research (Margalit *et al.*, 2004a; Kumar *et al.*, 2010; Singh *et al.*, 2010; Awasthi *et al.*, 2011; Foss *et al.*, 2011). So far, most of the chemical inhibitors described for the cell division apparatus act on FtsZ (Jennings *et al.*, 2004; Margalit *et al.*, 2004a; Stokes *et al.*, 2005a; Kumar *et al.*, 2010). FtsZ, a tubulin-like protein, assembles at a prospective division site, where it forms a 'Z-ring'. The Z-ring then recruits the other cell division proteins and templates the formation of a functional divisome. FtsZ forms polymers *in vitro* and binds and hydrolyzes GTP. These quantifiable activities have been used to develop assays for compound screens, resulting in the discovery of PC190723, PC58538, viriditoxin, the zantrins and several other candidate inhibitors (Wang *et al.*, 2003; Margalit *et al.*, 2004b; Stokes *et al.*, 2005b; Haydon *et al.*, 2008). Unlike all other organisms in which this process has been investigated to date, cell division in the filamentous bacterial genus *Streptomyces* is dispensable for viability (McCormick *et al.*, 1994; McCormick *et al.*, 1996; Flardh *et al.*, 2000; Bennett *et al.*, 2007; Mistry *et al.*, 2008). The growth of these bacteria takes place in the context of a complex life cycle involving morphologically distinct developmental stages (Hopwood, 2007). Colony growth initiates with spore germination and the formation of a vegetative colony of filamentous 'substrate hyphae'. Following this, a layer of reproductive 'aerial hyphae' grows up from the colony surface to impart a white, "fuzzy" appearance to it. Cell division is relatively rare in the substrate hyphae; these cells therefore, consist of long chambers containing multiple chromosomes. In contrast, a developmentally regulated round of cell division takes place in the aerial hyphae that divides each filament into a

chain of uni-nucleoid compartments that subsequently develop into spores (Chater,1998). In one of the best-characterized streptomycetes, *S. coelicolor*, the last visually observable step is the deposition of a gray spore pigment, the biochemical product of the *whiE* locus in the spore wall. This imparts a readily assessable gray phenotype to wild-type colonies (Davis *et al.*, 1990). Most mutations that block the maturation of spores in the aerial hyphae ultimately prevent *whiE* expression. These mutations are, therefore, referred to as *whi* for 'white' because they fail to develop the typical gray pigmentation associated with mature, wild-type spores (Davis *et al.*, 1990). These visual cues make it possible to distinguish mutations that block the maturation of spores in those cells.

2.3 Results

In previous work, we conducted a screen of 30,569 compounds against *S. coelicolor* and thereby identified chemical inhibitors of many steps of the organism's life cycle (Craney *et al.*, 2012). In particular, we identified 196 compounds that conferred white colony morphology – reminiscent of the *whi* mutants. Here we show that compounds that inhibit sporulation in *S. coelicolor* are similarly active against *Streptomyces venezuelae*, which unlike *S. coelicolor* sporulates in liquid culture as well as on solid medium. Furthermore, we find that these compounds are enriched for inhibitors of cell division in the rod-shaped bacterium *B. subtilis.* Consistent with the essentiality of cell division in *B. subtilis*, we show that the three of these compounds that we have explored in greatest detail exhibit antimicrobial activity against it. Finally, these compounds also affect endospore formation in the rod-shaped bacterium *B. subtilis*, a mechanistically distinct

developmental process that also requires cell division machinery. (Errington,1991; Margolis *et al.,* 1991). In sum, our data suggest that the described phenotypic distinctions can be used as a means of the directed screening of compound libraries for chemical probes of the divisome.

2.3.1 Inhibiting cell division blocks sporulation in *Streptomyce*s

We first explored the direct perturbation of cell division in *S. coelicolor* with genes and known chemical inhibitors to determine whether this conferred a developmental block. The *Escherichia coli* gene *sulA* and the *Mycobacterium tuberculosis* gene *chiZ* encode inhibitors of cell division (Huisman *et al.,* 1984; Chauhan *et al.,* 2006) that are expressed in response to damage to the DNA as part of the SOS response. Both of these inhibitors act via conserved divisome components: SulA binds FtsZ while ChiZ binds and FtsI/Q (Jones *et al.*, 1985; Vadrevu *et al.*, 2011). Since these divisomal proteins are conserved in the streptomycetes, we predicted that expressing *sulA* and *chiZ* in *Streptomyces* would block septation and sporulation. We, therefore, created expression constructs placing each gene under the control of a thiostrepton-inducible promoter and introduced the resulting constructs into *S. coelicolor*.

As shown in figure 2.1a while the empty vector had no effect on the grey pigment production in *S. coelicolor*, the expression of *sulA* and *chiZ* conferred a white phenotype. Scanning electron microscopy confirmed a block in spore formation (figure 2.1a). This indicates that the inhibition of FtsZ (by SulA) and FtsI/Q (by ChiZ) blocked sporulation in *S. coelicolor* without compromising viability, consistent with the previous work

(McCormick *et al.*, 1994; McCormick *et al.*, 1996; Bennett *et al.*, 2007; Mistry *et al.*, 2008).

We then tested the effects of previously reported chemical inhibitors of cell division on *S. coelicolor* development. As with genetic inhibition, treatment of *S. coelicolor* with totarol, berberine and PC190723 resulted in the formation of white colonies indicative of the sporulation block (figure 2.1b).

DNA damage blocks cell division in most prokaryotes through various mechanisms that are activated as part of the SOS response (Huisman *et al.*, 1984; Chauhan *et al.*, 2006; Ogino *et al.*, 2008; Modell *et al.*, 2011; Vadrevu *et al.*, 2011). We compared the effect of the DNA damaging compound mitomycin C and the translation inhibitors tetracycline and kanamycin on the colony phenotype in *S. venezuelae* (figure 2.1c). All the compounds tested exhibited antimicrobial effect as observed by the zone of inhibition.

However, at a concentration sub-inhibitory for growth, mitomycin C conferred a white phenotype, consistent with a specific block in spore formation, whereas tetracycline and kanamycin did not. Using light microscopy, we found that this phenotype was accompanied by a clear defect in completion of sporulation (figure 2.1c). In contrast, sporulation was normal in the presence of sub-inhibitory concentrations of tetracycline and kanamycin.

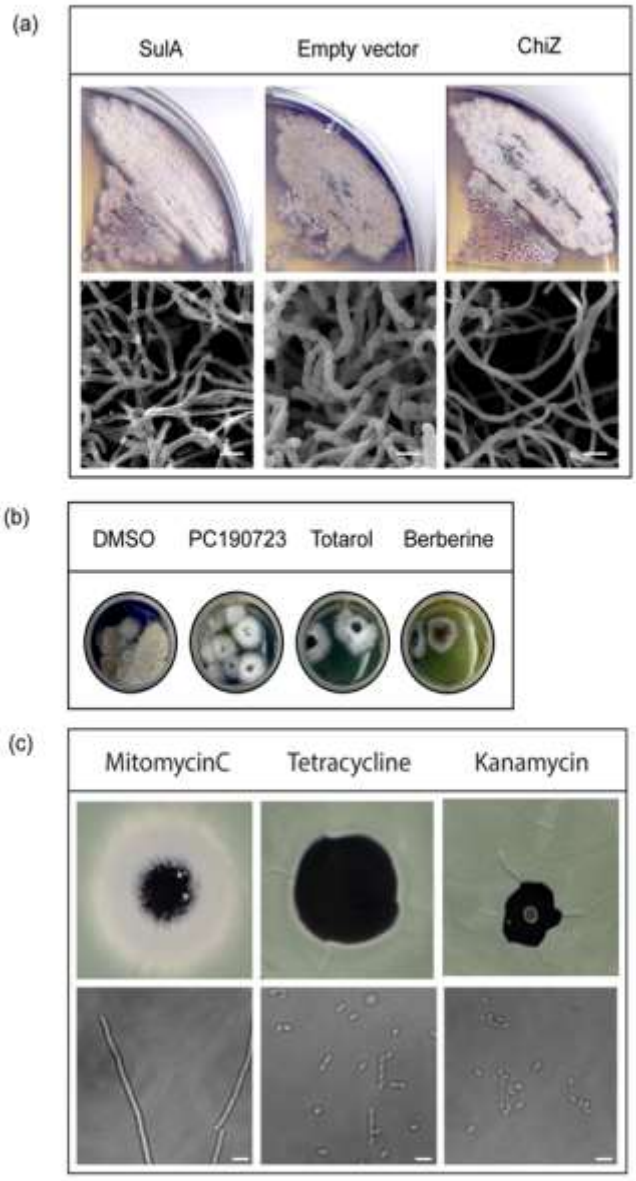


Figure 2.1 The inhibition of cell division confers a white phenotype on *Streptomyces*: (a) The proteinaceous inhibitors of FtsZ; SulA and FtsW; ChiZ were expressed from thiostrepton inducible promoter. The colony morphology on solid media was observed after incubation at 30 ^{o}C for four days. The expression of *sulA* and *chiZ* resulted in inhibition of sporulation and

formation of white fuzzy colonies. The empty vector containing strain underwent normal sporulation and produced grey-pigmented colonies. (b) Treatment of *S. coelicolor* with chemical inhibitors of FtsZ activity; totarol, berberine, PC190723, resulted in a sporulation block and the formation of white colony and DMSO treated colony appeared grey. (c) MitomycinC, tetracycline and kanamycin were spotted on *S. venezuelae* on solid media. The black zone indicated absence of growth and white zone indicated inhibition of sporulation. Indirect cell division inhibition by the DNA damaging antibiotic mitomycinC resulted in a sporulation block, whereas tetracycline and kanamycin had no effect on development.

These data indicated that genetic and chemical inhibition of cell division either by direct perturbation of the divisome or by DNA damage, prevent the appearance and maturation of spores in streptomycetes. This agrees well with previous work (McCormick *et al.*, 1994; McCormick *et al.*, 1996; Bennett *et al.*, 2007; Mistry *et al.*, 2008). We note that this is the first time that DNA damage has been demonstrated to block spore maturation in a streptomycete.

2.3.2 Identification of small molecule inhibitors of cell division

Our screen against the *S. coelicolor* life cycle identified 196 molecules that impaired sporulation (table 2.1) (Craney *et al.*, 2012). We hypothesized that some of these molecules might block cell division.

In contrast to *Streptomyces*, *B. subtilis* demonstrate a phenotypic change upon cell division inhibition that is distinct from the phenotype of sporulation inhibition. In the absence of cell division, *B. subtilis* forms long filamentous cells, in contrast to their usual

rod-shaped morphology. Thus, using *B. subtilis* would allow us to assort the molecules inhibiting cell division from those affecting the developmental pathways. We, therefore, applied these molecules to the Gram-positive, rod-shaped bacterium, *B. subtilis* and observed their effect using the light microscopy.

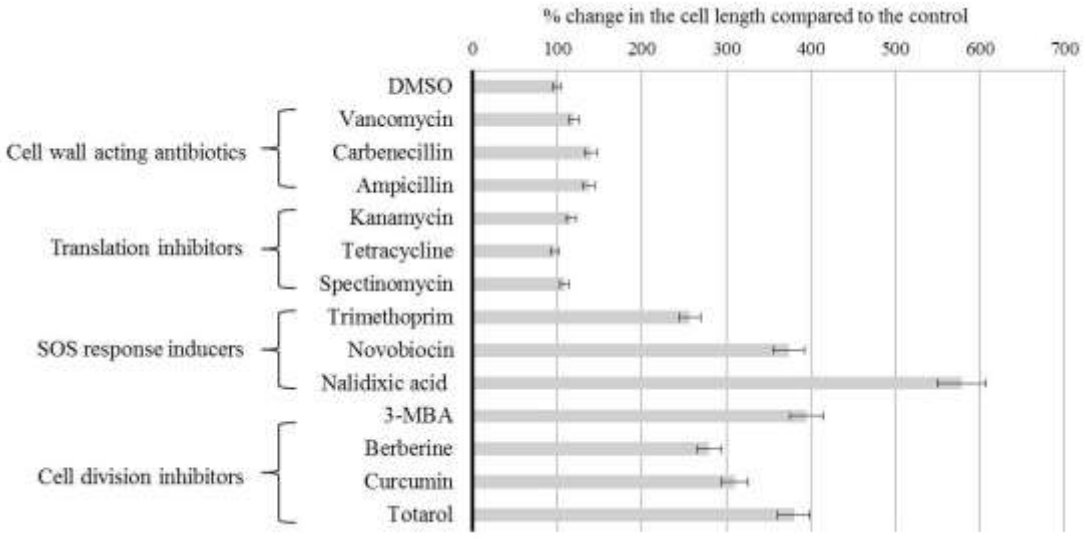


Figure 2.2 Filamentation of *B. subtilis*: Antibiotics with known mode-of-action were tested for their effects on the cell length. Antibiotics known to inhibit bacterial cell division significantly increased the cell length compared to the control. Other molecules did not have a substantial effect on the cell length.

I tested antibacterial molecules with a known mode of actions for filamentation of *B. subtilis* before testing the unknown compounds for the same. Since the test molecules were available in the limited quantities, to achieve maximum penetrance I used actively growing culture of *B. subtilis* at low cell density. Thus, *B. subtilis* cells at OD_{600} = 0.05 was treated with various concentrations of cell wall acting antibiotics: vancomycin,

ampicillin, carbenicillin, translational inhibitors; tetracycline, kanamycin, spectinomycin, DNA damaging molecules; trimethoprim, novobiocin, nalidixic acid and known FtsZ inhibitors; 3-MBA, berberin, curcumin and totarol. I saw significant increases in the cell length after the treatment with SOS inducing molecules and FtsZ inhibitors. Cells treated with the solvent (DMSO), cell wall inhibitors and translational inhibitors did not show significant filamentation (figure 2.2).

Of the 196 compounds that impaired sporulation in *S. coelicolor*, 22 conferred a filamentous phenotype in *B. subtilis* (table 2.1). Of these, three induced filamentation with potency suitable for further study (figure 2.3). Based on the filamentous phenotype conferred by these molecules we named them as Fil-1, Fil-2 and Fil-3.

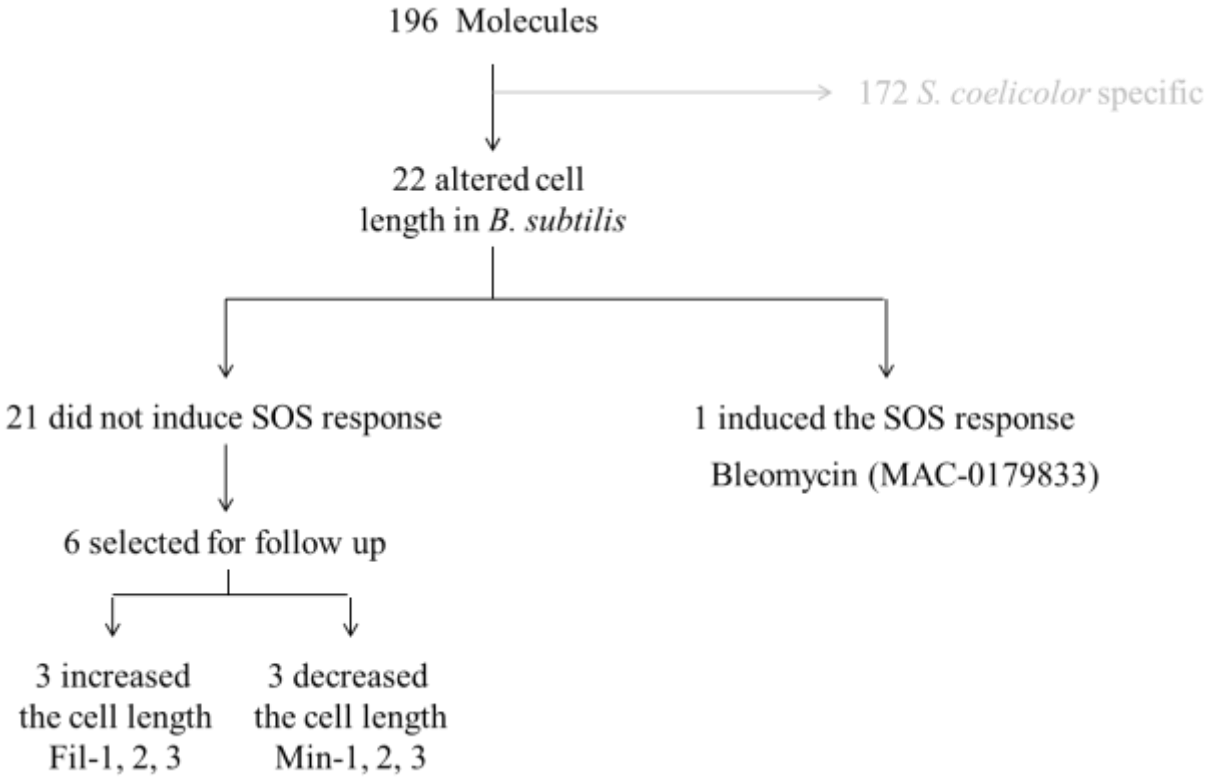
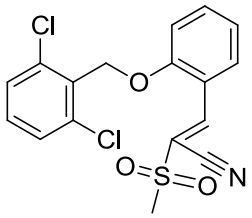
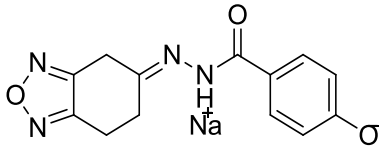
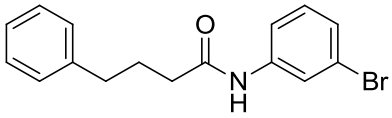
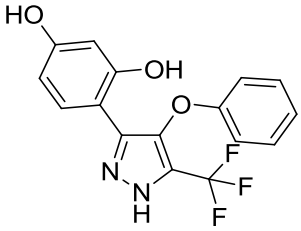
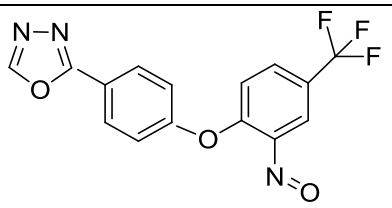
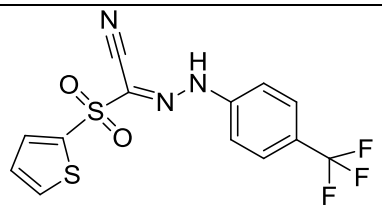
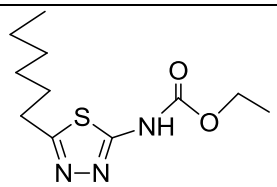
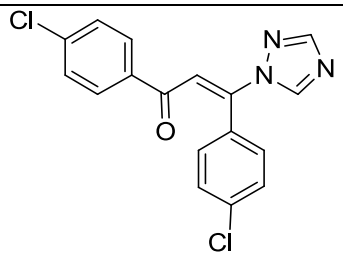
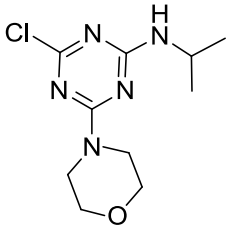
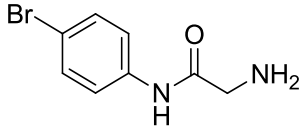
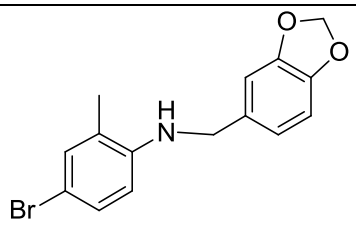
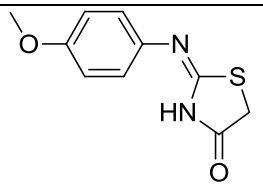
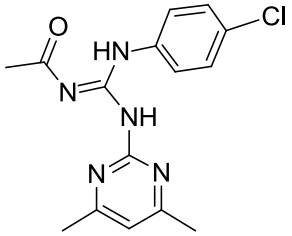
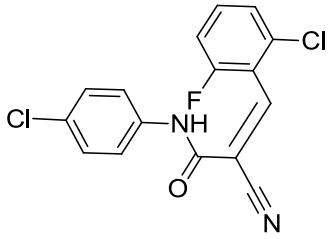
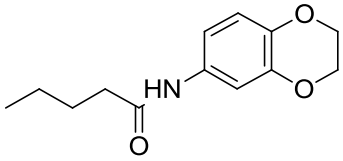
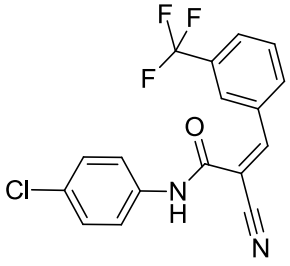


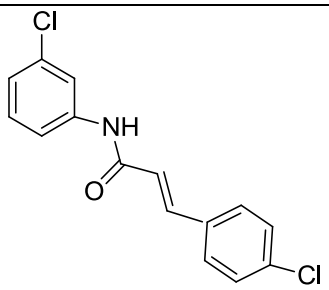
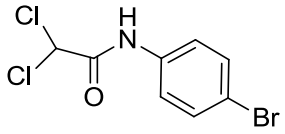
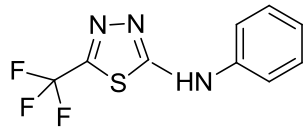
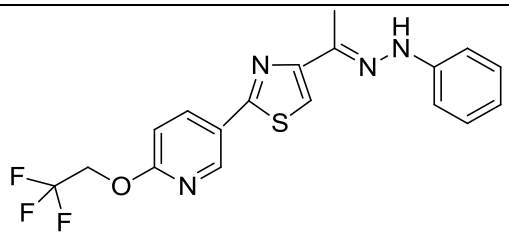
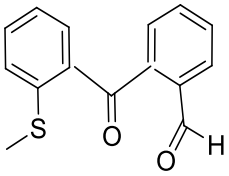
Figure 2.3 The secondary screen: The primary screen of 30,569 molecules performed in *S. coelicolor*, identified 196 molecules to inhibit sporulation. These molecules were grouped based on their phenotypic effects on *B. subtilis.*

Table 2.1 Structure of 21 molecules (bleomycin not shown) that affected the cell length of *B. subtilis*

Molecule	Structure	MIC (*B. subtilis*) µg/ml
Fil-1		96
Fil-2		36.7
Fil-3		76.6
Min-1		5
Min-2		6.6

Min-3		88
C19 (5312560)		>180
C26 (5543857)		>180
C25 (5468061)		>180
C27		>180
C28		>180

C29		>180
C30		>180
C31		>180
C32		>180
C33		>180

M4		>180
M21		>180
M23		>180
M43		>180
M20		>180

2.3.3 Fil-1, 2 and 3 do not act via DNA damage or cell wall damage

To determine whether any of the 22 compounds that induced filamentation in *B. subtilis* were DNA damaging agents, we applied them to *B. subtilis* strain YB5018 (*dinC*: Tn971*lacZ*), in which a *lacZ* reporter is under the control of the DNA-damage inducible promoter *dinC* (DNA damage-inducible) (Love *et al.*, 1985). The formation of a blue zone in the presence of X-gal is evidence for the induction of the SOS response. We grew this strain on LB-agar containing 8µg/ml X- gal and spotted 2 µl of 1 mM solutions of each of the 22 compounds that induced filamentous growth in *B. subtilis* 168. Only one of the 22 compounds, MAC-0179833, which was later identified as bleomycin, induced the SOS response. This is consistent with the fact that this compound is the well-known DNA damaging agent bleomycin (figure 2.4b).

We also tested the 22 compounds against the *B. subtilis* 168, which contains a *liaI-lacZ* reporter fusion. The *liaI* gene is induced during a stress response to cell wall damage (Mascher et al., 2004). Again, while *liaI* was induced by vancomycin, the remaining compounds had no such effect (figure 2.4b). These data indicate that most of these compounds induce filamentous growth through a mechanism other than the SOS response. The most likely explanation is that they act via the divisome or by a mechanism that ensures its proper placement or stability.

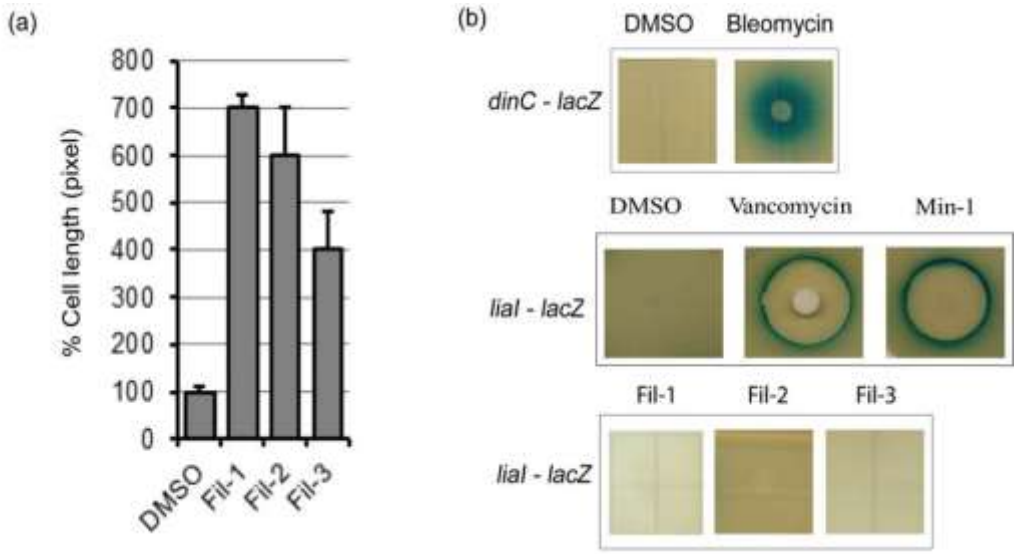


Figure 2.4 Fil-1, 2 and 3 inhibit cell division in the SOS independent manner: (a) *B. subtilis* cells were grown in the presence of Fil-1, 2 and 3, for 6 hrs. at 37 ^{o}C. Changes in the cell shape were observed by light microscopy. Increased cell length in the presence of Fil-1, 2 and 3 suggests a block in cell division. (c) *B. subtilis* strain containing *dinC-lacZ* fusion was spread on solid media containing 8 μg/ml X-gal. 2 μl of 10 mM molecules were spotted on the media. *lacZ* expression seen as the blue zone, indicated induction of DNA damage. Fil-1, 2 and 3 did not induce the DNA damage. Similarly, strain containing *liaI-lacZ* fusion was used as an indicator of cell wall damage. Vancomycin was used as a positive control. Fil-1, 2 and 3 did not induce *liaI* expression, which indicates they may not damage cell wall in *B. subtilis.*

2.3.4 Fil-1, 2 and 3 disrupt cell division at different stages

We explored the effects of Fil-1, 2 and 3 on the cell membrane, chromosome segregation and peptidoglycan synthesis in *B. subtilis* cells using light microscopy. We stained the membrane with the cationic lipophilic dye, FM 4-64 and the DNA was stained with DAPI (4', 6-diamidino-2-phenylindole).

As shown in figure 2.5, Fil-1 treated cells showed characteristic cell elongation along with complete inhibition of septum formation. Staining with FM4-64 showed distinct cross walls in the untreated cells, but the same were absent in the cells treated with Fil-1. The compound also blocked chromosome segregation. Blue fluorescently stained chromosomes showed a continuous, diffused localization along the length of the filamentous cells (figure 2.5a – D, E, & F). The observations are consistent with a block in the early stage of cell division.

In contrast, chromosome segregation appeared unaffected in Fil-2 treated cells, but the abnormalities in septum formation were clearly evident (figure 2.5a – G, H & I). FM 4-64 staining revealed the elongated cells with irregularly placed cross-walls and occasional twists (figure 2.5a – G, H & I). This phenotype suggests a role of Fil-2 in influencing the divisome function, placement or cell envelope synthesis. The defects in these processes can often confer a similar cell twisting phenotype (Adams *et al.*, 2011).

Fil-3 treated cells showed little or no defect in the early stages of cell division such as chromosomal segregation or septum formation (figure 2.5a – J, K & L). Rather, they appeared as long chains of cells having regular septation and were blocked in cytokinesis

(figure 2.4a – J, K & L). These observations suggest defects in cell separation, possibly by inhibition or impaired localization of a peptidoglycan hydrolase.

We used a fluorescently labeled vancomycin probe - Van-BODIPY, to investigate the effects of the molecules on peptidoglycan biosynthesis. Vancomycin is a glycopeptide antibiotic that binds to D-ala-D-ala terminal end of the pentapeptide chain in peptidoglycan (Tiyanont *et al.*, 2006). We stained Fil-1, 2 and 3 treated cells with Van-BODIPY and compared the localization of peptidoglycan synthesis by fluorescent microscopy. Consistent with our observations of membrane staining with FM 4-64, Van-BODIPY staining revealed the prevention of cross-wall formation by Fil-1, irregular cell wall formation in Fil-2 treated cells and normal septum formation in otherwise filamentous cells by Fil-3 (figure 2.4b).

These data suggest that Fil-1, 2 and 3 impair cell division at three different stages. Fil-1 blocks an early event in cell division, Fil-2 causes aberrantly placed divisomal function and Fil-3 prevents cell separation.

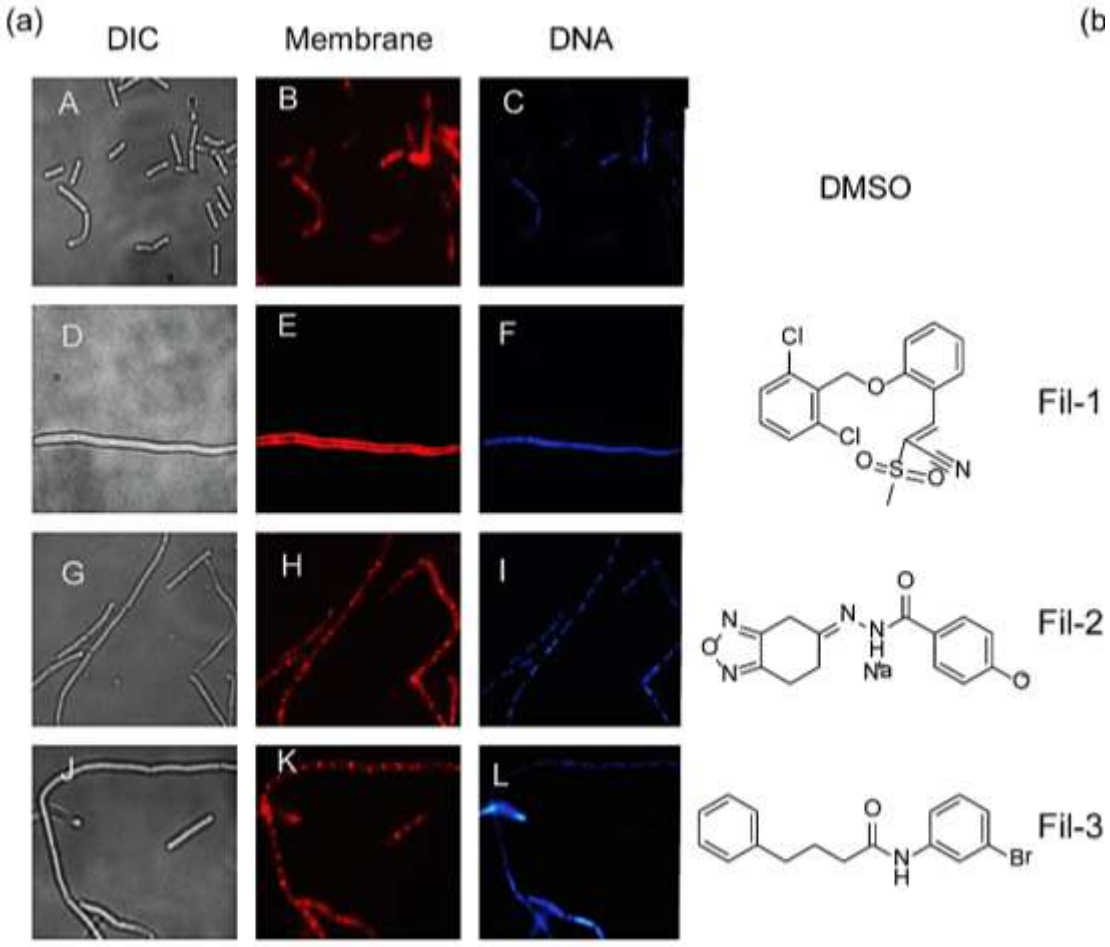
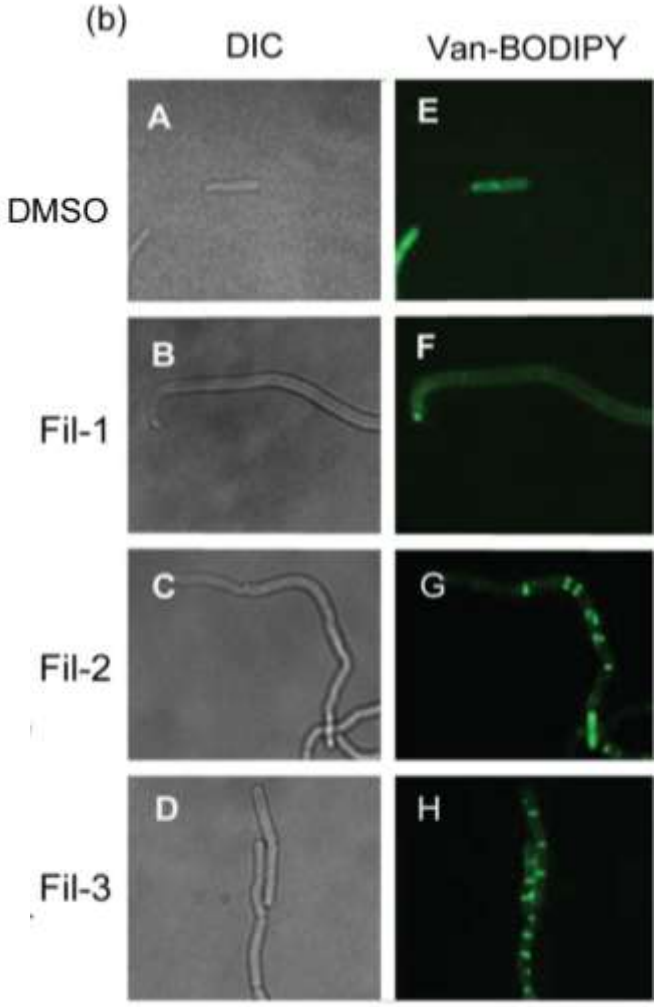


Figure 2.5 Fil-1, 2 and 3 confers distinct cell division block on *B. subtilis*: (a) Fil-1, 2 and 3 treated *B. subtilis* cells were stained with FM 4-64 and DAPI to visualize the membrane and DNA. Fil-1 treated cells (D, E and F) showed the absence of cross walls and DNA segregation; Fil-2 treated cells (G, H and I) showed the presence of irregular septa and Fil-3 treated cells (J, K and L) had no obvious defect in DNA segregation or septum formation, but grew as chains.



(b) *B. subtilis* cells treated with the molecules were stained with Van-BIDOPY at room temperature for 5 min. DMSO treated cells (A and E) accumulated fluorescent signal at the midcell, Fil-1 treated cells (B and F) showed absence of signal at the cross walls, Fil-2 treated cells (C and G) showed the formation of irregular septa and Fil-3 treated cells (D and H) showed the fluorescent signal at regular interval indicating normal synthesis of peptidoglycan.

2.3.5 Fil-1, Fil-2 and Fil-3 inhibit sporulation in *B. subtilis*

We argued that the ability of Fil-1, 2 and 3 to affect cell division in vegetative cells could also extend to sporulation in *B. subtilis*, an endospore-forming bacterium. Its developmental program is distinct from that of the streptomycetes. It does however; like streptomycetes, involve a developmentally regulated cell division event. The hallmark of this program is the formation of an asymmetrically positioned septum, creating a sporangium with a smaller forespore and a larger mother cell (Stragier *et al.*, 1996; Errington, 2010). The forespore goes on to develop into a heat-resistant endospore supported by the mother cell, which eventually dies.

We tested the effect of Fil-1, 2 and 3 on their ability to block sporulation in *B. subtilis*. For this, we measured the sporulation frequency of the cultures treated with increasing concentrations of each molecule. We observed that Fil-1, 2 and 3 could significantly decrease the sporulation frequency in a concentration dependent manner, completely stalling the formation of viable spores at ~50 µM (figure 2.6a). Fil-2 treated cells showed the distorted cell shape under the microscope. A detailed examination of Fil-2 treatment using light microscopy showed anomaly in the sporangium formation. The sporangia lacked the essential asymmetric septation (figure 2.6b) as well as exhibited severe structural abnormalities including cell twisting that was reminiscent of some effects on vegetative cells (figure 2.6b).

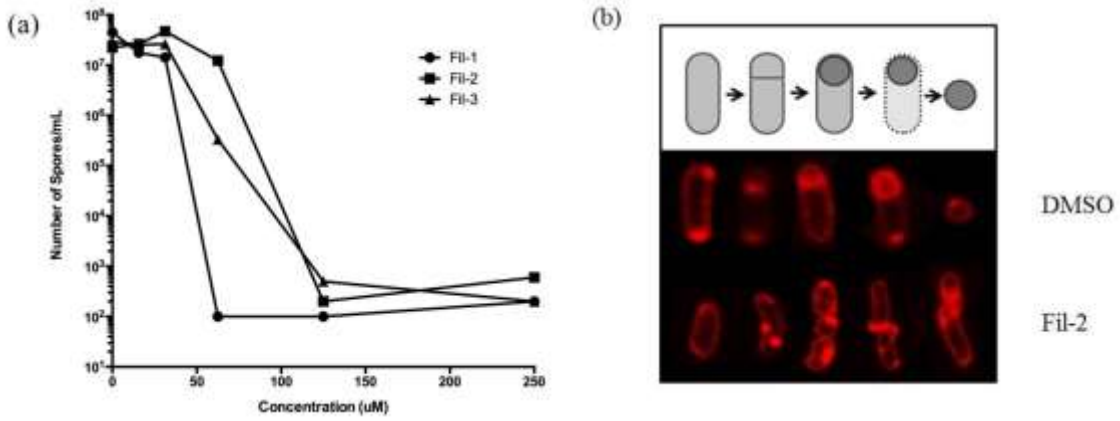


Figure 2.6 Fil-1, 2 and 3 inhibit sporulation in *B. subtilis*: *B. subtilis* cells undergoing sporulation were treated with various concentrations of Fil-1, Fil-2 and Fil-3. Sporulation was initiated by resuspending the overnight grown culture of *B. subtilis* in the sporulation media. Immediately following resuspension, varying concentrations of the molecules were added and allowed to incubate overnight at 37^{o} C. Following 30 minutes of heat treatment at 80^{o} C to kill any remaining vegetative cells the samples were serially diluted and plated to LB agar. After incubation, the colony forming units were counted. A significant decrease in the number of heat resistant spores was observed for all molecules in a concentration dependent manner, with Fil-1 being the most potent. IC_{50} values for Fil-1, Fil-2 and Fil-3 are 41.4, 61.8 and 60.2 respectively.

These data suggest that Fil-1, 2 and 3 prevent the normal functioning of the divisome so as to cause aberrant septation in vegetative and sporulating cells – these are both lethal events.

2.3.6 Effects of Fil-1, 2 and 3 on the expression of the developmental genes in *S. coelicolor*

In *Streptomyces* developmental pathway and cell division are tightly linked. Decision to undergo development is regulated mainly at the transcriptional level by a complex network of cascading signals (Claessen *et al.*, 2006; Flardh *et al.*, 2009; McCormick *et al.*, 2012). Completion of the earlier developmental event is a prerequisite and a signal for entry into the next stage of development. Unlike *E. coli* or *B. subtilis*, cell division in *Streptomyces* faces an additional level of control – gene expression regulated by developmental regulators. Transcription of the *ftsZ* gene in *S. coelicolor* is under the control of three different promoters: *ftsZ*p1, *ftsZ*p2 and *ftsZ*p3 (Flardh *et al.*, 2000). While *ftsZ*p1 and *ftsZ*p3 are expressed constitutively in vegetative hyphae and aerial mycelium, *ftsZ*p2 is developmentally regulated and is active only in aerial hyphae prior to the sporulation (Flardh *et al.*, 2000).

Since interfering with the gene expression cascade could also result in a sporulation block; we tested if any of the Fils are affecting the expression of developmental and/or cell division regulators. We chose to examine the activity of the promoter regions of the key developmental genes involved in each stage of the development; *ramC*, *whiE* and the developmentally regulated promoter of the cell division gene *ftsZ*.

ramC – *ramC* is the first gene of the operon *ramCSAB* together encoding the morphogenetic peptide SapB. SapB is a lantibiotic like secreted peptide that coats the surface of aerial hyphae and helps in erection of aerial mycelium. SpaB is essential for

the formation of aerial mycelium in rich media. Expression of *ramC* cluster is regulated by other regulatory signals like *bldB* and *ramR*. Inhibition of expression of *ramC* will not allow the development to proceed beyond the substrate mycelium formation (O'Connor *et al.*, 2002).

whiE – *whiE* gene cluster encodes for a polyketide synthase involved in the synthesis of spore associated grey polyketide pigments. Just like *ramC* cluster, activation of *whiE* cluster depends on the success of earlier developmental events (Davis *et al.*, 1990). Since the deposition of grey pigments is the last visually observable step of the *Streptomyces* development, activity of *whiE* could be used as an indicator of successful completion of sporulation. *whiEp1* is one of the divergent promoters of the *whiE* gene cluster and was used in this study as the indicator of the sporulation process (Kelemen *et al.*, 1998).

hrdB – *hrdB* encodes for the major vegetative sigma factor in *S. coelicolor*. Expression of *hrdB* was used as a measure of the effects of Fils on the growth of *Streptomyces* colony.

*frsZ*p2 – FtsZ protein forms the first step of the divisome assembly in most bacteria. We used the developmentally controlled promoter of *ftsZ*; *ftsZp2*, to test the effects of Fils on the divisomal proteins from and the developmental regulators.

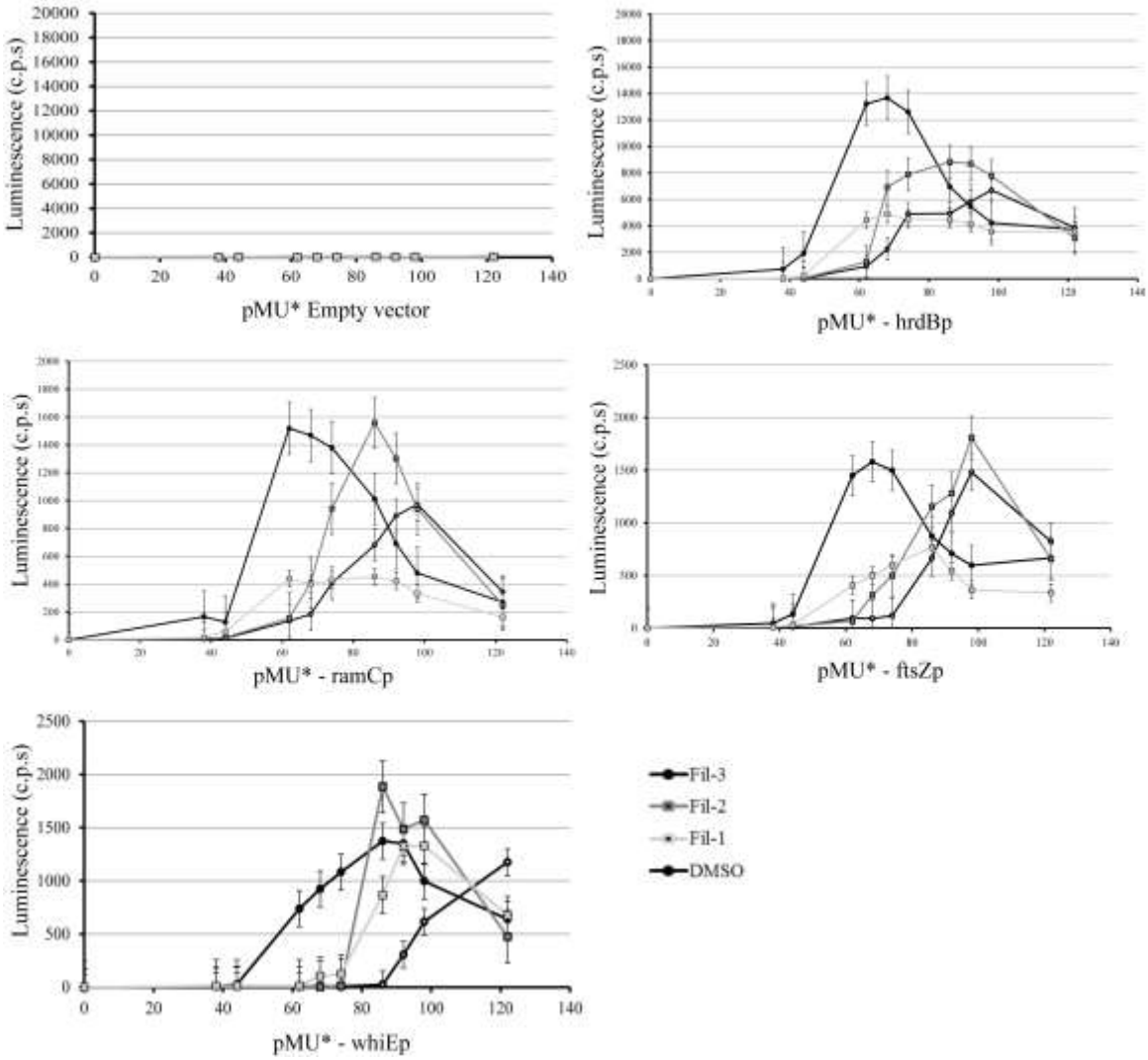


Figure 2.7 Effects of Fil-1, 2 and 3 on expression of developmentally regulated genes in *S. coelicolor*: Expression from the promoter region of some of the key developmental genes, cell division gene *ftsZ* was used to test the effect of Fil-1, Fil-2 and Fil-3 *Streptomyces* development. The vegetative sigma factor *hrdB* was used as an indicator of the growth defect. The promoter regions of the selected genes were cloned in a vector containing *luxABCDE* genes. Luminescence from the resulting *S. coelicolor* strain was measured and used as an indicator of the gene expression.

The changes in the expression were measured by fusing the promoter region of these genes in the vector pMUI-superstar. *ramCp*, *whiEp* and *hrdBp* constructs were kindly provided by Craney AR (Craney *et al.*, 2007). To construct the reporter for the *ftsZ* gene expression, the promoter region p2 was amplified from the genomic DNA of *S. coelicolor* using the primer sequences shown in table 2.2. The promoter fragment was cloned in the vector pMU-superstar and the construct was transferred into *S. coelicolor* by conjugation.

As shown in figure 2.7 Fil-1, 2 and 3 did not affect expression of developmental genes tested. Some delay in the gene expression was observed, which could be attributed to the reduced growth rate in the presence of the sub-inhibitory concentration of Fils.

2.3.7 Effect of Fil-1, 2 and 3 on FtsZ activity *in vitro*

To determine whether any of the molecules act on the core divisomal protein FtsZ, the most conserved cell division protein, we explored their effects on its enzymatic and biophysical functions *in vitro*. Similar to its eukaryotic homolog, FtsZ has concentration dependent GTPase activity and GTP-dependent capacity for polymerization. To determine whether Fil-1, 2 or 3 can influence FtsZ function, I purified untagged FtsZ from *B. subtilis* and measured its GTPase activity as well as its ability to polymerize in the presence of the molecules. We found that the GTPase activity of FtsZ was not affected in the presence of Fil-1 and Fil-3, however the addition of Fil-2 resulted in a significant decrease in the GTPase activity (figure 2.8a).

I used negative-staining electron microscopy, to directly visualize inhibition of FtsZ polymer formation by Fil-2. Purified FtsZ monomers were incubated with DMSO or Fil-2 and the polymerization was induced by addition of 1mM GTP. In the DMSO treated sample, clear FtsZ filaments were observed which were absent in the Fil-2 treated sample, providing direct evidence of the effect of Fil-2 on FtsZ (figure 2.8b).

To further confirm these results and observe the effects of Fil-2 on steady state polymer formation, we carried out a sedimentation assay. Purified FtsZ monomers were treated with DMSO, Fil-1, Fil-2 and Fil-3. The polymerization of FtsZ monomers was induced by addition of GTP. The FtsZ polymers were sedimented by ultracentrifugation. Inhibition of FtsZ polymerization would result in a decreased amount of the protein in the pellet and increased amount in the supernatant. The protein concentration in the pellet, as well as the supernatant fraction, was measured by SDS-PAGE. The amount of protein in the pellet fraction was found to decrease with increasing the concentration of Fil-2. Fil-1 and Fil-3 had no effect on sedimentation of FtsZ (figure 2.8c).

This clearly shows that Fil-2 inhibits FtsZ activity *in vitro*, whereas Fil-1 and Fil-3 appears to be non-FtsZ cell division inhibitors.

However, it has been suggested by Feng B.Y. and colleagues that small molecule inhibiting enzyme activity at higher concentrations may be doing so by forming larger colloidal aggregate (McGovern *et al.*, 2003; Feng *et al.*, 2006). Thus, they could be acting by inhibiting enzyme activity non-specifically. Covalent modification of the protein by non-specific nucleophiles has been suggested as another mechanism of non-specific enzyme inhibition in small-molecule screens by Blanchard *et al.* (Blanchard et al., 2004).

DTT being a strong reducing agent can prevent such covalent modification of the enzyme.

Since Fil-2 inhibits the FtsZ activity at higher micromolar concentrations, we decided to test the effects of detergent and DTT on FtsZ inhibition in the presence of the molecule. A high-performance liquid chromatography based assay using an enzymatic conversion of α–^{32}P-GTP to α–^{32}P-GDP was developed and performed in Dr. Eric Brown's lab by Dr. Amrita Bharat (Bharat *et al.*, 2013).

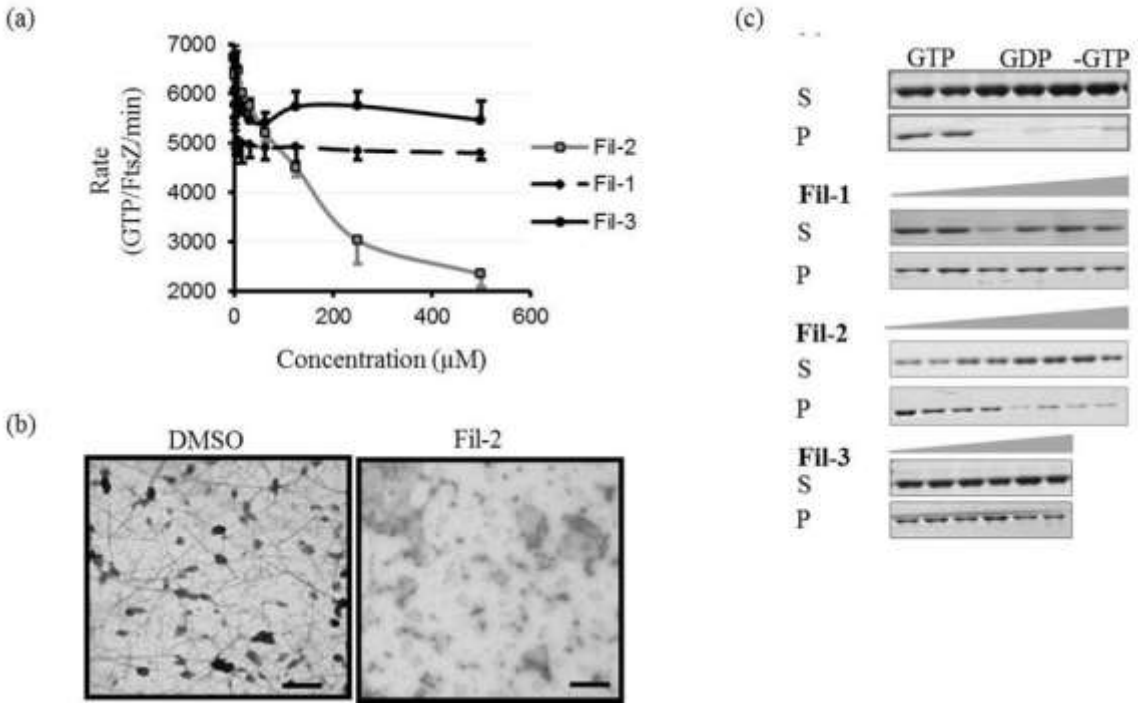


Figure 2.8 Fil-2 inhibits FtsZ activity: (a) GTPase activity of FtsZ was measured by pyruvate kinase/lactate dehydrogenase coupled assay, in the presence of Fil-1, 2, 3 and DMSO. The reaction was initiated by the addition of 1mM GTP and decrease in absorbance at 340 nm, resulting from the oxidation of NADH was measured for 30 min. Addition of Fil-2 decreased the rate of GTP hydrolysis, whereas Fil-1 and Fil-3 had no significant effects. (b) To observe FtsZ

polymerization by negative electron microscopy, the protein was incubated with Fil-2 and DMSO in MES buffer at pH = 6.5, for 5 min at room temperature and 1mM GTP was added to initiate the polymerization. The reaction sample was stained with 0.1 % uranyl acetate and observed under transmission electron microscope. Inhibition of FtsZ polymerization by Fil-2 was observed. (c) Purified *B. subtilis* FtsZ was incubated with Fil-1, Fil-2 and Fil-3 and DMSO. Polymerization was induced by addition of 1 mM GTP and the samples were sedimented by ultracentrifugation. Amount of protein in the pellet and the supernatant fractions were analyzed by SDS-PAGE. Increasing the concentration of Fil-1 and Fil-3 has no effect on polymerization of FtsZ, whereas Fil-2 inhibits FtsZ polymerization.

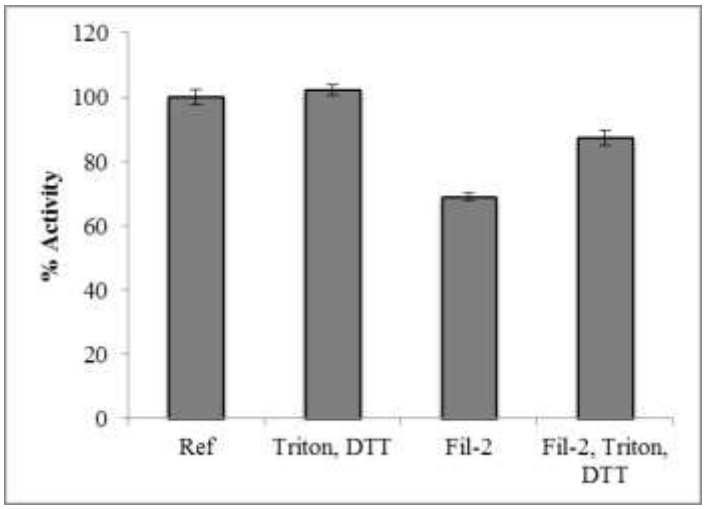


Figure 2.9 Inhibition of the GTPase activity of FtsZ by Fil-2 is relieved in the presence of triton and DTT: GTPase activity of FtsZ was measured in the presence of Fil-2, Triton and DTT. Addition of triton and DTT relieved inhibition of GTPase activity by Fil-2.

As shown in the figure 2.9, the inhibitory effect of Fil-2 on the GTPase activity of FtsZ was abolished in the presence of a detergent, tritonX and DTT (figure 2.9). This suggests that FtsZ inhibition observed by Fil-2 in the earlier experiments was non-specific and

primarily due to the formation of large colloidal aggregate by Fil-2 at higher concentrations.

2.3.8 Electron cryotomography of *B. subtilis* in the presence of Fil-2

Our light microscopy experiments showed that while Fil-1 treated cells completely lacked cross walls and Fil-3 treated cells formed normal cross-walls, Fil-2 caused the formation of septa and partial septa having abnormal morphology and positioning. To further investigate the effects of this compound on cell wall formation at higher resolution, we collaborated with Dr. Grant Jensen at California Institute of Technology. Cryotomograms in the presence of different concentrations of Fil-2 using the *B. subtilis* Δ*ponA* strain were collected. This strain is narrower than other *B. subtilis* strains, making it a more suitable subject for cryotomography. At concentrations of 50 μM and below the growth rate of the cells appeared unaffected. Consistent with the antimicrobial activity of this molecule, at 83 μM growth was impaired and at concentrations above 100 μM cells failed to grow altogether. We, therefore, examined the cell morphology in the presence of 83 μM Fil-2. When imaged with light microscopy, the cells appeared filamentous and FM4-64 staining showed the presence of irregular division septa as observed with wild-type *B. subtilis*.

Imaging of *B. subtilis* with electron cryotomography (ECT) revealed several structural insights. In vegetative cells, the peptidoglycan appeared much thinner in the presence of Fil-2, irregular in thickness and dissociated from the cytoplasmic membrane (figure 2.10

A and B). Some cells displayed filamentous structures underneath the cytoplasmic membrane reminiscent of FtsZ. However, no membrane invagination was observed at those instances (data not shown). In several cases, the peptidoglycan surrounding the division septa appeared loose suggesting that the synthesis or maintenance of peptidoglycan could have been affected by Fil-2 (figure 2.10C). We observed some cells with regular division septa suggesting that at 83 μM not all cells were affected by the molecule (figure 2.10D). Dividing cells showed abnormalities at the septal sites that appeared as membrane invaginations surrounded by a thin layer of peptidoglycan (figure 2.10 E and F).

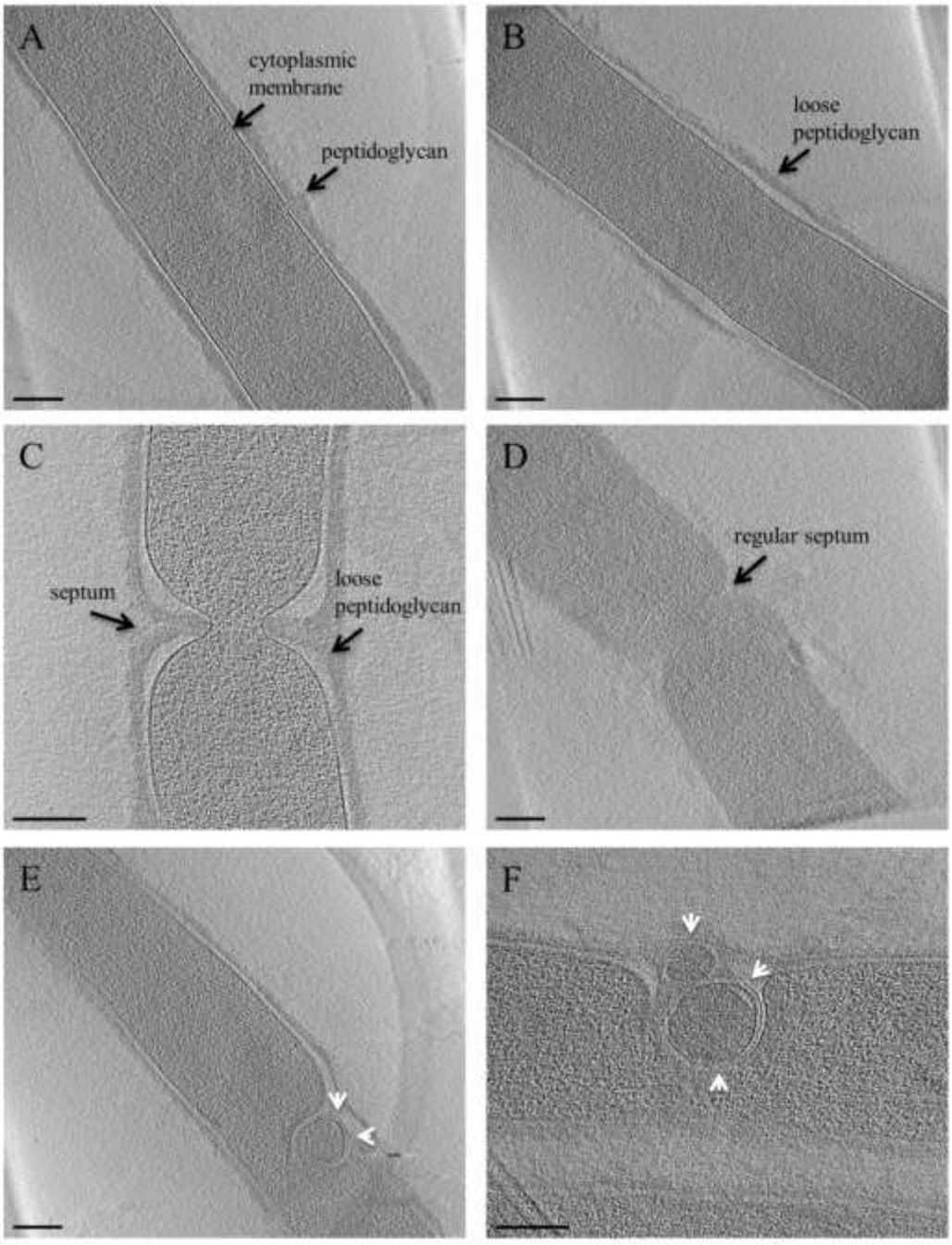


Figure 2.10 Electron cryotomography of *B. subtilis* showing the effects of Fil-2 on cell morphology: A and B) Tomographic slices through vegetative cells showing irregular and loose synthesis of peptidoglycan; C) Loose peptidoglycan synthesis was also observed at the site of vegetative septa; D) A regular-appearing vegetative septum; E and F) Irregular septa showing

cytoplasmic membrane and peptidoglycan blebs indicative of unsuccessful division sites (black arrowheads).

2.3.9 Mins – Molecules that decreased the cell size

As mentioned earlier, in addition to the molecules that increase the length of *B. subtilis* cells we also found three molecules that reduced the length of the rod-shaped bacterium. Although the presence of such molecules was unanticipated, involvement of cell division machinery in producing this phenotypic effect can be explained. I examined the microscopic effects of the molecules on the *B. subtilis* cell wall and chromosome

Staining of Min-1, 2 and 3 treated *Bacillus* cells with the membrane staining dye FM4-64 and the DNA binding dye DAPI showed no defect in DNA segregation (figure 2.11). Formation of mini cells in *E. coli* and *B. subtilis* has been shown to occur in the absence of MinCD (Bi E. *et al.*,1993). This protein pair is involved in the inhibition of FtsZ ring formation at the poles. However, unlike the Min-1, 2 and 3 treated cells, a fraction of the mini cells resulting from the absence of the gene *minCD* are anucleate because of the formation of division septa near the poles. Since we did not see this defect in the chromosome segregation in Min-1, 2 and 3 treated cells, we ruled out the inhibition of activity of MinCD or any such mechanism regulating the septum placement by Min-1, 2 and 3.

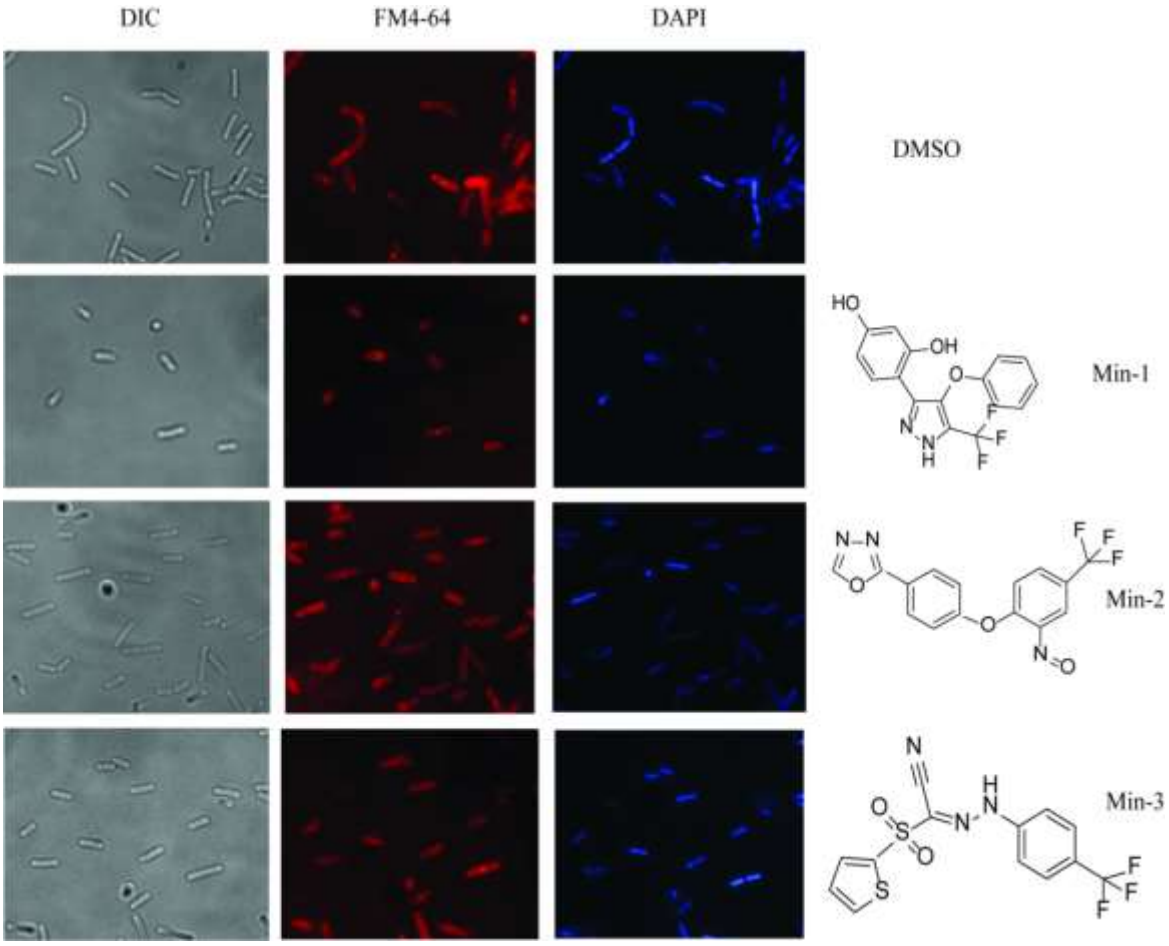


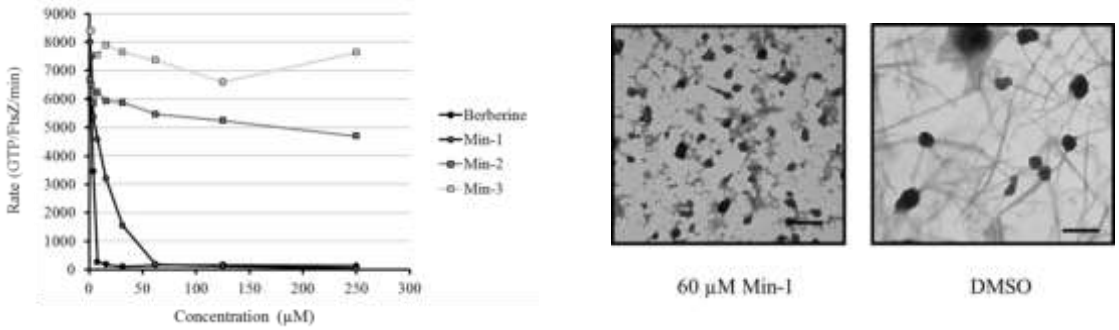
Figure 2.11 Effects of Min-1, 2 and 3 on chromosome segregation and cell membrane of *B. subtilis*: Min-1, 2 and 3 treated *B. subtilis* cells were stained with FM 4-64 and DAPI to visualize the membrane and DNA. The cells had no obvious defect in DNA segregation or septum formation.

Although, Min-1, Min-2 and Min-3 had no effect on DNA damage response demonstrated by the lack of *dinC-lacZ* expression, one of the molecules, Min-1 induced *liaI* expression. *liaI* is an indicator of the cell wall damage, typically induced by vancomycin and bacitracin (Mascher *et al.*, 2004). Effects of Min-1 on cell wall stress

might be indirect, probably via altering the divisome function and needs to be explored further.

Since the modulation of FtsZ activity has been shown to decrease cell size in bacteria, in attempts to understand the mechanism of Min's action, I tested their effects on FtsZ enzyme activity and polymerization as described earlier. One of the molecules, Min-1, was found inhibit polymerization of FtsZ, at least in the absence of detergent, by inhibiting its GTPase activity (figure 2.12). These molecules are being followed up by an incoming graduate student to decipher their mode of action in detail.

(b)



(c)

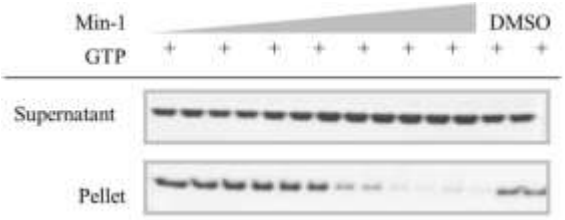


Figure 2.12 Effects of Min-1, 2 and 3 on the FtsZ activity: (a) GTPase activity of FtsZ was measured by pyruvate kinase/lactate dehydrogenase coupled assay, in the presence of Min-1, 2, 3 and DMSO in the absence of detergent. The reaction was initiated by the addition of 1mM GTP and decrease in absorbance at 340 nm, resulting from the oxidation of NADH was measured for 30 min. The addition of Min-1 decreased the rate of GTP hydrolysis, whereas Min-1 and Min-3 had no significant effects. (b) To observe FtsZ polymerization by negative electron microscopy, the protein was incubated with Min-1 and DMSO in MES buffer at pH = 6.5, for 5 min at room temperature and 1mM GTP was added to initiate the polymerization. The reaction sample was stained with 0.1 % uranyl acetate and observed under transmission electron microscope. Inhibition of FtsZ polymerization by Min-1 was observed. (c) Purified *B. subtilis* FtsZ was incubated with Min-1, Min-2 and Min-3 and DMSO. The polymerization was induced by the addition of 1 mM GTP and the samples were sedimented by ultracentrifugation. Amount of protein in the pellet and supernatant fractions was analyzed by SDS-PAGE. Increasing the concentration of Min-2 and Min-3 has no effect on polymerization of FtsZ, whereas Min-1 inhibits FtsZ polymerization.

2.4 Discussion

DNA replication, transcription, translation and cell wall biosynthesis have been widely exploited as antibacterial targets (Lock *et al.*, 2008). The prevalence of pathogenic strains that are resistant to these antibiotics suggests a need for new antimicrobial agents having new targets. Cell division is a good candidate for this because it is widely conserved and essential for growth in the majority of prokaryotes. Furthermore, the fact that the biochemical roles of some cell division proteins remain poorly understood suggests that chemical probes of the divisome could be valuable tools for understanding the divisome function as they have done previously for other essential structures. Indeed, pioneering work in this area by researchers at Merck laboratories, Jeff Errington, Debabrata RayChaudhuri and others supports the growing interest in this endeavor (Margalit *et al.*, 2004a; Stokes *et al.*, 2005b; Tsao *et al.*, 2006; Haydon *et al.*, 2008) .

Many compound libraries are enriched in compounds that have antimicrobial activity against bacteria. Determining whether any of these compounds target the cell division process can be a challenge, particularly at high throughput. The microscopic visualization of cells using a robotically-driven microscope could be employed to detect the induction of filamentous growth by antimicrobial compounds. However, filamentation could be due to DNA or cell wall damage rather than by perturbation of the cell division apparatus. We suggest, therefore, that the disruption of the developmental phenotypes in *S. coelicolor* or *S. venezuelae,* is an efficient way of identifying the candidate inhibitors of the divisome. This approach is particularly powerful when supplemented with genetic tests for the

induction of various stress responses, many of which have been characterized in *B. subtilis* (Love et al., 1985).

The biochemical targets of Fil-1, 2 and 3 are of great interest. While we have not identified their molecular targets in this work, *in vitro* FtsZ polymerization studies and enzymatic assays showed that these molecules are not targeting FtsZ activity.

The effect of Fil-1, Fil-2 and Fil-3 on endospore formation in *B. subtilis* is also significant. It has been demonstrated previously that sporulation is impaired in the presence of cell division inhibitors. The fact that each Fil molecule impairs both endospore formation and vegetative cell division is also consistent with a molecular target that is either part of the divisome (though clearly not FtsZ) or in the apparatus that positions or stabilizes it.

Using cryo-electron tomography we further showed that Fil-2 treatment results in cell wall defects in *B. subtilis* indicating a divisomal protein other that FtsZ as the possible target. This could be supplemented by facile secondary screens for DNA-damaging or cell wall-damaging agents as we have done here.

We suggest that this approach is complementary to and can be used as a pre-screening strategy before performing *in vitro* biochemical tests for inhibitors of specific cell division proteins.

Additionally, the secondary screen using *B. subtilis* identified three molecules, Min-1, Min-2, and Min-3 that decreased the cell length compared to the DMSO-treated cells. A decrease in the cell size similar to that of the Min treated cells has been observed earlier

in *E. coli* and *B. subtilis*. Such a phenotype reported so far are caused by the genetic mutations and did not involve any defect in chromosome segregation. In 1983, Lutkanhaus observed that the overexpression of FtsZ leads to reduced cell size (Ward *et al.*, 1985). Overexpression of FtsZ and FtsA simultaneously and an FtsA mutant that binds tightly with FtsZ, also decreases the cell size (Geissler *et al.*, 2007). It has been recently reported that deletion of a two-component system, YycGF, that regulates cell division in *B. subtilis*, also results in decreased cell size (Dubrac *et al.*, 2008). Moreover, deletion of a metabolically regulated inhibitor of FtsZ, *utgP* results in around 20% decrease in the size of *B. subtilis* (Weart *et al.*, 2007). A similar protein governing the cell size in response to the nutrient condition has been discovered in *E. coli* (Hill *et al.*, 2013). All of the above reports indicate that the gain-of-function of the cell division machinery, can lead to decrease in the size of the rod-shaped bacteria. In addition to this, observation that over-expression of FtsZ in *S. coelicolor* inhibits sporulation, links the mini cell formation in *B. subtilis*, back to the white phenotype in *S. coelicolor*, observed in the primary screen (van Wezel *et al.*, 2000). Exploring to the mechanism of action of these molecules could help understand the functioning of divisome placement.

Finally, our work is suggestive of the possible utility of chemical probes for dissecting the various roles of the divisome and its interaction with other cellular components. Future work aimed at identifying the target of these molecules would help explain the divisome in greater detail.

2.5 Materials and methods

2.5.1 Bacterial growth and culture

B. subtilis 168 strain was grown at 37°C in Luria-Bertani (LB) media. *Streptomyces* strains were grown in R5M, R2YE, MYM or MS media at 30^{o} C. Thiostrepton at the concentration of 30µg/ml was used for induction of gene expression. Mitomycin C was used at 2µg/ml, kanamycin and tetracycline was used at 50µg/ml concentrations. *E. coli* XL-blue strain was used for cloning purpose and was grown at 37^{o}C.

2.5.2 Cloning

Oligonucleotides used to amplify *sulA* from *E. coli* genome and *chiZ* from *M. tuberculosis* and *ftsZp2* form *S. coelicolor* genome are listed in data table 2.2 The amplified region of DNA were digested with restriction endonucleases NdeI and KpnI and ligated in to the vector pIJ6902 digested with the same restriction enzymes (Huang *et al.*, 2005). In the case of ftsZ-2 the amplicon was digested with BamHI and KpnI and ligated to pMU-superstar digested with the same enzymes. The resulting constructs were transformed in *E. coli* ET12567 strain containing pUZ8002 and introduced into *S. coelicolor* by conjugation.

2.5.3 *B. subtilis* filamentation assay

To test the induction of filamentation, overnight culture of *B. subtilis* was diluted to O.D. $_{600}$ = 0.05 and 10 µM of the molecules was added. After six hours of incubation at 37^{o} C the cells were observed under a light microscope.

Table 2.2 Oligonucleotides used to amplify *sulA, chiZ and ftsZp2*

gene amplified	Primer name	Oligonucleotide sequence
chiZ	*chiZ* Forward	ATCGACATATGACACCGGTCCGGC
	chiZ Reverse	ATCGGGTACCTCAGCCAACCGGAGCGA
sulA	*sulA* Forward	ATCGACGTCATATGTACACTTCAGGCTATG
	sulA Reverse	ATCGGGTACCTTAATGATACAAATTAGAGTGAA
ftsZp2	*ftsZp2* Forward	ATCGGGATCCCGCCCGACGCGCGGCACTTCG
	ftsZp2 Reverse	ATCGGGTACCTCGAAGGCCTCTCGCCTCGAG

2.5.4 Microscopy

For fluorescence microscopy, cultures grown in presence of the molecules were pelleted by centrifugation at 5000 rpm for 15 seconds and re-suspended in 200 µl of saline with 0.5 µg/ml FM4-64 (Molecular probe), 0.2 µg/ml DAPI or 2 µg/ml of Van-FL (vancomycin -BODIPY conjugate from molecular probe) and were incubated for 5-10 min in dark. Cells were washed with saline once and mounted on microscope slides covered with a thin film of 1.5% agarose in water prepared using the gene frame (Thermo Fischer). Images were acquired with a Hamamatsu Orca ER-AG camera attached to a Leica DMI 6000 B microscope. The images were analyzed with IPLab software.

2.5.5 Electron cryotomography

B. subtilis Δ*ponA* cells were grown in liquid LB medium supplemented with 0, 50, 83, 100 and 150 μM of Fil-2. EM grids were prepared by plunge freezing cells in nitrogen-cooled liquid ethane. Data were collected on an FEI Polara (FEI Company, Hillsboro, OR) 300 kV FEG transmission electron microscope equipped with a Gatan energy filter and a lens-coupled 4k x 4k UltraCam (Gatan, Pleasanton, CA). Samples were imaged at a dosage of 200 $e^{-}/Å^{2}$ with a defocus of -10μm and a tilt range from -60 to +60°. Three-dimensional reconstructions and segmentations were produced with IMOD (Kremer *et al.*, 1996).

2.5.6 *B. subtilis* sporulation assay

Sporulation assays were performed on *B. subtilis* strains inoculated into Difco sporulation medium (DSM) and incubated with aeration for 24 h at 37 °C. Half of the cultures samples were incubated for 30 min at 80 °C and serially diluted. Dilutions of heat treated and untreated cultures were plated on LB agar to determine the percentage of heat resistant cells (spores). Sporulation frequency was calculated as the ratio of heat resistant C.F.U. to C.F.U. of the untreated sample.

2.5.7 FtsZ sedimentation assay

FtsZ sedimentation assay was performed as previously described by (Mukerjee and lutkenhous). FtsZ (10 μM) and molecules (Fil-1, 2, 3) were incubated for 5 min at room temperature in 200 μl MES buffer. FtsZ polymerization was initiated by the addition of 1

mM GTP and samples were incubated for 5 min at 30°C. The polymerized FtsZ was spun down for 10 min, 80 000 r.p.m., 25°C in a Beckman ultracentrifuge with a TLA-100 rotor. Supernatant and pellet fractions were separated by SDS–PAGE followed by Coomassie-blue staining.

2.5.8 FtsZ GTPase assay

Time dependent decrease in GTPase activity of purified *B. subtilis* FtsZ in presence of Fil-1, 2 and 3 was measured by a pyruvate kinase/lactate dehydrogenase coupled enzyme assay (Margalit *et al*., 2004).The reaction mixture contained 1 mM PEP, 200 μM NADH, 68 units/ml pyruvate kinase, 68 units/ml l-lactate dehydrogenase and 5 μM FtsZ in reaction buffer (50 mM 4-morpholinepropanesulfonic acid, pH 6.5, 50 mM KCl, 5 mM $MgCl_2$). A 175 μl reaction mixture was dispensed per well of half-area UV transparent 96-well plates. The reactions was initiated at 25°C by adding 1 mM GTP to the wells and monitored over 45 min by the decrease in absorbance at 340 nm.

2.5.9 Overexpression and purification of FtsZ *B. subtilis*

B. subtilis FtsZ protein was used for testing the inhibition of the FtsZ activity by Fils and Mins. The protein was overexpressed and purified form the *E. coli* strain W1110 (pBS52)(pCXZ) containing the $ftsZ_{B.\ subtilis}$ constructed previously by Lutkenhaus (Wang and Lutkenhaus, 1993). The plasmid containing the ftsZ gene (pCXZ) was transformed in *E. coli* BL21 cells. A single colony form the transformants on the LB-agar plates containing ampicillin was inoculated in LB media. After overnight incubation, the culture was diluted 1:100 with LB. The resulting culture was grown at 37^oC until OD_{600} of 0.6

was achieved and expression of ftsZ was induced by addition of IPTG ant the final concentration of 1mM. After growing the culture at 37^{o}C for four hours cells were harvested by centrifugation at 5000Xg for 10 min at 4^{o}C. The pellet was washed with FtsZ induction buffer (recipe below). After washing, the pellets can be stored at -80^{o}C for at least one year.

For the purification of FtsZ, the frozen pellet was thawed on ice in the presence of a protease inhibitor and 10 ml FtsZ induction buffer. The pellet was resuspended and the final volume of 30 ml was achieved by addition of FtsZ induction buffer. The cells were sonicated on ice 5X for 10 sec intervals with 30 sec rest in between. The resulting lysate was cleared by centrifugation at 160,000Xg for 45 min at 4^{o}C.

FtsZ was precipitated with the saturated solution of ammonium sulfate. At first, ammonium sulfate equal 30% of the supernatant volume was added dropwise. The lysate was incubated on ice for 20 min and the protein was pelleted by spinning at 10,000Xg for 10 min at 4^{o}C. The first ammonium sulfate precipitation at 30% removes several protein but not FtsZ. After spinning the supernatant, was transferred to a new tube and FtsZ was precipitated by bringing the ammonium sulfate concentration to 40%. After incubating the lysate for 20 min at 4^{o}C the protein was pelleted by centrifugation at 10,000Xg for 10 min.

2.5.10 Negative electron microscopy

Polymerization of FtsZ in the presence of Fil-1, 2, 3 and Min-1, 2, 3 was observed by electron microscopy. The purified protein was incubated with the molecules and DMSO

as a control in MES buffer at pH = 6.5, for 5 min at room temperature. Polymerization was induced by 1mM GTP. The sample was stained with 0.1 % uranyl acetate and was observed under transmission electron microscope.

2.5.11 Induction of *lacZ*

Stain containing *dinC*-*lacZ* fusion YB5018 (*dinC*: Tn971*lacZ*) was kindly provided by Yasbin RE and *liaI*-*lacZ* (Em trpC2 *liaI*::pMUTIN attSPbeta) fusion strain was provided by *Bacillus* Genetic Stock Centre (BGSCID - 1A980). The strains containing *lacZ* fusion were spread on LB-agar containing 8 µg/ml X- gal and molecules were spotted on the plate at various concentrations.

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CHAPTER 3

EFFECTS OF DNA DAMAGE ON CELL DIVISION AND GLOBAL GENE EXPRESSION IN *STREPTOMYCES*

3.1 Abstract

In earlier work, we screened 30,569 compounds for biological activity against *Streptomyces coelicolor* and identified 196 molecules that inhibit sporulation, conferring the so-called "white" phenotype. One of these compounds was a known DNA damaging agent, bleomycin, a well-studied DNA cleaving glycopeptide antibiotic produced by *Streptomyces verticillus*. Bleomycin's action leads to the induction of a DNA damage response in bacteria, known as the SOS-response (Umezawa *et al.,* 1966; Ishizuka *et al.,* 1967). One of the attributes of the SOS-response includes cessation of the cell division machinery that may be partly or solely responsible for the white phenotype observed in the primary screen. To understand the inhibition of cell division by the DNA damage response I explored the global transcriptomic changes of *Streptomyces venezuelae* after the treatment with mitomycin C. For this, I carried out a pan-genome transcriptional profiling experiment in *S. venezuelae* after the DNA damage and characterized the SOS response. I combined computational modeling along with mRNA sequencing approaches to understand these changes.

I observed a significant overlap between the SOS genes predicted by computational search and those expressed due to the DNA damage according to the RNA-seq data. Several known SOS genes were found to be induced as a result of the DNA damage as well as putative SOS genes identified through the *in silico* search, confirming the search strategy. The mechanism of regulation of the SOS-response that is dependent on the RecA protein is widespread in bacteria. In actinomycetes, in addition to the RecA-

dependent mechanism another RecA-independent mechanism has been proposed. In both - computational and RNA-seq data, I observed a greater reliance of the *S. venezuelae* SOS response on the non-conventional RecA-independent regulation mechanism.

To understand the regulation of cell division during the SOS response I analyzed changes in the expression of genes related to cell division. I observed down-regulation of some key cell division genes accompanied by upregulation of certain cell wall hydrolase genes previously shown to be involved in cell division inhibition in other actinomycetes. This paves the way for a better understanding of cell division regulation in DNA-damaged *Streptomyces* cells.

During the analysis of RNA-seq data, I also found that among the most significantly upregulated genes were those that can be grouped under a functional category of genes involved in iron homeostasis. I hypothesize this may have a role in programmed cell death as reported earlier in *E. coli*.

3.2 Introduction

In nature, bacteria are constantly exposed to diverse environmental conditions. It is vital for cell survival that changes in the environmental conditions are perceived as cellular signals and responded to appropriately. This is achieved by dynamically regulating expression of genes necessary to survive under varying conditions. The mechanism that bacteria deploy as an immediate response to DNA damage is called the SOS response (Radman,1975; Janion,2008). This response is also observed when the replication of DNA is stalled. The SOS response involves a cascade of events that shut down the cell division machinery and thereby allow the cell to activate DNA repair mechanisms (d'Ari,1985; Favre *et al.*, 1985; Autret *et al.*, 1997).

The SOS-response has been extensively studied in *E. coli* (Peterson *et al.*, 1988). Damage to the DNA caused by a physical (UV), chemical (toxins) or physiological (a replication block) agent activates RecA-LexA system that triggers a feedback reaction. This is in the form of either induction or upregulation of ~40 genes involved in various cellular processes including DNA damage repair and cell division inhibition (Favre *et al.*, 1985).

The DNA damage response is less well understood in *Streptomyces* - an actinomycete that is studied in depth for antibiotic production and as a model of bacterial development. *Streptomyces* are prolific producers of secondary metabolites and undergo a complex developmental cycle. So, unlike *E. coli*, when streptomycetes encounter DNA damaging agents, they must respond by modulating several processes such as cell division, developmental pattern as well as secondary metabolite production. Although the *recA* and *lexA* genes in *Streptomyces* have been studied earlier, the global response to DNA

damage has not been explored. Therefore, to characterize the yet unknown and interesting features of the *Streptomyces* SOS-response and to understand how it affects the process of cell division, I studied the changes in phenotype and analyzed the transcriptome for changes in global gene expression in response to the DNA damage.

3.3 Results

In recent years draft genome sequences of >120 *Streptomyces* strains have been reported. Four of these have been successfully assembled to completion (*S. coelicolor, S. avermitilis, S. griseus, S. venezuelae*) and carefully annotated - a prerequisite for transcriptomics-based experiments. This makes *S. venezuelae* a suitable candidate for our transcriptomic study. Moreover, the unusual ability of *S. venezuelae* to sporulate in the submerged conditions, in contrast to *S. coelicolor*, which only sporulates on solid medium, provides an essential advantage for conducting the experiments using the broth cultures (Bibb *et al.*, 2012). For these reasons, we selected *S. venezuelae* over *S. coelicolor* for characterizing the DNA damage response. This study provides an opportunity to further explore a possible link between sporulation and the SOS response, a potentially novel feature for the streptomycetes.

I begin this chapter by describing a computational approach for analyzing the transcriptomic changes after the DNA damage by identifying the putative DNA-damage response genes. In the second part, I described *in vivo* aspects of this response wherein I elaborate on the transcriptional changes observed by mRNA sequencing in *S. venezuelae.*

3.3.1 *In silico* analysis of the DNA damage response pathways

The results from the studies involving DNA damage in various bacteria show that the effect leads to a global response by the genes having common and highly conserved upstream regulatory sequences. The purpose of this exercise was to determine the signature sequence which can then be used as a tag to identify the putative SOS response

genes in *Streptomyces*. Such tags popularly known as the sequence motifs have been identified in the non-coding sequences present upstream of the groups of genes involved in various pathways in prokaryotes as well as eukaryotes. These sequences are generally recognized by a regulatory protein or other molecules (e.g. a riboswitch), which thereby mediates transcriptional control of the target genes. Analyses of these sequences allow us to identify functionally related genes and predict the differential expression of the genes in a pathway. These predictions can be done independent of the knowledge regarding the putative role of the gene. Thus, identification of the promoter motifs involved in the SOS-response will serve as a marker to identify potential genes that are expected to respond in the event of DNA damage.

To initiate this work, I analyzed the upstream sequences of genes known to be involved in the DNA damage response in *S. venezuelae*. Using this information, I constructed a putative SOS response sequence motif and then used the information gained to identify other candidate SOS genes by searching the non-coding sequence of the *S. venezuelae* genome.

Several actinomycetes have been shown to encode two types of SOS responsive motif – RecA-dependent and RecA-independent. To gain the best possible understanding of the transcriptional changes after DNA damage, I analyzed motifs belonging to both the groups.

Tools used for the majority of this *in silico* work are listed in table 3.1.

Table 3.1 Tools used of computational prediction of the SOS genes

Name of the tool	Description	Used in this study for	Link	Reference
MEME – suit	Bundle of online tools containing MEME and FIMO		http://meme.nbcr.net/meme/	(Bailey *et al.*, 2009)
MEME	Allows construction of a motif from a group of DNA, RNA of protein sequences	Constructing the RecA-dependent and RecA-independent motif from the upstream sequences of known SOS genes	http://meme.nbcr.net/meme/cgi-bin/meme.cgi	(Bailey *et al.*, 1994)
FIMO	Allows to scan the large collection of sequences for the presence of the motif constructed by MEME	Identifying unknown genes containing the motifs in the upstream region of the *Streptomyces* genome	http://meme.nbcr.net/meme/cgi-bin/fimo.cgi	(Grant *et al.*, 2011)
RAS-tool	Searches the query DNA pattern (motif) in the collection of sequences for the presence of the motif	Identifying the genes containing the RecA-dependent motif in the upstream region of the *Streptomyces* genome with higher stringency	http://rsat.ulb.ac.be/genome-scale-dna-pattern_form.cgi	(Thomas-Chollier *et al.*, 2011)
KEGG pathway search	Searches the functional pathway in which the query gene might be involved	Functional grouping of the putative SOS-genes	http://www.genome.jp/kegg/pathway.html	(Kanehia M. *et al.*, 2000)

3.3.1.1 Identification of a putative RecA-dependent motif and putative RecA-dependent SOS genes in *S. venezuelae*

The presence of a RecA-dependent sequence motif in the upstream region of the DNA damage inducible genes has been documented in diverse groups of bacteria (Cheo *et al.*, 1991). The motif has been named as the SOS-box and is known to be directly associated with the DNA damage response (Peterson *et al.*, 1988; Cheo *et al.*, 1991; Yasbin *et al.*, 1991). The motif is recognized by a repressor protein, LexA that remains bound to the

SOS-box in the absence of the DNA damage. The onset of the DNA damage initiates a series of events where RecA bound to the ssDNA activates auto-proteolytic activity of LexA. Thus, LexA commits auto-cleavage resulting in the release of the protein bound to the SOS box; enabling the expression of the downstream SOS genes (refer chapter 1 for more detail) (d'Ari,1985; Sedgwick,1986; Shinagawa,1996). The consensus sequences of the SOS-box vary significantly between Gram-positive and Gram- negative bacteria (table 3.2).

Table 3.2 SOS-box sequences in various classes of bacteria

Phylogenetic Group	Sequence	Reference
Alpha Proteobacteria	GAAC(N)7GAAC	(Fernandez de Henestrosa *et al.*, 1998; Tapias *et al.*, 1999)
	GTTC(N)7GTTC	(Tapias *et al.*, 1999)
Betaproteobacteria	CTGT(N)8ACAG	(Erill *et al.*, 2003)
Gammaproteobacteria		
Deltaproteobacteria	CTRHAMRYBYGTTCAGS	(Campoy *et al.*, 2003)
Gram-positive Bacteria	CGAACRNRYGTTYC	(Winterling *et al.*, 1998b; Davis *et al.*, 2002a)
Cyanobacteria	RGTAC(N)3DGTWCB	(Mazon *et al.*, 2004)

The SOS-box in *Streptomyces* was first identified in the promoter of the *recA* in *Streptomyces lividans*, as the sequence **GAAC**ATCC**ATTC** (Vierling *et al.*, 2000). This sequence, although close to the consensus sequence of *B. subtilis* SOS-box; CGAACRNRYGTTYC, is not a perfect palindrome (Winterling *et al.*, 1998a; Vierling *et al.*, 2000).

I analyzed the upstream region of the known RecA-dependent SOS genes; *recA*, *ruvC*, *uvrA* and *lexA* from *S. venezuelae*. The intergenic sequences of *S. venezuelae* required for the analysis were kindly provided by G. Chandra (John Innes Centre, Norwich, UK). I used the open access search tools, described in table 3.1 and materials and methods, to detect putative LexA binding sequence motifs in these intergenic sequences.

The DNA sequences 500 bp upstream from the translational site of known the RecA-dependent SOS genes were used as the query sequence for the construction of a palindromic motif. The motif thus constructed was used to scan the genome of *S. venezuelae*. The genome scan was designed for low stringency to enrich the collection to include as many sequences as possible at the same time to eliminate the bulk of the sequences that showed no sign of such motif. For this reason the results of the scan showed a higher degeneracy with a scope for several false positive motif sequences.

The results of the primary scan were further refined using the online program for **R**egulatory **S**equences **A**nalysis called the RSA-tool (http://rsat.ulb.ac.be/) to reduce the number of false positives (Thomas-Chollier *et al.,* 2011). The "DNA-pattern-matching" program of RSA-tool searches for sequence match to the query sequences with the selected number of deviation at each position. This is different from the tool used earlier in the study (MEME-suit), which searches for motifs based on the probability of the presence of a particular nucleotide at each position. RSA-tool thus serves to yield only the sequences with a higher confidence level.

In the second round of the motif construction, I combined the information gained by both the tools as illustrated in the figure 3.1. The motif constructed this way is shown in figure 3.2. I used this motif sequence for searching the putative RecA-dependent SOS genes in *S. venezuelae* (table 3.3) and other *Streptomyces* genomes (data not shown).

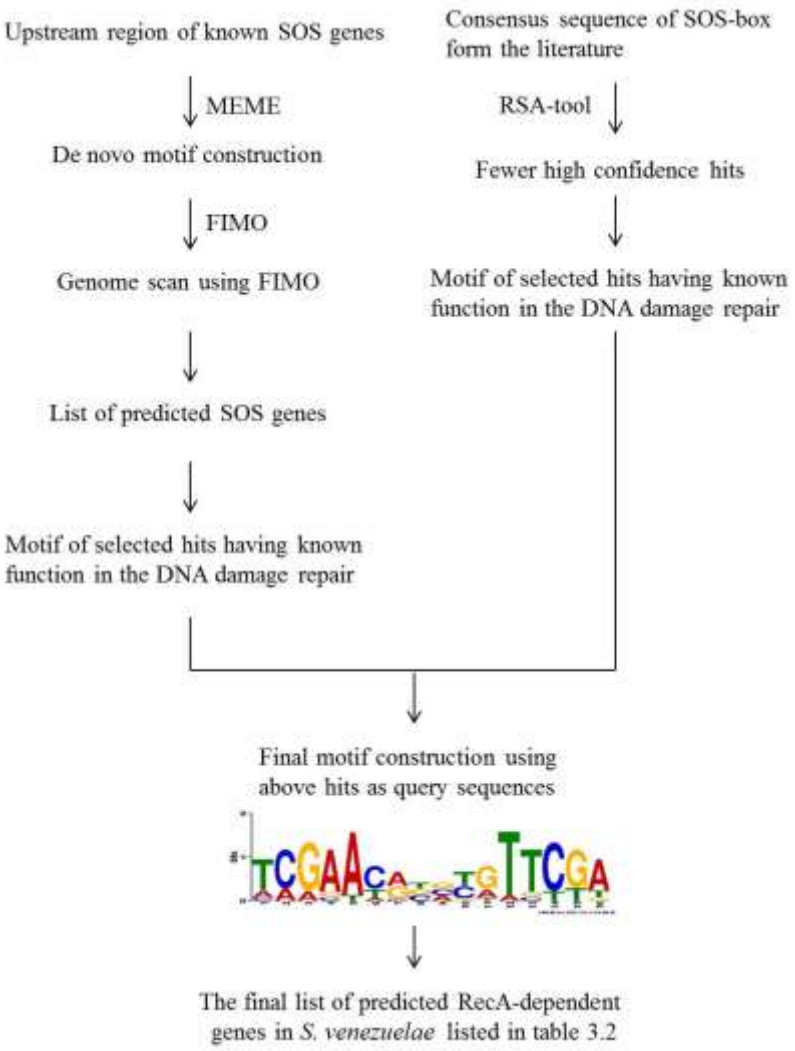

Figure 3.1 Scheme used for constructing and refining the RecA-dependent motif of *S. venezuelae.*

In general, I found the individual sequences of the SOS box in *Streptomyces* to deviate from the consensus. Most of the candidate motifs are not perfect palindromes. For example the motif generated for known SOS genes *recA* (TC**GAAC**ATCC**ATTC**TC), *lexA* (TC**GAAC**GTGT**GTTT**GG) and *ruvC* (AC**AAAC**AGGT**GAGC**GA) were found to deviate from the palindrome at 11^{th}, 15^{th} and 3^{rd}, 14^{th} as well as 15^{th} position respectively. This degeneracy was shown earlier in the SOS-box of *recA* gene of *Streptomyces lividans* (Vierling et al., 2000). Thus, based on our analysis it was determined that observation made earlier in one of the SOS genes of *S. lividans* is a common feature of SOS-box motifs in *S. venezuelae* (table 3.3) as well as across different *Streptomyces* strains (data not shown).

By taking this approach, I identified 83 genes in the *S. venezuelae* genome with a putative SOS-box motif in their upstream sequences. These genes were grouped based on the functional role assigned by KEGGS pathway search tool (http://www.genome.jp/kegg/pathway.html).

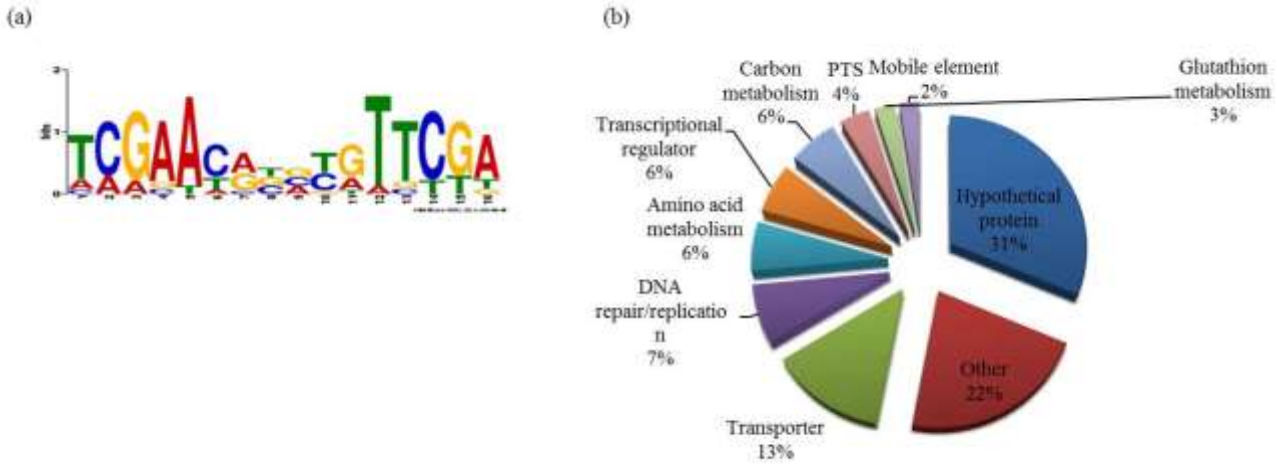

Figure 3.2 *S. venezuelae* genes adjacent to putative SOS-Boxes: (a) The SOS box motif of *S. venezuelae* as determined by MEME. (b) Functional grouping of *S. venezuelae* genes containing the putative SOS-box.

Genes involved in the repair of DNA replication are expected to be under the influence of the SOS-box. 7% of the total putative RecA-dependent genes fall into the category of genes known to be involved in DNA damage repair and regulation. These genes included *recA, lexA, ruvC* which are known SOS-box containing genes and were used as query sequences to construct the motif (Shinagawa,1996; Janion,2008). Interestingly, *alkB* gene was one of the DNA damage repair gene identified as the putative RecA-dependent SOS gene. *alkB* is involved in repairing the DNA damage caused by the alkylating agents (Nieminuszczy *et al.*, 2007). The SOS-gene *alkB* is regulated by the transcriptional regulator *ada* in *E. coli* and does not contain the SOS-box (Sedgwick *et al.*, 2002). *S.*

venezuelae does not encode an *ada* homolog and according to our data *alkB* may be regulated by LexA binding to the putative RecA-dependent promoter.

As summarized in figure 3.2, more than half of the genes identified in our search were either uncategorized or were classified under 'others' and 'hypothetical' categories respectively. A relatively large number of the genes are coding for the transport related proteins, suggesting an increased mobilization of molecules across the membrane.

Two genes, SVEN_7218 annotated as a homolog of EBNA-1 protein and SVEN_5662 annotated as IstB ATP-binding protein, fall into the category of putative mobile elements. EBNA-1 is a multifunctional viral protein important for viral replication of Epstein-Barr virus (Sivachandran *et al.*, 2012). IstB ATP binding proteins are associated with the insertion element IS21 and are thought to be involved in the transposition (Schmid *et al.*, 1999). The presence of an SOS-box in the upstream region of these genes might be affecting the rate of transposition or mobility of a fragment of chromosome in a DNA-damaged cell. An increased transposition in the cells under stressful conditions has been reported before (Permina *et al.*, 2002; Auchtung *et al.*, 2005; Ubeda *et al.*, 2005). The presence of the phage λ under the control of SOS response and its mobilization by the response was reported previously (Rozanov *et al.*, 1998). Additional reports have observed other mobile elements containing genes for antibiotic resistance and virulence under the control of DNA damage response as well (Beaber *et al.*, 2004; Auchtung *et al.*, 2005; Matsui *et al.*, 2005; Ubeda *et al.*, 2005; Ubeda *et al.*, 2005). Our computational analysis indicates that in *Streptomyces*, such mobilization of insertion sequences may be under the influence of the DNA damage response. It will be interesting to confirm these

predictions *in vivo* and to understand the function of the mobile elements regulated by the SOS response

Table 3.3 Genes containing RecA-dependent promoter motif

	Sequence Name	Distance from the start site (nt)	Matched Sequence	Function	Gene expression after MMC addition		
					20 min	40 min	80 min
DNA damage repair/replication	SVEN_5479	40	TCGAACGTGTGTTTGG	SOS-response repressor and protease lexa	1.0	1.1	2.1
	SVEN_1356	34	TCGTACGGACGTTCGA	DNA polymerase III alpha subunit	1.1	1.2	2.9
	SVEN_1120	91	ACAAACAGGTGAGCGA	Crossover junction endodeoxyribonuclease ruvc	3.3	4.5	5.6
	SVEN_5424	145	TCGAACATCCATTCTC	Reca	0.4	2.1	2.5
Transcriptional regulators	SVEN_6566	85	TCGCTCATGCGTTCGG	Alkylated DNA repair protein alkb	0.7	1.5	3.4
	SVEN_3904	11	TAAAACGTCCGACCGA	Polyphosphate kinase	0.1	0.7	0.8
	SVEN_5209	1	TAGAACGTACGAGTGA	Transcriptional regulator, tetr family	1.8	2.0	2.3
	SVEN_5498	51	CCGATCACCCTTTCGT	Transcriptional regulator, gntr family	0.9	0.9	2.9
	SVEN_5546	89	CCGCACGGGCGACCGA	DNA-binding response regulator kdpe	1.1	2.2	3.0
	SVEN_3001	91	TCGCTCAGGCGATCGG	Transcriptional regulator, tetr family	2.2	7.9	14.3
	SVEN_3227	30	ACGTACGGCCTTTCGA	Transcriptional regulator, meci family	0.8	1.3	1.6
Amino acid metabolism	SVEN_1588	35	TAGCACGCACATTCGA	Maleylpyruvate isomerase, mycothiol-dependent	0.5	0.5	1.2
	SVEN_1842	42	CCGATCGGGCGTGCGT	Dihydrolipoamide dehydrogenase of branched-chain alpha-keto acid dehydrogenase	0.1	0.3	0.4
	SVEN_0293	84	TCGGTCGTCCGTTTTA	Phenylacetate-coenzyme A ligase paaf	0.4	0.8	1.1
	SVEN_1174	43	TCGATCGGGCGGTCGT	N-acetyl-gamma-glutamyl-phosphate reductase	0.1	0.1	0.1
	SVEN_1175	59	ACGACCGCCCGATCGA	Argininosuccinate synthase	0.1	0.1	0.1
	SVEN_3145	51	ACGAGCGGGCGATCGA	Putative broad specificity phosphatase	0.3	0.4	0.7
Transporter	SVEN_1957	7	CAGCACGTGGGTTCGG	Ferrous iron transport permease efeu	1.0	1.6	7.9
	SVEN_1049	24	CCGGACGCGCGTCCGG	Putative uracyl permease	0.5	0.7	0.8

	SVEN_3247	6	CCGAACGCATGTGTGA	L-proline glycine betaine ABC transport system permease protein prov	1.6	1.6	2.9
	SVEN_3246	77	TCACACATGCGTTCGG	Binding-protein-dependent transport systems inner membrane component	1.4	1.4	1.9
	SVEN_2586	11	TCGCTCACCCGTTCGG	Putative transport protein	1.9	3.4	4.8
	SVEN_0916	60	ACGCACGGCCTTTCGT	Major facilitator family transporter, putative	1.2	3.1	3.5
	SVEN_2723	73	CCGAACAGGTGTTGGA	Putative transport protein	0.3	0.5	1.2
	SVEN_5772	66	ACGGAACGCCGTTCGT	Tellurium resistance protein	1.6	2.6	4.3
	SVEN_3247	6	CCGAACGCATGTGTGA	L-proline glycine betaine ABC transport system permease protein prov	1.6	1.6	2.9
	SVEN_1225	56	CCGGACGCCTGTCCGA	Twin-arginine translocation protein tata	0.2	0.6	1.3
	SVEN_4360	30	TCGCACAGAGGTTCGA	Hypothetical protein	on	On	1
	SVEN_1357	23	TCGAACGTCCGTACGA	Hypothetical protein	0.7	0.8	2.9
	SVEN_0171	6	CAGAACACGCGTCCGA	Hypothetical protein	0.7	1.1	2.3
	SVEN_4360	6	TCGAACAGATGTTCCA	Hypothetical protein	on	On	1
	SVEN_0717	42	ACGAACACATGTCCGG	Hypothetical protein	on	On	1
	SVEN_6443	95	TCGATCATGGTTTCGA	Hypothetical protein	0.3	0.3	0.3
	SVEN_0817	47	TCGAACAGCCGACCGG	Hypothetical protein	0.8	4.0	5.0
	SVEN_0320	95	TCGAACAGACGCTCTA	Hypothetical protein	0.5	0.6	0.9
	SVEN_0463	46	AAACACGTGTGTTCGA	Hypothetical protein	0.3	0.8	1.9
Hypothetical protein	SVEN_2345	100	TCGCACGGGGGTGCGT	Hypothetical protein	1.5	2.0	2.8
	SVEN_2873	22	ACGAATACCCGACCGA	Hypothetical protein	on	on	on
	SVEN_0116	44	CCGAACGGCCGGTCGA	Hypothetical protein	0.3	0.9	1.0
	SVEN_4963	33	TCGAATGGGTTTCCGG	Hypothetical protein	0.1	0.1	0.4
	SVEN_2169	4	TCGGGCACATGTTCGA	Hypothetical protein	1.7	2.2	2.4
	SVEN_2983	70	CCGATCACACATCCGA	Hypothetical protein	0.1	0.2	1.1
	SVEN_4806	56	CCGGACGGGTGATCGT	Hypothetical protein	0.6	0.8	1.9
	SVEN_4224	43	TCACACGTGTGTGCGG	Hypothetical protein	0.4	0.9	1.1
	SVEN_1256	23	CCGCACGGGTTTTCGG	Hypothetical protein	0.2	0.3	0.6
	SVEN_3436	11	CCGGACAGGCTTGCGA	Hypothetical protein	0.1	0.3	1.6
	SVEN_1314	83	CCGGACGGAGATTCGT	Hypothetical protein	0.3	1.8	5.0

	SVEN_3412	66	TCGCAGGCGTGTTCTA	Hypothetical protein	0.5	0.7	1.6
	SVEN_3088	88	TCGATCAGGTGAGCGG	Hypothetical protein	0.2	0.8	2.2
	SVEN_4366	53	TCGGACCGGCGTACGA	Hypothetical protein	1.1	2.1	2.6
	SVEN_4253	20	TAGAACGTACGGCCGA	Hypothetical protein	0.9	2.7	4.2
	SVEN_4976	55	CCGGACGGCTGTTCGC	Hypothetical protein	0.4	1.3	1.5
	SVEN_4782	1	CAGAACGGCTGATCGA	N-succinyl-L,L-diaminopimelate aminotransferase alternative	0.5	0.6	1.1
	SVEN_4210	72	TCGAATGCGAGTTCGA	Hydrolase	0.6	0.9	1.1
	SVEN_5517	7	TCGGACCGCCGTTCGG	Acyl-coa synthetase	0.5	0.7	0.7
	SVEN_6824	56	TCGGTCCCGCGTTCGA	Putative oligosaccharide deacetylase	0.2	0.9	2.0
	SVEN_0816	34	CCGGTCGGCTGTTCGA	Cytochrome P450	0.5	0.7	1.1
	SVEN_3143	95	CCAAACGCCCATGCGA	Thiol:disulfide oxidoreductase	0.3	0.6	0.7
	SVEN_5665	80	CCGGATGGGCGTCCGA	HAD-superfamily hydrolase subfamily IA, variant 3	On	On	On
Others	SVEN_3717	84	CCGCACGCCCGTGCGT	Hydrolase	1.4	1.5	1.7
	SVEN_6979	6	TCAAACCCCTGATCGG	Putative acyl-coa dehydrogenase	0.4	0.4	0.4
	SVEN_1709	36	AAGAACGGCTGTACGA	Putative membrane-associated oxidoreductase	0.1	0.2	0.4
	SVEN_0225	9	CCGCTCGGCTGTGCGA	Putative secreted ribonuclease	32.0	3.6	11.3
	SVEN_1150	33	TCGATTCCGGGTGCGA	Putative serine or threonine protein kinase	0.7	1.2	1.3
	SVEN_3873	96	ACGGACGGCGGTGCGT	NADH dehydrogenase	0.5	0.6	1.0
	SVEN_4257	36	TCACTCGCATGTTTGA	Menaquinone via futalosine step 1	0.2	0.3	0.3
	SVEN_3437	47	TCGCAAGCCTGTCCGG	Putative integral membrane protein	0.4	0.6	1.9
	SVEN_2589	36	CCGGACGCCGGTTTGT	Putative secreted sugar hydrolase	1.2	2.4	4.1
	SVEN_2529	55	TCGAAGAGGTGTCCGA	Secreted protein	on	On	On
	SVEN_4646	18	TCGAACGGAGGTGTGG	Secreted protein	on	On	On
Glutathion metabolism	SVEN_3354	39	TCGAAAGTGCGTGTGG	Lactoylglutathione lyase	0.6	1.1	1.5
	SVEN_0797	41	CCGGACGCGGAATCGA	Glutathione peroxidase	0.5	0.6	0.9
	SVEN_4772	83	TCGAATATCCGACCGA	Putative small membrane protein	0.2	0.4	0.9
Carbon metabolism	SVEN_2781	60	TCGCACGGACGACCGA	N-acetylglucosamine-1-phosphate uridyltransferase or Glucosamine-1-phosphate N-acetyltransferase	1.5	3.1	4.3

PTS	SVEN_0975	58	ACGACCGCGGGTTCGA	Phosphoenolpyruvate-protein phosphotransferase of PTS system	0.4	0.5	1.4
	SVEN_5516	22	CCGAACGGCGGTCCGA	Phosphocarrier protein of PTS system	0.4	0.6	0.9
	SVEN_4059	25	CCGAACGGCTGACCGA	PTS system, N-acetylmuramic acid-specific IIB component or PTS system, N-acetylmuramic acid-specific IIC component	0.2	0.4	0.4
Mobile element	SVEN_7218	83	CCGGATCCCCGTTCGG	EBNA-1 protein	1.2	1.5	1.7
	SVEN_5662	81	TCGACCCCACGTCCGA	IstB ATP-binding protein	0.2	0.5	2.3

3.3.1.2 *In silico* analysis of genes containing a putative RecA-independent motif

Although the RecA-LexA-SOS-box system was accepted as the sole mechanism regulating the DNA damage response in bacteria for many years, the resistance to various DNA damaging agents in mutants expressing a non-functional *recA* had been reported in *E. coli* as early as 1991 (Goodson *et al.*, 1991). Later in 1998 the induction of λ prophage in *E. coli*, which is expected to require the functional *recA*, was observed in the *recA* negative background (Rozanov *et al.*, 1998). These and other observations suggested the existence of a RecA-independent mechanism of regulation of the SOS response. More recently, the interest in understanding this mechanism arose in *Mycobacterium* and *Acenatobacter* due to the fact that both appeared to have DNA damage-inducible responses independent of the RecA-LexA pathway (Rozanov *et al.*, 1998; Davis *et al.*, 2002b). Rand and coworkers in 2003 carried out a detailed microarray analysis of wild-type and *recA* mutant strains of *M. tuberculosis* treated with MMC. As a result, they observed that the induction of SOS-genes in *Mycobacterium* is in stark contrast to the established notion of RecA-dependent induction of the SOS-genes. The majority of mycobacterial SOS-genes are expressed independently *recA* (Rand et al., 2003). Using the upstream sequences of these genes Gamulin *et al.* identified a promoter motif and named it the RecA-independent promoter motif or RecA-NDp (Gamulin *et al.*, 2004). The consensus sequence for the motif in *Mycobacterium* was determined to be tTGTCRgtg-8nt-TAnnnT (Gamulin *et al.*, 2004). A similar motif was also found in the upstream region of known SOS-genes in other actinomycetes, but was absent in non-actinomycete bacteria (Gamulin *et al.*, 2004).

A RecA-independent motif has also been identified in *S. coelicolor* (Studholme *et al.*, 2004). The motif identified in *Streptomyces*, ttgtCAGTGN(13)TGGA, was slightly different from the one identified in *Mycobacterium* and other actinomycetes (figure 3.3) (Gamulin *et al.*, 2004).

Figure 3.3 Multiple alignment of RecA-independent motif generated in three independent studies

Gamulin *et al*, 2004 TTGTCRGTGNNNNNNNNTANNNT--- 23
This study TTGTCRGTGNNNNNNNNYANNNT--- 23
Studholme *et al*, 2004 TTGTCAGTGNNNNNNNNNNNNNTGGA 26

To identify a putative RecA-independent SOS motif I used the tools described in table 3.1 and the materials and methods. 500 bp sequences upstream of the known RecA-independent genes *recA, ruvC, recQ, uvrD* were used as the query sequences (Gamulin *et al.,* 2004). The putative RecA-independent motif of *S. venezuelae* is constructed independently of the previously published work on *Streptomyces* and is closely related to the one found by Gamulin *et al.* and by Studholme *et al.* (figure 3.3). Using this motif as the query, putative RecA-independent SOS genes were identified and the list was further enriched based on certain conserved features of this motif (figure 3.4).

Criteria for the enrichment of RecA-independent motif

The genome scan for the motif search was first performed on *S. venezuelae* upstream sequences. The RecA-independent regulatory sequence motif is 26 nt long with ten highly conserved nucleotides (figure 3.4). To reduce the rate of false positive hits, three rounds of genome scans were performed. The high confidence hits (with lowest p value) and known DNA damage repair related genes from the previous search were used as a query to construct a new motif in the next round of search. Based on the sequence of the query motif from known RecA-independent SOS genes, high confidence hits from the genome scan and the motif described in other published studies, the following criteria were established for the consideration of a hit as the putative

RecA-independent motif: the first four nucleotides TGTC, A at the 19th and T at the 23rd positions.

Only the motifs following these criteria were included in the final list (table 3.4). Based on the analysis of the motifs of known SOS-genes, motifs with a TA at 18th and 19th position and/or GTG at the 6th, 7th and the 8th position were grouped as high confidence hits. Functional analysis of these hits identified that the majority of these genes as either known or predicted DNA damage repair genes in bacteria, confirming the selection criteria described above. The putative SOS genes identified in such a way are listed in the table 3.4.

Functional grouping of the putative RecA-independent SOS genes

Following the grouping pattern earlier employed for RecA-dependent genes, the hits obtained from the computational search for RecA-independent genes were also clustered based on their functional attributes: DNA damage repair genes, transcriptional regulators, enzymes, export related genes, hypothetical proteins and others. Though very stringent criteria for refining the putative RecA-independent motif sequence were used, the search returned 137 hits. This is 65% more that the RecA-dependent genes, which could be consistent with a more pervasive reliance on the RecA-independent DNA damage response mechanism in *S. venezuelae.*

Functional analysis of the genes revealed that 28 of these genes were involved in DNA repair or replication, which is a relatively large fraction compared to just 5 of the RecA-dependent genes falling into this category. This indicates that, as in *Mycobacterium*, the majority of SOS genes in *Streptomyces* may be regulated through RecA-independent mechanism (Rand *et al.*, 2003). The genes falling into this category included the known SOS-genes used as the query, DNA helicase, error-prone DNA polymerase, endonucleases, topoisomerases and other known DNA repair

genes. Out of 28 putative RecA-independent genes, orthologs of 26 genes were found in *S. coelicolor*. 18 (69%) of the *S. coelicolor* orthologs also contained a RecA-independent motif in their promoter region. This supports the idea that the phenomenon of greater reliance of the SOS-response on the RecA-independent mechanism may be common among streptomycetes.

Similarly, to the RecA-dependent pathway, the largest fraction of the putative RecA-independent genes was uncharacterized hypothetical proteins. Some of these could be false positives. However, this could also be explained by the fact that, unlike the *E. coli* and *B. subtilis* genome, the streptomycetes genomes are not as extensively annotated. Another significant fraction of the genes fall into the category named "others", as these genes, although well annotated in the database did not group into any functional category by the KEGG pathway search, nor did the homology search revealed any information about the functional class they belong to.

10% of the hits were transcriptional regulators. Although this number is not as large as other categories, these genes can have a significant impact on the transcriptome through indirect effects on the expression of other genes.

Interestingly, 2% of the genes were involved in folate metabolism. Folate, also known as folic acid or vitamin B12, is essential for the synthesis of DNA and RNA. It is involved in the synthesis of dTMP and dUMP (Crider *et al.*, 2012). In fact, an inhibitor of dihydrofolate reductase, trimethoprim, is a known SOS-response inducing agent (Shaw *et al.*, 2003). Thus, the presence of the RecA-independent motif in the promoter region of the folate biosynthesis genes is consistent with their roles in DNA repair and validates the computational search strategy.

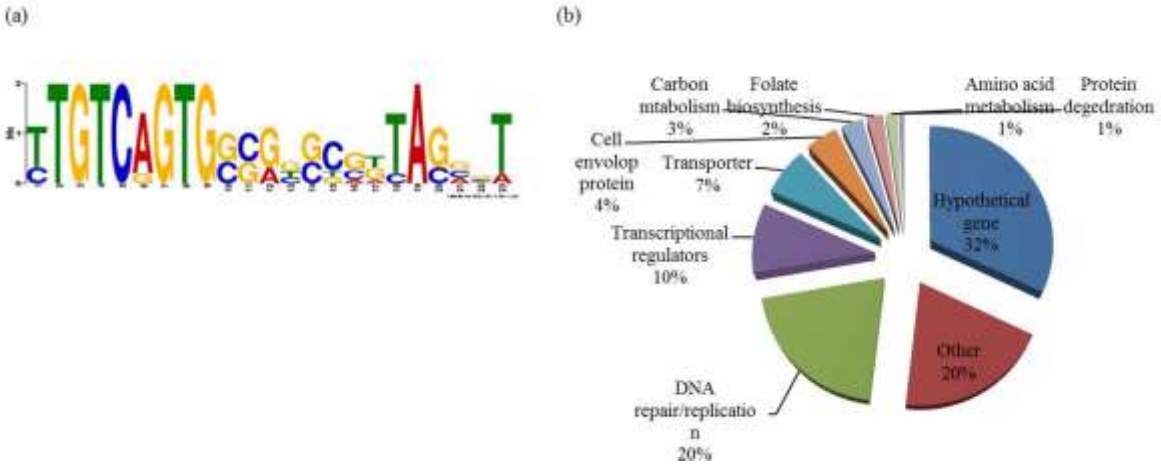

Figure 3.4 *S. venezuelae* genes containing putative RecA independent regulatory motif: (a) The RecA-NDp motif of *S. venezuelae* as determined by MEME. (b) Functional grouping of *S. venezuelae* genes containing the motif.

Table 3.4 Genes containing RecA-independent motif in *S. venezuelae*. *S. coelicolor* orthologs in bold indicates the presence of RecA-independent motif in the upstream region of the genes.

Functional group	*S. venezuelae* gene	Motif	Gene annotation	Distance from the start site (nt)	Fold change in expression after MMC addition			
					20 min	40 min	80 min	S. coe orthologous
DNA damage and replication	SVEN_5478		DinG family ATP-dependent helicase YoaA	90	1.159	1.735	2.154	**SCO5802**
	SVEN_3210		DNA repair protein RadA	92	1.53	2.343	2.981	**SCO3351**
	SVEN_6525		ATP-dependent DNA helicase RecQ	93	0	1.005	1.286	**SCO4577**
	SVEN_1600		Methylated-DNA--protein-cysteinemethyltransferase	93	0	1.888	2.216	**SCO1969**
	SVEN_1120		Crossover junction endodeoxyribonuclease RuvC	67	3.27	4.536	5.6	**SCO1520**
	SVEN_4474		ATP-dependent DNA helicase UvrD or PcrA	90	0	1.592	1.944	**SCO4797**
	SVEN_1266		RecD DNA helicase YrrC	21	0	1.179	1.547	SCO1671
	SVEN_5902		putative DNA polymerase III epsilon subunit	61	2.233	4.853	6.483	**SCO6084**
	SVEN_3340		Endonuclease III	93	0	0	0	SCO3569
	SVEN_5424		RecA protein	56	0	2.068	2.51	**SCO5769**
	SVEN_0903		Uracil-DNA glycosylase, family 1	57	0	1.954	2.193	**SCO1343**
	SVEN_1581		Excinuclease ABC subunit C	92	0	1.043	1.25	SCO1953
	SVEN_0964		DNA polymerase IV	92	1.596	2.63	3.459	**SCO1380**
	SVEN_3302		DNA polymerase III delta prime subunit	72	0	1.59	1.684	**SCO3541**
	SVEN_5743		Helicase	1	1.051	1.06	1.896	-

	SVEN_1718		DNA polymerase III alpha subunit	56	1.336	3.022	3.745	SCO2064
	SVEN_5927		DNA topoisomerase IB (poxvirus type)	15	0	0	1.658	-
	SVEN_1223		putative helicase	89	0	0	0	**SCO1631**
	SVEN_0741		Uracil-DNA glycosylase, family 1	91	0	1.013	1.177	SCO1114
	SVEN_5511		Topoisomerase IV subunit A	54	0	1.437	2.092	**SCO5836**
	SVEN_5493		ATP-dependent DNA helicase RecQ	93	-1.19	2.16	3.151	**SCO5815**
	SVEN_3655		DNA polymerase III beta subunit	51	0	1.245	2.034	**SCO3878**
	SVEN_1618		Helicase, SNF2 or RAD54 family	52	2.808	7.36	9.054	**SCO6262**
	SVEN_1071		Helicase PriA essential for oriC or DnaA-independent DNA replication	92	1.109	1.717	1.81	**SCO1475**
	SVEN_4557		Cytidine deaminase	57	0	0	1.32	SCO4889
	SVEN_2717		Non-specific DNA-binding protein HBsu	22	0	0	0	**SCO2950**
	SVEN_2258		Deoxyguanosinetriphosphate triphosphohydrolase	93	1.926	3.565	4.521	SCO2470
	SVEN_4720		Exodeoxyribonuclease VII large subunit	93	1.488	1.552	1.775	SCO5056
	SVEN_4999		Hypothetical protein	58	1.127	2.994	5.123	-
	SVEN_7438		Hypothetical protein	48	On	on	On	-
	SVEN_0209		Hypothetical protein	83	On	On	On	-
	SVEN_5742		Hypothetical protein	43	1.428	1.804	1.952	-
Hypothetical protein	SVEN_2015		Hypothetical protein	55	1.753	10.12	10.84	-
	SVEN_1572		Hypothetical protein	91	-0.93	1.977	2.692	-
	SVEN_0817		Hypothetical protein	92	-0.82	4.003	5.042	**SCO3065**
	SVEN_3825		Hypothetical protein	46	1.116	1.771	1.817	**SCO4069**
	SVEN_2733		Hypothetical protein	58	-0.56	-0.82	-0.8	**SCO2975**

						3	
SVEN_6863		Hypothetical protein	55	1.444	8.962	9.315	**SCO3050**
SVEN_6311		Hypothetical protein	92	3.286	2.902	9.399	**SCO6478**
SVEN_6098		Hypothetical protein	62	On	On	On	-
SVEN_3315		Hypothetical protein	93	0	0	0	SCO3545
SVEN_0855		Hypothetical protein	93	2.229	3.796	4.05	**SCO1256**
SVEN_0976		Hypothetical protein	63	1.831	3.357	4.715	**SCO1392**
SVEN_7390		Hypothetical protein	16	0	4.799	6.062	**SCO6686**
SVEN_0756		Hypothetical protein	27	1.085	2.118	2.416	-
SVEN_4750		Putative membrane protein	33	1.023	2.771	7.536	SCO5105
SVEN_0565		Hypothetical protein	43	0	0	0	SCO2641
SVEN_1754		Hypothetical protein	51	On	On	On	-
SVEN_0740		Hypothetical protein	59	0	1.951	2.465	**SCO1113**
SVEN_4023		Hypothetical protein	93	0	2.406	3.13	**SCO4268**
SVEN_7392		Hypothetical protein	48	1.301	3.964	5.347	-
SVEN_3443		Hypothetical protein	17	On	On	On	-
SVEN_4482		Hypothetical protein	61	1.061	4.491	7.256	**SCO4803**
SVEN_0041		Hypothetical protein	66	0	0	0	SCO7653
SVEN_2169		Hypothetical protein	46	1.728	2.15	2.43	-
SVEN_0023		Hypothetical protein	93	1.502	4.204	5.373	-
SVEN_0427		Hypothetical protein	61	On	On	1	SCO7336
SVEN_2171		Hypothetical protein	54	On	On	On	-
SVEN_1866		Hypothetical protein	37	0	0	3.069	**SCO2205**

	SVEN_0995		Putative membrane protein	92	0	0	1.369	**SCO1409**
	SVEN_0421		Hypothetical protein	66	On	On	On	**SCO6674**
	SVEN_4925		Hypothetical protein	93	1.955	4.185	4.671	SCO1297
	SVEN_7434		Hypothetical protein	93	On	On	On	-
	SVEN_1338		Hypothetical protein	40	-0.33	-0.58	-0.66	SCO1721
	SVEN_7166		Hypothetical protein	65	-0.36	1.003	2.321	-
	SVEN_1622		Hypothetical protein	16	0	0	0	SCO1997
	SVEN_3154		Hypothetical protein	93	0	0	0	**SCO2353**
	SVEN_0024		Hypothetical protein	90	1.476	1.899	2.907	-
	SVEN_0027		Hypothetical protein	93	0	1.983	2.334	-
	SVEN_2011		Hypothetical protein	93	0	1.944	3.103	SCO0080
	SVEN_3396		Hypothetical protein	54	1.511	2.207	2.231	-
	SVEN_4042		Hypothetical protein	51	0	0	2.312	-
	SVEN_7233		Hypothetical protein	9	1.339	1.342	3.63	SCO0104
	SVEN_3867		Hypothetical protein YgaF	78	0	0	1.306	**SCO4113**
	SVEN_6521		4-carboxymuconolactone decarboxylase domain or alkylhydroperoxidase AhpD family core domain protein	45	On	On	On	SCO0469
Enzymes	SVEN_7394		ADP-ribose pyrophosphatase	29	1.069	1.848	2.442	SCO6960
	SVEN_1107		ATPase, AAA family	97	1.68	2.228	2.372	**SCO1506**
	SVEN_0306		Beta-lactamase (Cephalosporinase)	59	0	0	0	SCO7243

SVEN_6733		Beta-xylosidase	90	1.684	2.405	4.178	SCO0118
SVEN_3739		Cytochrome oxidase biogenesis protein, putative copper metallochaperone	24	0	0	0	SCO3966
SVEN_5468		Diaminopimelate epimerase	29	0	0	0	SCO5793
SVEN_0211		Dienelactone hydrolase	38	1.423	9.9	11.07	-
SVEN_2392		Dihydrofolate synthase or Folylpolyglutamate synthase	93	0	0.771	1.126	SCO2614
SVEN_3252		Dihydroneopterin aldolase	29	0	0	0	**SCO3400**
SVEN_6040		Enolase	56	1.385	1.851	3.951	SCO7638
SVEN_3805		GCN5-related N-acetyltransferase	92	2.916	6.661	7.536	-
SVEN_6585		Guanosine-3,5-bis(Diphosphate) 3-pyrophosphohydrolase	25	0	-0.95	-1.17	SCO2623
SVEN_3389		N-acetylmuramoyl-L-alanine amidase	33	0	0.298	0.678	-
SVEN_4273		NADH-ubiquinone oxidoreductase chain I	68	0	1.293	8.547	SCO4570
SVEN_2323		Phosphate starvation-inducible protein PhoH,predicted ATPase	52	0	0	0	**SCO2532**
SVEN_5970		Possible NAD-dependent epimerase or dehydratase	63	1.448	1.511	1.771	SCO5893
SVEN_6350		Pterin-4-alpha-carbinolamine dehydratase	80	-0.88	1.62	1.703	SCO6540
SVEN_1632		Putative formate dehydrogenase oxidoreductase protein	57	0	0	0	**SCO2004**
SVEN_7333		Putative hydrolase	53	0	1.051	1.432	-
SVEN_6281		Putative inositol monophosphatase	93	0	0	0	**SCO6445**
SVEN_1507		Putative oxidoreductase	11	1.186	2.634	2.73	**SCO7586**
SVEN_2745		Putative phosphatase	93	0	0	1.317	**SCO3000**
SVEN_5141		Salicylate hydroxylase	73	0	0	1.225	SCO3245
SVEN_3592		Serine or threonine protein kinase	46	0	1.078	1.156	**SCO3821**

	SVEN_1262		Magnesium and cobalt transport protein CorA	21	1.008	1.074	1.465	SCO1667
	SVEN_3246		Binding-protein-dependent transport systems inner membrane component	53	1.423	1.429	1.92	-
Transporters	SVEN_6602		Predicted rhamnose oligosaccharide ABC transport system, permease component 2	45	0.245	0.56	0.736	SCO1538
	SVEN_5111		putative ABC transporter ATP-binding protein	91	2.913	4.39	5.877	**SCO5451**
	SVEN_3533		Multidrug ABC transporter permease	24	0.236	0.299	0.652	SCO3825
	SVEN_4908		RNA polymerase sigma factor	24	0.238	0.467	0.602	SCO5243
	SVEN_1459		Molybdenum cofactor biosynthesis protein MoaA	59	0.4	0.439	0.561	SCO1821
	SVEN_1846		Transcriptional regulator, DeoR family	93	0.322	0.503	0.578	**SCO2184**
	SVEN_6231		LysR family transcriptional regulator	35	1.184	1.747	2.435	-
	SVEN_1764		Putative transcriptional regulatory protein	93	0.529	0.711	0.755	**SCO2105**
	SVEN_1377		Transcriptional regulator, DeoR family	92	1.353	1.747	2.03	-
Transcriptional regulators	SVEN_0319		Iron-chelator utilization protein	61	On	On	1	-
	SVEN_4562		RNA polymerase sigma-70 factor	44	1.341	2.224	3.782	**SCO4895**
	SVEN_6880		Putative LacI-family transcriptional regulator	31	0.699	0.96	1.426	SCO0886
	SVEN_3874		Serine phosphatase RsbU, regulator of sigma subunit	21	0.452	0.794	0.876	SCO4120
	SVEN_4595		Transcriptional regulator, TetR family	17	0.603	0.866	0.869	SCO5951
	SVEN_3482		Putative transcriptional regulator	93	1.12	2.486	3.311	-
	SVEN_3308		Permeases of the major facilitator superfamily or Thymidylate kinase	93	1.299	1.55	3.377	**SCO3542**
	SVEN_4229		RNA polymerase ECF-subfamily sigma factor	53	0.244	0.33	1.025	SCO4409
	SVEN_4209		Iron-dependent repressor IdeR or DtxR	61	0.432	0.596	0.7	**SCO4394**

							02	
	SVEN_3682		SSU ribosomal protein S6p	29	0.163	1.483	1.811	SCO3906
	SVEN_1597		Tellurium resistance protein TerD	36	0.652	0.881	2.309	**SCO1965**
	SVEN_4788		Putative secsecreted protein	60	0.711	0.714	0.932	SCO5142
	SVEN_0647		Spore photoproduct lyase	21	1.761	2.243	2.448	SCO7402
	SVEN_2598		Putative secreted protein	92	1.379	2.418	3.105	SCO2808
	SVEN_5329		Protein T24A6.7	92	1.267	1.433	1.91	**SCO5665**
	SVEN_0594		Phage tail assembly protein	61	1.181	4.719	5.35	-
	SVEN_6857		Gas vesicle synthesis protein	60	On	On	On	-
Other	SVEN_6267		MbtH protein	58	On	On	1	-
	SVEN_7162		Peptidase M23B	36	0.337	1.357	3.637	-
	SVEN_4417		Lipoprotein	93	0.631	1.296	2.085	SCO4739
	SVEN_2407		putative integral membrane protein	91	0.844	1.39	1.754	SCO2624
	SVEN_6919		Alpha-glucosides-binding periplasmic protein AglE precursor	48	2.02	2.558	3.092	-
	SVEN_2188		LrgA-associated membrane protein LrgB	92	2.1	3.953	5.296	SCO2373
	SVEN_2417		Putative aminopeptidase	64	0.754	1.289	2.399	SCO2635
	SVEN_6236		acyl carrier protein	61	On	1	1	-
	SVEN_2403		ATP-dependent Clp protease proteolytic subunit	23	0.061	0.631	0.68	SCO2619

3.3.2 Analysis of changes in the global gene expression by RNA-seq

The computational analysis described above identified 79 putative RecA-dependent and 137 putative RecA-independent SOS genes. To independently identify genes induced by the DNA damage, I sequenced total mRNA of *S. venezuelae* following treatment with the DNA damaging agent Mitomycin C (MMC). MMC is a potent DNA cross-linker isolated from *Streptomyces caespitogus* and *Streptomyces lavendulae* and it is widely used as a chemotherapeutic drug. After the intracellular activation, MMC alkylates guanine nucleoside at sequence 5'-CpG-3' (Iyer *et al.*, 1963; Takagi,1963). This stalls the replication fork and induces the SOS response.

To determine the time point of induction of the SOS genes after the MMC addition, I measured the expression of the representative genes known to respond to DNA damage in *Streptomyces*, by qRT-PCR analysis.

The choice of genes for qRT-PCR analysis included the known SOS gene *recN* with RecA-dependent motif, *umuC* with RecA-independent motif and *recA* which is known to contain both the motifs (Gamulin *et al*., 2004). Two standard housekeeping genes of *S. venezuelae* (*rpoB* and *hrdB*) routinely used in qPCR experiments were used to normalize the expression data. A 16 fold increase in the relative expression of *recA* and 8 fold increase in *recN* expression as well as a drastic ~200 fold induction of *umuC* gene was observed by the addition of 0.25 µg/ml MMC at 20, 40 and 80 min after the induction (figure 3.5). The aliquots saved from the same cultures were used to extract RNA for transcriptome sequencing. The samples were sent for sequencing to Otogenetics Co., GA, USA. The criteria used for the sequencing are described in materials and methods. The result of the mRNA sequencing was obtained as the fold difference in the gene expression of ~7500 *S. venezuelae* genes in the absence of MMC and 20, 40 and 80 min after the addition of MMC.

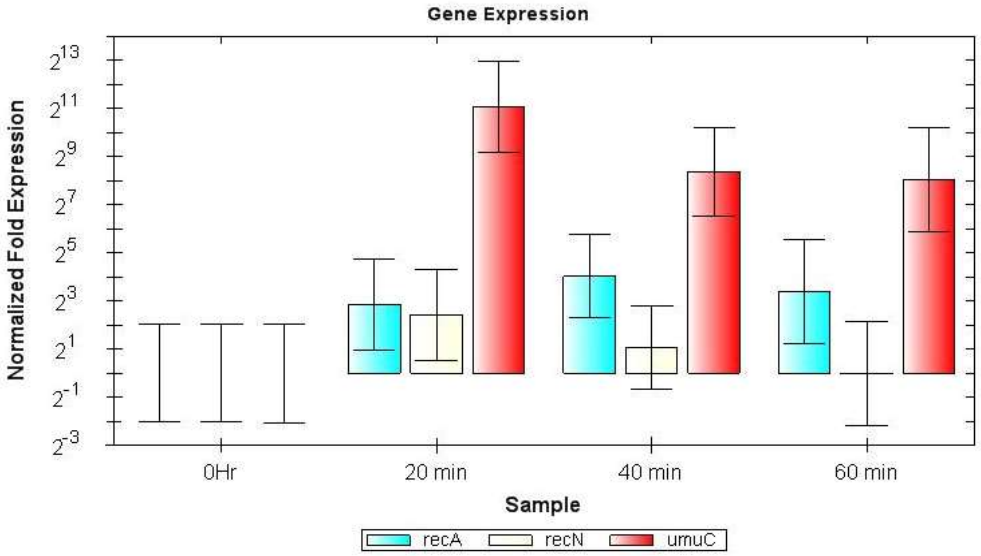

Figure 3.5 qRT-PCR analyses of known SOS genes of *S. venezuelae*: Expression of three known SOS genes, *recA, recN* and *umuC* was measured prior to performing mRNA sequencing. RNA was harvested at time-points 20, 40 and 80 min after the addition of MMC. As expected, the expression of all three SOS genes was upregulated.

To validate the RNA-sequencing results the expression of the computationally predicted SOS genes was analyzed. 40% of the computationally predicted RecA-dependent genes and ~60% of the predicted RecA-independent SOS genes were found to be upregulated two fold or more as a result of MMC treatment. As noted earlier 7% of the computationally predicted RecA-dependent genes and 20% of RecA-independent genes are known or predicted to be involved in DNA damage repair. A five of the six predicted RecA-dependent genes and 14 of 28 predicted RecA-independent genes were found to be up-regulated by the MMC treatment in *S. venezuelae* (table 3.3 and 3.4, figure 3.6). The observed overlap between the *in silico* and *in vivo* data validates the computational predictions and *vice versa* and confirms that many of these genes are involved in the streptomycetes SOS response.

(a) (b)

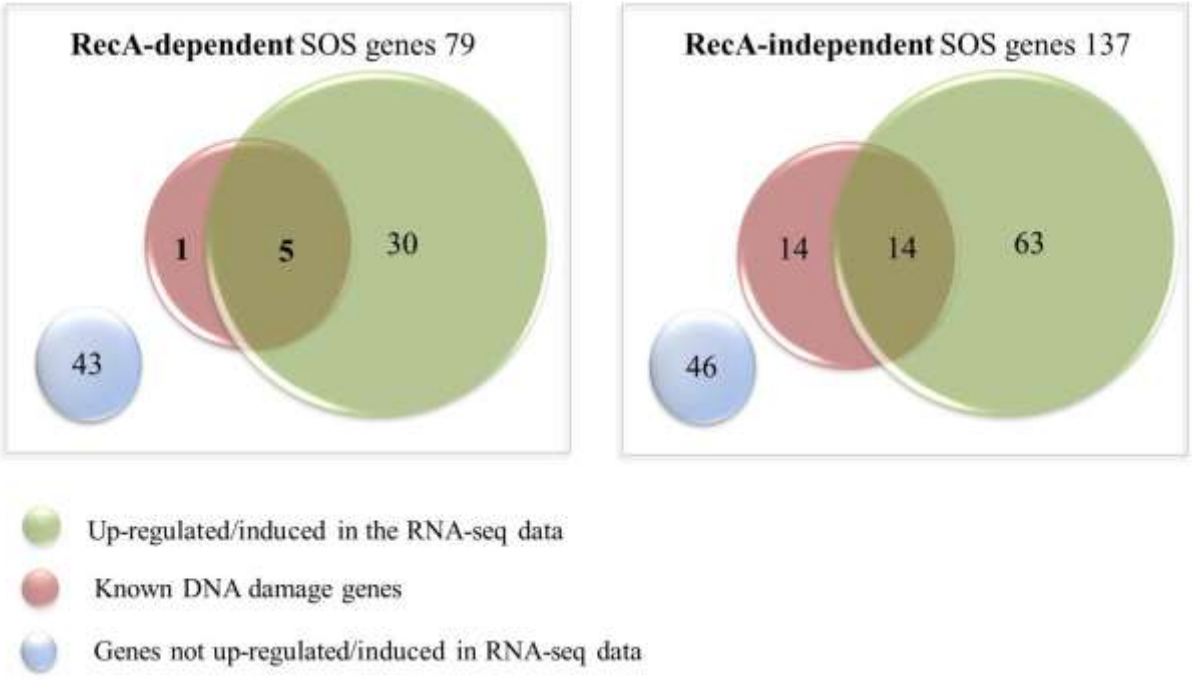

Figure 3.6 Transcriptomic changes after the DNA damage in *S. venezuelae*: (a) 79 genes were predicted to have the RecA-dependent SOS-box motif by *in silico* analysis. This contained 6 known SOS genes, of which 5 were also induced in RNA-seq experiments. 30 other genes containing the predicted SOS-box were induced by the MMC treatment, whereas 43 were unaffected. (b) 137 genes containing the predicted RecA-independent motif in the upstream region were identified of which 77 were induced by the MMC treatment. Of which 14 are known SOS genes.

The data was further analyzed by observing and grouping the genes with the most significant changes in expression and genes belonging to various functional categories. Some of them are discussed below.

3.3.2.1 Inhibition of cell division by the DNA damage response

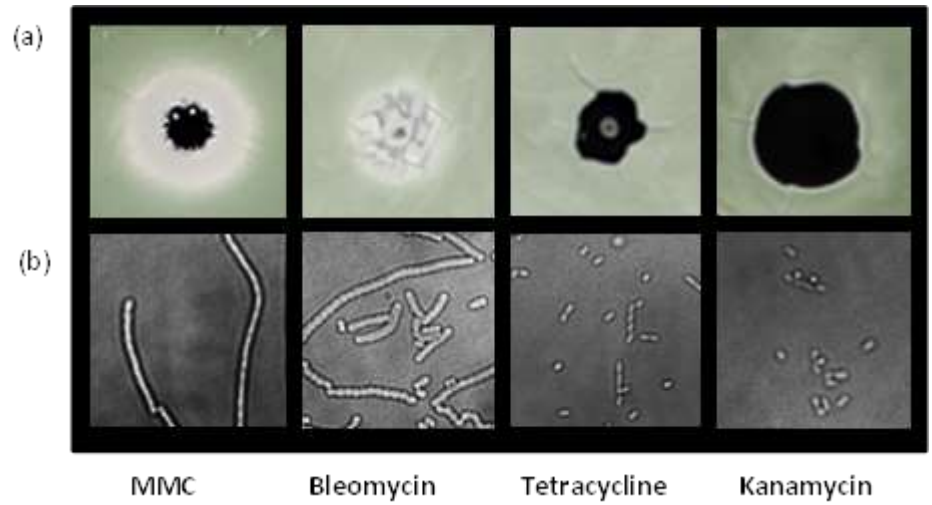

Figure 3.7 DNA damaging molecules inhibit sporulation in *Streptomyces* (adapted form Chapter 2): (a) MMC and bleomycin inhibits sporulation on solid media, whereas tetracycline and kanamycin do not. Two DNA damaging molecules (MMC and bleomycin) and translation inhibitors (tetracycline and kanamycin) were spotted on solid media containing spores of *S. venezuelae.* After 24 hrs of incubation a white zone where the sporulation was inhibited was produced by MMC and bleomycin, but not by tetracycline and kanamycin. (b) DIC images of the cells around the zone of inhibition of growth. Inhibition of sporulation is seen in cells treated with MMC and bleomycin. Tetracycline and kanamycin treated cells were able to undergo normal sporulation.

As discussed in chapter 2, we had earlier observed the phenotypic effects of the DNA damage response on cell division in *S. venezuelae*. To recapitulate, I tested the effect of DNA damage on the colony morphology of *S. venezuelae*. I used two antibiotics, namely mitomycin C and bleomycin, known for their ability to cause damage to the DNA (Iyer *et al.*, 1963; Takagi,1963; Umezawa *et al.*, 1966; Ishizuka *et al.*, 1967). Tetracycline and kanamycin that bind to the ribosome and stall translation served as the negative controls for the experiment (Rose *et al.*, 2006; Lambert,2012). As shown in figure 3.7, both the DNA damaging agents produced a halo

of white colored aerial mycelia surrounding the center of the spotting where high concentration of the molecule completely inhibited the bacterial growth. The 'white phenotype' results from the inhibition of sporulation in the aerial mycelia. No such effect could be observed in the control group where the kill zone, seen as a black circle in the figure 3.7, is surrounded by a lawn of characteristic green spores. A light microscopic analysis of the bacterial cells from the region of sub-inhibitory drug concentration also showed only chain of filaments in the test strains indicating inhibition of spore formation in these cultures (figure 3.7a and b).

Thus, the phenotype of the DNA damage induced cell division inhibition in *S. venezuelae* is manifested by sporulation inhibition. However, at the level of gene expression the observed changes in phenotype could result from one or more of the following:

1. Down-regulation of the sporulation-specific genes
2. Down-regulation of the cell division genes
3. Up-regulation of dedicated cell division inhibitor(s)

To understand the process better I first analyzed the expression pattern of the sporulation and cell division genes.

(1) Expression of sporulation-specific genes

The sporulation genes in *Streptomyces* have been well studied by isolating mutants defective in spore formation in the model strains (Chater,1998; Flardh,2003). Since these mutants have a white fuzzy mass of aerial mycelium, they were termed 'white mutants'. The sporulation genes identified by analysis of such mutants were termed *whi* genes. Five such *whi* genes, *whiA, whiB,*

whiG, whiH, whiI are essential for spore formation. All these *whi* genes encode transcription factors (Claessen *et al.*, 2006; Flardh *et al.*, 2009; McCormick *et al.*, 2012)**.**

To determine if the observed white phenotype in the presence of MMC is due to impaired sporulation in the presence of the DNA damage, I analyzed the expression of the above mentioned, known sporulation genes as well as the genes annotated as sporulation genes by NCBI. I did not observe any significant change in the overall trend on either side of expression values that can be attributed to the sporulation pathway. There were sporadic changes observed at different time points as observed in for *whiD, whiB* and *whiI* expression (table 3.5). Our analysis, however, indicates that the sporulation machinery of *S. venezuelae* may not be directly regulated by the SOS response at least at the level of transcription. The unaltered expression of *whi* genes, indicates that the white phenotype observed is certainly not due to the inhibition of known regulators of sporulation.

Table 3.5 Expression of sporulation genes after addition of MMC

		Fold change in expression		
		20 min	**40 min**	**80 min**
whiG	SVEN_5300	1.9	1.2	-1.3
whiA	SVEN_1578	-1.7	1.3	-1.2
whiH	SVEN_5498	-1.3	2.8	-1.1
whiD	SVEN_4452	4	2.6	1
whiB	SVEN_2776	3.2	2.8	1.5
whiE ORFiv	SVEN_6795	no test	no test	2.8
whiI	SVEN_5827	2.3	6.5	3.5
whiE ORF VIII	SVEN_6799	no test	no test	3.5
whiE	SVEN_6739	no test	no test	On
whiE ORFV	SVEN_6793	On	On	On
whiE ORF III	SVEN_6796	no test	no test	On

(2) Expression of cell division genes

A decrease in the expression of cell division genes after the DNA damage has been documented in several bacteria. In *E. coli*, the SOS response decreases expression of *ftsQA* and in *Caulobacter cresentus* DNA damage decreases expression of *ctrA*, an activator of *ftsA* and *ftsQ* expression (Modell *et al.*, 2011b). However, additional proteins that inhibit the cell division apparatus directly have also been identified in both the organisms (Modell *et al.*, 2011a).

To see if there is any decline in the transcription of cell division genes in *S. venezuelae* I analyzed the RNA-seq data. I observed a significant decrease in several cell division genes. For example, there was a 12-fold decrease in *ssgB* expression and a fourfold decrease in the expression of *ssgA* (table 3.6). SsgA is the first protein to localize to the future septum and mark the site for septum formation prior to the chromosome condensation. SsgA then interacts with SsgB which recruits FtsZ to the site of septation. Null mutants of *ssgA* and *ssgB* are impaired in sporulation specific cell division (Traag *et al.*, 2008; Xu *et al.*, 2009; Willemse *et al.*, 2011). A decrease in *ssgA* and *ssgB* levels may contribute to a reduction of cell division after the DNA damage.

Table 3.6 Changes in the expression of cell division genes after MMC addition

		Fold change in the expression after the MMC addition		
Genes name	**SEVN number**	**20 min**	**40 min**	**80 min**
ssgB	SEVN_1140	-1.7	-5.3	-12.1
ftsH	SVEN_3256	-1.5	-1.1	-4
ssgA	SVEN_3705	-0.8	-2.6	-4
Sigh	SVEN_4908	-1.6	-2	-4
ftsZ	SVEN_1737	-1.7	-1.4	-3.7
	SVEN_5849	-1	-1.3	-3
cell division trigger factor	SVEN_2404	-3.7	-1.3	-2.5

ftsI	SVEN_1745	-1.2	-1.2	-2.3
ftsW	SVEN_3630	-1.1	-1	-2.3
ftsQ	SVEN_1738	-1.3	-1	-2
ftsE	SVEN_2458	-2.5	-3.2	-1.9
ftsW	SVEN_1740	-1.2	-0.8	-1.6
ftsK	SVEN_5398	-0.9	-1	-1.6
ftsX	SVEN_2726	-1.5	-0.7	-1.5
ftsI	SVEN_2386	-1.1	-0.8	-1.4
Cell division initiation	SVEN_5262	-1.1	-0.8	-1.4
	SVEN_2681	-1.3	-1.2	-1.3
ftsE	SVEN_2727	-1.1	-0.5	-1.1
ssgD	SVEN_6402	-1	-1.9	-0.9
ftsI	SVEN_4995	2.8	2.5	2.8
	SVEN_5283	1.2	1.3	2.8
ftsW	SVEN_4996	8	4.9	7.5
crgA	SVEN_3640	0	0	0

In addition, four-fold decreases in the expression of *ftsZ, ftsH* and *sigH* were observed, which may further contribute towards the observed inhibition of the cell division. The table 3.6 shows a clear down-shift in the expression of the divisomal genes. Though, this indicates the involvement of cell division genes in producing the phenotypic effects observed earlier, it may not be the sole mechanism involved in imparting this phenotype. As seen in other bacteria, the involvement of a dedicated cell division inhibitor gene may not be ruled out.

To identify candidates for such putative cell division inhibitors I gathered information obtained from the computational search, RNA-seq data as well as information from known DNA damaged induced cell division inhibitors in other bacteria.

(3) A putative cell division inhibitor - cell wall hydrolase

Proteins directly involved in the cell cycle arrest from DNA damage have been identified in several bacteria including SulA in *E. coli*, YneA in *B. subtilis*, ChiZ in *M. tuberculosis* and SidA in *C. crescentus* (Huisman et al., 1984; Chauhan et al., 2006; Ogino et al., 2008; Mo et al., 2010; Modell et al., 2011a; Vadrevu et al., 2011). Such an inhibitor has not been identified in *Streptomyces.* In attempts to identify a potential inhibitor, I utilized information obtained from our transcriptomic data.

It is a peculiarity of this response that none of the cell division inhibitors listed above are homologs of one another nor do they act by a common mechanism. The mechanism of action is known for SulA, which directly binds the FtsZ protein and ChiZ which interacts with FtsQ/I (Chauhan *et al.*, 2006; Huisman *et al.*, 1984; Mo & Burkholder, 2010). The SOS-activated inhibitors of cell division identified in Gram-positive bacteria do, however, share several features: they are all localized to the membrane, they all possess peptidoglycan hydrolase activity and many of them possess a LysM-type peptidoglycan binding domain (Ogino *et al.*, 2008; Mo *et al.*, 2010; Vadrevu *et al.*, 2011). In addition to these features, the genes that encode these cell division inhibitors are in many cases located in syntenic positions on the chromosome and, of course, all of these genes are induced in response to DNA damage (table 3.7). I used these common characteristics as a guideline for the identification of a putative cell division inhibitor in *S. venezuelae.* As a first step, I examined all of the 41 genes containing peptidoglycan hydrolase domains.

Peptidoglycan (PG) is an important part of the cell wall, which provides a rigid encapsulation to the cell and protects it from the osmotic rupture. It is a polysaccharide made up of alternating N-

acetyl-glucosamine and N-acetyl-muramic-acid. The polysaccharide strands are linked to each other by a short peptide bridge. During growth and cell division, to accommodate the changes in cell shape, this exoskeleton undergoes remodeling. This is mediated by tandem action of PG synthetic machinery and PG degrading machinery in a very tightly regulated fashion (Typas *et al.*, 2011; Wang *et al.*, 2013; Cava *et al.*, 2014). Most bacterial cells comprise of a collection of PG hydrolase enzymes that are involved in the PG degradation (van Heijenoort,2011; Wyckoff *et al.*, 2012). These hydrolases may intuitively appear as a destructive force, but their activity is vital for growth and cell division. The importance of these hydrolases in cell separation is well known for example.

Table 3.7 SOS induced cell division inhibitors in bacteria

Organism	SOS-induced cell-division inhibitor	Localization	Domain	Target	References
E. coli	SulA	Cytosolic	-	FtsZ	(Huisman *et al.*, 1984)
B. subtilis	YneA	Membrane protein	LysM	?	(Mo *et al.*, 2010)
Listeria monocytogenes	YneA	Membrane protein	LysM	?	(van der Veen *et al.*, 2010)
M. tuberculosis	Rv2719c	Membrane protein	LysM, cell wall, hydrolase	FtsI, FtsQ	(Chauhan *et al.*, 2006)
C. glutamicum	DivS	Membrane protein	Cell wall hydrolase	?	(Ogino *et al.*, 2008)
C. cresentus	SidA, DidA	Membrane protein	-	FtsW	(Modell *et al.*, 2011b)
Streptomyces	**?**	**?**	**?**	**?**	

Recent studies in *Mycobacterium* and *Corneybacterium* have shown that during the SOS-response the induction of specific cell wall hydrolases, *chiZ* and *divS*, respectively, leads to the inhibition of cell division (Ogino *et al.*, 2008; Vadrevu *et al.*, 2011). At present the mechanism by which this takes place is not well understood: it could be something as simple as the septally localized disassembly of peptidoglycan to reverse an already initiated cell division event, or it

could be something more complex. Nevertheless, their role in the process is now well-documented.

The cell division inhibitors from these actinomycetes and from *B. subtilis* contains the peptidoglycan binding protein domain LysM (Ogino *et al.*, 2008; Mo *et al.*, 2010; Vadrevu *et al.*, 2011). The LysM domain is found in secreted proteins, lipoproteins, membrane proteins and proteins that non-covalently attach to the peptidoglycan. In bacteria, a LysM domain is most often found in cell wall hydrolases (Buist *et al.*, 2008). Particularly in actinobacteria, LysM domain is found in proteins of the resuscitation promoting factors (Rpf) family - a family of cell wall hydrolase involved in spore germination (Mukamolova *et al.*, 2002).

Streptomyces is related to *Mycobacterium* and *Corneybacterium* (all three are members of the high-GC, Gram-positive Actinobacterial phylum) so it is possible that the SOS cell division inhibitor in *Streptomyces* is a cell wall hydrolase containing the LysM domain. Based on the results of the homology search, I identified 41 cell wall hydrolases from various functional categories in *S. venezuelae* (table 3.8). Using HMMR and PSI-BLAST, I identified the LysM domain containing proteins in *S. venezuelae*. The amino acid sequence of LysM domain from ChiZ in *M. tuberculosis* was used as the query sequence for both. The bioinformatics screen yielded seven candidates cell wall hydrolases containing this domain as listed in the table 3.8.

I examined the status of these genes in the RNAseq data at different time points and indeed I observed induction at varying degrees for different hydrolases. I also observed three additional hydrolases that did not contain a LysM domain or the SOS response motif (RecA-dependent or RecA-independent), yet were significantly overexpressed, perhaps as an indirect effect of one of

the SOS-induced transcriptional regulators. The induction of these cell wall hydrolases merits their candidature for further study.

Table 3.8 Cell wall hydrolase of *Streptomyces*

Hydrolase group or subgroup	*S. coelicolor* Gene	*S. venezuelae* gene	expression 20, 40, 80 min (Fold)	Domain	Conservation	Localization
***N*-Acetylmuramidase/*N*-acetylglucosaminidase (including lysozyme, lytic transglycosylase, Slt, and GEWL-like domains)**	SCO0543	-	-	-	-	-
Cleaves between MurNAc and GlcNAc (muramidase) and GlcNAc and MurNAc (glucosaminidase)	SCO0591	-	-	-	-	-
	SCO1805	SVEN_1440	no change	Lysozyme like	++	
	SCO2001	SVEN_1628	no change	Lysozyme like	+++	Secreted
	SCO2409	-				
	SCO4132	SVEN_3890	no change/down	Lysozyme like and NLP_P60	++++	
	SCO4582	SVEN_7324	on	LysM & lysozyme	+	
	SCO4820	-				
	SCO5092	SVEN_4696	3, 12, 5	Lysozyme like	+++	Secreted
	SCO5286	SVEN_4980	2	GH25 muramidase	+++	Secreted
	SCO5466	-				
	SCO5997	-				
Resuscitation-promoting factor (Rpf) subgroup	SCO0974	SVEN_6803	no change	lysM+transglycosylase+peptidaseM23	+++	
	SCO2326	-				
	SCO3097	SVEN_2900	16, 62, 33	LysM	+++	Secreted
	SCO3098	SVEN_2901	1.7, 6.2,7	LysM + Transglycosylase	+	

	SCO3150	SVEN_2970	6, 3, 6	3DUF348 + G5 + transglycosylase	+++	
	SCO5092	SVEN_4696	3, 12, 5	Lysozyme like	+++	Secreted
	SCO7458	-				
Carboxypeptidase	SCO0088	-				
	SCO3408	SVEN_3260	no change	PBP4 + transpeptidase	++++	D-alanyl-D-alanine carboxypeptidase
	SCO3774	-				
	SCO3811	-				
	SCO4439	SVEN_3076	1.6, 3.8, 1.4	DacC	Dac domain conserved	
	SCO4847	SVEN_4519	no change			
	SCO5467	SVEN_715	no change			
	SCO5660	SVEN_5322	no change			
	SCO6131	-				
	SCO6489	SVEN_6323	no change			
	SCO7050	SVEN_424	1.8, .3, 4.6			
	SCO7607	SVEN_2911	no change			
Endopeptidase	SCO2835	SVEN_2614	2, 4, 2			
	SCO3368	SVEN_3221	no change			
	SCO3949	-				
	SCO4082	SVEN_3837	down			
	SCO4132	SVEN_3890	no change			
	SCO4561	SVEN_4264	11, 21, 11		++	
	SCO4672	-				
	SCO4798	SVEN_4475	2.5, 5, 2.5			
	SCO5623	SVEN_5302	no change/down			

	SCO5839	SVEN_5514	12, 20, 10	peptidaseM23	+++	
	SCO6773	SVEN_6458	on	LysM		
NlpC/P60 subgroup	SCO1240	SVEN_841	7, 22, 6	NLP_P60	+++	
	SCO2135	SVEN_1797	2.5, 4, 2.5			
	SCO2136	SVEN_1798	0.8, 2.7, 1.8			
	SCO3511	-				
	SCO4108	SVEN_3863	3, 5, 3	peptidaseM23	+++	
	SCO4202	SVEN_3950	0.9, 2.3, 1.3			
	SCO4793	SVEN_4472	2, 2.7, 0.7			
	SCO4796	-				
	SCO4795	SVEN_7235	on	TDT like	+	IGM
	SCO5294	SVEN_4989	no change			
	SCO6884	SVEN_5634	on	NLP_P60	++	Lipoprotein
	SCO7021	-				
Amidase	SCO1172	-				
	SCO2116	SVEN_1777	0.3, 4, 1.8			
	SCO2313	SVEN_2027	0.7, 3, 1.1			
	SCO5487	SVEN_5158	0.9, 4, 1.3			
	SCO7179	-				
	SCO7250	SVEN_0268	on	PGRP	+	

The seven candidate hydrolases were PCR amplified using sequence-specific primers using genomic DNA of *S. venezuelae*, and cloned in the plasmid pIJ6902 under the expression from a thiostrepton inducible promoter (Huang *et al.,* 2005). Each sequence-verified clone was then

introduced in *S. venezuelae* and *S. coelicolor* via intergenic conjugation from *E. coli* ET12567/pUZ8002.

If any of these hydrolases is responsible for the cell division inhibition after DNA damage, overexpression of the hydrolase by thiostrepton would mimic the DNA damage response and confer white phenotype to the *Streptomyces* colonies.

Figure 3.8 Phenotype of *S. venezuelae* overexpressing selected cell wall hydrolase: Seven cell wall hydrolase upregulated by MMC treatment were cloned and overexpressed in *S. venezuelae* under the control of thiostrepton induced promoter. Of these three SVEN_2900, SVEN_2901 and SVEN_7235 conferred white phenotype.

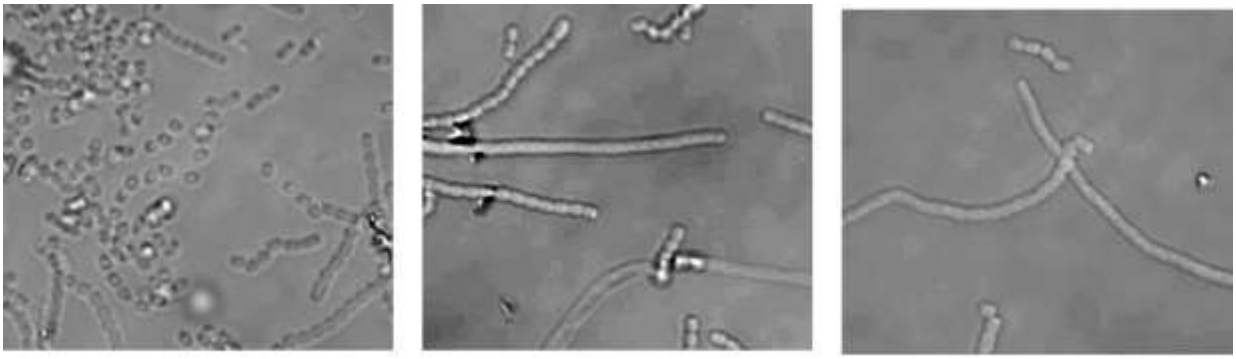

Figure 3.9 Microscopic analysis of aerial hyphae of *S. venezuelae* strains expressing cell wall hydrolase SVEN_2901 and SVEN_7235: DIC images showed incomplete sporulation in strains expressing SVEN_2901 and SVEN_7235, whereas the empty vector containing strain was able to undergo normal sporulation.

As shown in the figure 3.8, three of the seven cell wall hydrolase, SVEN_2900, SVEN_2901 and SVEN_7235 conferred a white phenotype to *S. venezuelae* whereas the control strain containing the empty vector was able to sporulate and produce green pigment. The inhibition of sporulation was also observed by light microscopy as well (figure 3.9).

If any of these cell wall hydrolases are important components of the SOS response, deletion mutations in their cognate genes might influence their response to DNA damaging agents. Colleagues in the Elliot laboratory had constructed null mutants in several of the orthologous hydrolase genes in *S. coelicolor* including SCO3098 (ortholog of SVEN_2901) and SCO3097 (ortholog of SVEN_2900). I, therefore, analyzed the effects of MMC on these mutants, in comparison with their parent strains. Cells were grown on Bennet's media and a paper disk containing 5µl of 2mg/ml MMC was overlaid on the culture. As shown in the figure 3.10 the *S. coelicolor* strains M145 (wild type control) and ΔSCO3097 exhibited white rings around the

MMC disk, indicating the inhibition of sporulation in the sublethal zone of the DNA damaging agent. The similar prominent white ring was not seen when the MMC disk was placed on ΔSCO3098.

Figure 3.10 Effect of DNA damage on sporulation of *S. coelicolor* cell wall hydrolase mutants: *S. coelicolor* strains of cell wall hydrolase mutants, ΔSCO3908 and ΔSCO3097, as well as wild-type control were grown on solid rich media with MMC containing disks at the center. Wild-type and ΔSCO3097 showed the presence of a white zone around the MMC disk which was missing from ΔSCO3098.

This suggests that SCO3098 in *S. coelicolor*, an ortholog of SVEN_2901 in *S. venezuelae*, may be involved in cell division inhibition as a result of the MMC treatment. SVEN_2901 in addition to showing the phenotypic changes showed certain other criteria of an SOS response induced cell division inhibitor- it contains LysM domain and the peptidoglycan hydrolase domain, its expression is upregulated in the presence of the DNA damaging molecule MMC, its overexpression confers white phenotype to *S. venezuelae*. However, further experiments needs to be done to confirm its direct role in cell division inhibition.

3.3.2.2 Additional genes induced to high levels by Mitomycin C.

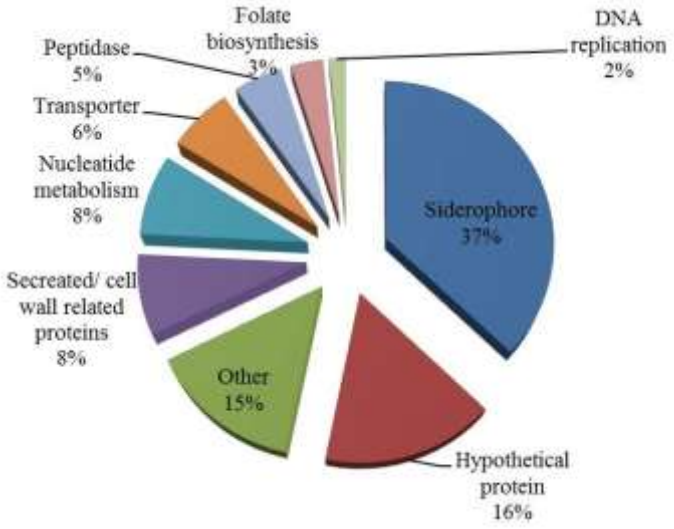

Figure 3.11 Genes highly upregulated by DNA damage: 62 genes upregulated 16 fold or more by the MMC treatment in *S. venezuelae* were grouped based on their functional category. 37% of these genes were involved in siderophore related functions.

In addition to analyzing the genes from specific functional categories, I also looked at the genes that were highly induced by DNA damage in the RNA-seq data. The transcriptome analysis showed over 16 fold upregulation of 62 genes at different time points under study, compared to their respective controls in the un-induced transcriptome (figure 3.11). This includes genes involved in the nucleotide metabolism and folate biosynthesis. During the synthesis of DNA, folate serves as an important cofactor. Some of the genes from this group show a RecA-independent motif in the upstream regulatory region as was identified in our *in silico* search. Thus, upregulation of genes from this group, observed in the RNA-seq data, again, substantiates our computational predictions.

The most surprising of all the upregulated genes are those involved in the iron siderophore biosynthesis. In fact, 37% of the genes induced by MMC are involved in iron homeostasis, making this the single largest group among the SOS-induced genes (figure 3.11). The genome-wide *in silico* search for the presence of natural product biosynthetic clusters in *S. venezuelae* was done using antiSMASH software dedicated for the purpose. The software identified four clusters predicted to be involved in the biosynthesis of siderophores (Medema *et al.*, 2011). According to our data, all these clusters were upregulated by MMC treatment. The induction of the siderophore genes and possible reasons for their up-regulation are discussed further in the following sections (table 3.9).

Other fractions of the up-regulated genes included hypothetical genes and other gene involved in the metabolic process, sensor kinases and enzyme of unknown function.

3.3.2.3 Iron homeostasis genes and DNA damage

Iron is an important metal involved in some vital processes in living organisms. It serves as a cofactor for enzymes involved in respiration and nucleotide biosynthesis among other processes (Andrews *et al.*, 2003). Iron is also important for virulence in pathogenic bacteria and probably for the same reason the host immune system limits the free iron available to bacteria by sequestering it in hemoglobin, ferritin and other iron binding proteins (Finkelstein *et al.*, 1983).

In nature, iron is abundantly available as the insoluble Fe^{+3} but the biologically absorbable Fe^{+2} state is not readily available. Bacteria must acquire iron from their environment such as soil (Wandersman *et al.*, 2004; Budzikiewicz,2010). For this, bacteria secrete a specially designed class of small molecules called siderophores. These molecules show an extraordinarily high affinity to

bind with iron and form a complex. This siderophore-iron complex is then imported in to the cell by active transport, usually via an ABC transporter. Siderophores are one of the strongest iron chelators produced by bacteria, fungi and plants to capture the free iron (Andrews *et al.*, 2003; Sandy *et al.*, 2009; Saha *et al.*, 2013). Bacteria also possess iron storage proteins that can reversibly bind and store iron. These storage proteins maintain iron homeostasis by regulating free iron concentration in the cell. They prevent cellular toxicity by sequestering surplus amounts of iron and release it when the levels in the cell deplete. (Andrews,1998; Carrondo,2003; Chiancone *et al.*, 2004) (figure 3.12).

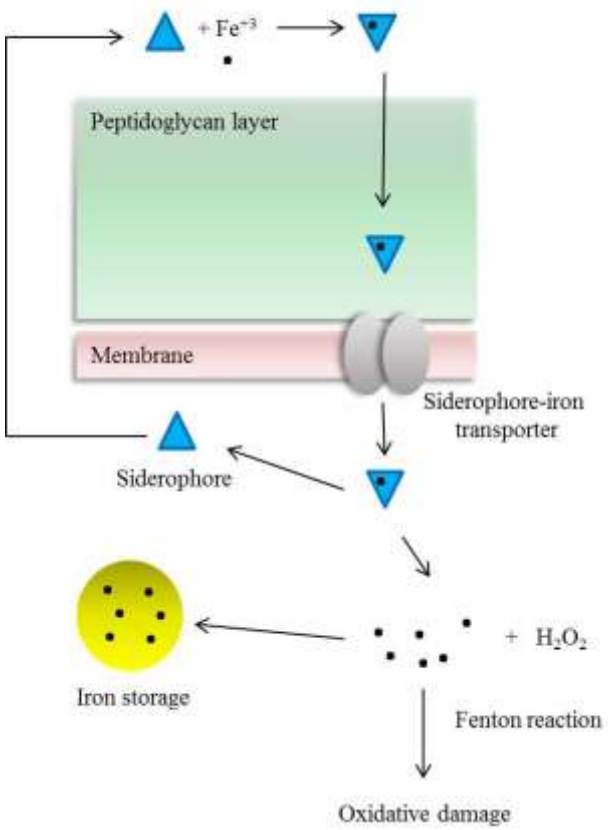

Figure 3.12 Simplified illustration of iron homeostasis in Gram-positive bacteria: During iron limiting condition bacteria produces and secrets iron chelators called siderophores. Siderophores bind extracellular Fe^{+3} that is imported in the cell by active transport. Inside the cell, iron is separated from the siderophore and utilized for biological functions. Siderophores are recycled for iron transport and excess iron is stored in iron storage proteins. If the excess iron is not sequestered by iron storage proteins, it can generate free hydroxyl radicle via Fenton reaction and can lead to oxidative stress.

Excess free intracellular iron can be lethal as the reactive free hydroxyl radicle generated by it can cause oxidative stress leading to severe DNA and protein damage (Cornelis *et al.*, 2011).

For this reason, iron homeostasis is highly regulated process in bacteria and adequate resources are deployed to continuously monitor the intracellular iron pool.

The bacterial 'ferrome' – a collection of all the proteins involved in iron homeostasis in the cell, can be broadly categorized into three major groups: (1) iron acquisition proteins that import iron from the environment in iron limiting conditions eg. siderophore biosynthesis and transport (2) iron storage proteins that sequester excess of intracellular iron, eg. ferritin, bacterioferritin and (3) regulatory proteins that sense the iron concentration and accordingly drive the expression of above mentioned pathways eg. Fur in *E. coli*, IdeR in *M. tuberculosis* (Andrews *et al.*, 2003).

The balanced expression of the first two groups of proteins is vital. An excess of iron storage protein and limitation of iron acquisition protein can lead to cell death by starvation as vital metabolic processes may not function. On the other hand, excess of iron acquisition proteins with low levels of iron storage protein can lead to cell death by oxidative damage.

As mentioned earlier, the genome mining of *S. venezuelae* using antiSMASH identified 29 natural product biosynthetic clusters on the chromosome including four siderophore biosynthetic clusters. In our RNA-seq data all four clusters were found to be up-regulated following the MMC treatment (table 3.9). Indeed, one of the induced siderophore clusters (from SVEN_5472 to SVEN_5477) contained the known SOS genes *lexA* and *nrdR* adjacent to it. Interestingly, a second cluster spanning from the gene SVEN_5414 to SVEN_5423 was also located in proximity to the most widespread regulator of the SOS-response, *recA* (SVEN_5424) (data not shown). Proximity of the siderophore clusters to the known SOS genes on the chromosome was found to be conserved in 10 other sequenced *Streptomyces* and points towards the possibility of a specific role of induction of siderophore genes after the DNA damage.

Supporting this surge in the expression of siderophore biosynthesis clusters, a significant increase in the expression of at least 25 other genes involved in siderophore function or iron assimilation was observed (table 3.11). This suggests that either the maintenance of iron homeostasis may be an important factor in the SOS response in *S. venezuelae*.

Table 3.9 Siderophore biosynthesis clusters

Genes	Function	Expression after MMC addition 20 min	40 min	80 min
	Cluster 1			
SVEN_2569	Hypothetical protein associated with desferrioxamine E biosynthesis	3.3	24.0	3.5
SVEN_2570	Desferrioxamine E biosynthesis protein DesA; L-2,4-diaminobutyrate decarboxylase	8.0	268.8	19.3
SVEN_2571	Desferrioxamine E biosynthesis protein DesB; monooxygenase	0.9	75.8	4.1
SVEN_2572	Desferrioxamine E biosynthesis protein DesC	0.4	42.6	2.7
SVEN_2573	Desferrioxamine E biosynthesis protein DesD	0.6	36.6	2.5
SVEN_2574	Beta-hexosaminidase precursor	0.8	6.1	1.4
SVEN_2575	Hypothetical protein	0.1	10.3	7.1
SVEN_2576	Glucosamine--fructose-6-phosphateaminotransferase	0.2	18.6	7.5
SVEN_2577	Hypothetical protein	0.3	1.2	0.4
	Cluster 2			
SVEN_5414	hypothetical protein	3.2	4.7	5.2
SVEN_5415	Cytochrome d ubiquinol oxidase subunit I	2.5	1.2	1.1
SVEN_5416	DNA-binding response regulator, LuxR family	1.4	3.0	4.1
SVEN_5417	Putative two-component system sensor kinase	1.3	1.7	2.1
SVEN_5418	Hypothetical protein	1.4	2.2	2.6
SVEN_5419	Siderophore synthetase superfamily, group A	1.1	1.1	1.2
SVEN_5420	Hypothetical protein	0.8	1.2	1.2
SVEN_5421	Hypothetical protein	2.6	2.1	1.7
SVEN_5422	Hypothetical protein	1.3	1.5	2.3
SVEN_5423	Hypothetical protein	0.4	0.5	0.9
	Cluster 3			
SVEN_5472	Putative secreted protein	0.5	1.5	4.1
SVEN_5473	Putative secreted protein	0.4	1.2	3.3
SVEN_5474	Siderophore biosynthesis diaminobutyrate--2-oxoglutarate aminotransferase	1.7	2.1	2.2
SVEN_5475	Siderophore synthetase superfamily, group A @ Siderophore synthetase large component, acetyltransferase	1.6	3.5	6.2
SVEN_5476	Siderophore synthetase small component,acetyltransferase	1.4	2.2	6.2
SVEN_5477	Siderophore synthetase component, ligase	3.8	10.2	10.5

SVEN_5478	DinG family ATP-dependent helicase YoaA	1.2	1.7	2.2
	Cluster 4			
SVEN_7067	Hypothetical protein	On	On	On
SVEN_7066	Hypothetical protein	3.3	18.4	1.6
SVEN_7065	ABC-type Fe3+-siderophore transport system,ATPase component	On	On	On
SVEN_7064	ABC-type Fe3+-siderophore transport system,permease 2 component	On	On	On
SVEN_7063	ABC-type Fe3+-siderophore transport system,permease component	6.1	31.6	6.0
SVEN_7062	ABC transporter protein, ATP-binding component	1.5	12.3	0.9
SVEN_7061	Putative ABC transporter transmembrane	2.3	13.9	0.8
SVEN_7060	MbtH protein	1.5	1.9	0.0
SVEN_7059	Siderophore biosynthesis non-ribosomal peptide synthetase modules @ Bacillibactin synthetase component F	10.0	31.1	1.7
SVEN_7058	Possible ABC Fe(3+) transporter binding protein	6.4	38.9	0.6
SVEN_7057	Siderophore biosynthesis protein, monooxygenase	2.8	40.5	0.7
SVEN_7056	Formyltransferase	7.4	88.3	3.5
SVEN_7055	Transcriptional regulator, MarR family	3.4	32.6	1.6
SVEN_7054	Transport protein	3.2	11.1	1.6
SVEN_7053	Hypothetical protein	On	On	On
SVEN_7052	Putative ABC transporter, periplasmic iron-siderophore binding protein precursor	3.7	60.8	3.3
SVEN_7051	ABC-type Fe3+-siderophore transport system,permease component	1.5	22.5	1.3
SVEN_7050	ABC-type Fe3+-siderophore transport system,permease component	3.6	31.9	2.4

The most striking effect in expression was observed in the desferrioxamine E biosynthetic cluster (table 3.9). Desferrioxamine E is a bacterial siderophore involved in sequestration of iron from the environment. The cluster for desferrioxamine biosynthesis consists of an operon of genes SVEN-2570 to SVEN_2573 (*desA-D*) and four other neighboring genes. Although a significant increase in the expression of all the genes of this operon was observed after the MMC treatment, the first gene of the operon, *desA*, was found to be up-regulated to the highest level; 268 fold higher than the controls. Interestingly, the repressor of the operon (*ideR or* SVEN_4209), in spite of containing the RecA-independent promoter motif, was not induced by DNA damage (discussed in detail below).

Moreover, in an independent and unbiased search of SOS genes using the computational approach (discussed above) I identified an iron transport gene containing the SOS-box in the upstream region, as well as an iron-dependent repressor and an iron chelator gene containing the RecA-independent sequences in the upstream region (table 3.10).

Table 3.10 Iron related genes containing the SOS response motif

				Gene expression			
Motif	**Gene**	**Motif sequence**	**Function**	**20 min**	**40 min**	**80 min**	***S. coelicolor* homolog**
RecA-Dp	SVEN_1957	CA**GCAC**GTGG**GTTC**GG	Ferrous iron transport permease EfeU	1	8	2	SCO2277
RecA-NDp	SVEN_4209	TGTCGGCGCGATGCGTCAGAAT	Iron-dependent repressor IdeR or DtxR	0	-1	-1	SCO4394
RecA-NDp	SVEN_0319	TGTCGGGAGGGGCGGGGAACGT	Iron-chelator utilization protein	on	on	on	-

The RecA-dependent sequence containing iron transporter SVEN_1957 (*efeU*) was up-regulated and an iron chelator utilization gene; SVEN_0319 was found to be induced in the presence of MMC, which suggests regulation of these genes through the SOS-response. However, a repressor gene, in spite of containing RecA-independent motif in the upstream region, was not induced by MMC. This gene, SVEN_4209 is a homolog of *M. tuberculosis* iron-dependent repressor *ideR* or *Corynebacterium diphtheria dtxR*, an iron-dependent repressor present in high-GC Gram-positive bacteria. *S. coelicolor* homolog of the repressor gene SVEN_4209 is named *desR* and is reported to regulate expression of desferrioxamine B biosynthetic cluster in *S. coelicolor* and *Streptomyces pilosus* (Tunca *et al.*, 2007). Induction of *desR* in *Streptomyces* would result in inhibition of the iron-assimilation genes. Consistent with this I have observed a dramatic up-regulation of the iron assimilation gene, especially *desR* regulated genes (*desA-desD*) and no induction of the iron-dependent repressor gene, *desR* (SVEN_4209).

Induction of the siderophore clusters and other iron-related genes as well as presence of the DNA damage specific motifs in the upstream regions of certain iron homeostasis related genes indicates a specific and significant role of iron in the response to the stress in *S. venezuelae*.

Table 3.11 Expression of iron homeostasis related genes after MMC addition

		Expression after MMC addition		
Genes	**Function**	**20 min**	**40 min**	**80 min**
SVEN_7440	hypothetical protein	1.0	1.3	3.2
SVEN_0506	Anthranilate synthase, aminase component	1.6	1.9	1.9
SVEN_0510	Long-chain-fatty-acid--CoA ligase	0.7	1.2	2.6
SVEN_0517	pyochelin synthetase F	1.4	7.5	24.4
SVEN_6190	phosphopantetheinyl transferase	on	On	On
SVEN_6269	4-phosphopantetheinyl transferase entD	1.3	1.4	1.9
SVEN_1504	putative acetyltransferase	1.5	1.8	15.0
SVEN_0777	Ferric hydroxamate ABC transporter , periplasmic substrate binding protein FhuD	1.2	2.6	32.4
SVEN_0914	phosphopantetheinyl transferase	1.5	1.7	2.8
SVEN_0319	iron-chelator utilization protein	1.0	On	On
SVEN_1502	Uncharacterized ABC transporter ATP-binding protein	1.8	2.8	29.7
SVEN_1219	Iron utilization protein	1.0	1.4	5.9
SVEN_0164	ABC transporter (iron.B12.siderophore.hemin) ,periplasmic substrate-binding component	1.6	3.0	5.5
SVEN_0165	ABC transporter (iron.B12.siderophore.hemin) ,permease component	1.4	2.3	3.7
SVEN_0166	ABC transporter (iron.B12.siderophore.hemin) ,ATP-binding component	0.8	1.7	2.1
SVEN_0516	putative reductoisomerase in siderophore biosynthesis	1.0	On	On
SVEN_2664	ABC transporter (iron.B12.siderophore.hemin) ,ATP-binding component	0.9	1.6	2.5
SVEN_2665	ABC transporter (iron.B12.siderophore.hemin) ,permease component	0.9	1.0	1.1
SVEN_2666	ABC transporter (iron.B12.siderophore.hemin) ,periplasmic substrate-binding component	0.3	0.3	0.4
SVEN_4132	ABC-type Fe3+-siderophore transport system,ATPase component	0.9	1.2	1.4
SVEN_4133	ABC-type Fe3+-siderophore transport system,permease 2 component	0.5	0.5	0.9
SVEN_4134	ABC-type Fe3+-siderophore transport system,permease component	2.4	2.4	2.9
SVEN_1423	ABC-type Fe3+-siderophore transport system,ATPase component	1.5	4.3	8.2

SVEN_1643	Siderophore biosynthesis protein, monooxygenase	5.2	6.2	20.0
SVEN_1644	Siderophore biosynthesis L-2,4-diaminobutyrate decarboxylase	On	On	on
SVEN_7379	ABC transporter (iron.B12.siderophore.hemin) ,periplasmic substrate-binding component	1.1	3.0	on
SVEN_7381	putative Fe3+-siderophore ABC transporter, ATPase component	1.0	On	on
SVEN_0490	Isochorismatase of siderophore biosynthesis	1.0	On	on
SVEN_1997	putative iron-siderophore uptake system exported solute-binding component	1.0	1.6	5.5

Possible reasons of the induction of siderophores by DNA damage?

Iron is an essential mineral for survival of all cells and yet the soluble form of this element is very scarce. Bacteria, fungi, plant and animal cells have designed specialized mechanisms to sequester soluble iron from their surrounding environment. However, cell also very tightly regulates iron-uptake as excess free iron can itself cause lethal DNA damage.

The up-regulation of iron acquisition genes and down-regulation of iron storage genes disturb the balance of intracellular iron and can lead to cell death via oxidative damage.

In our computational and RNA-seq study we have observed

(1) Upregulation of iron uptake genes

(2) Downregulation of iron storage genes (table 3.12)

(3) Lack of induction of the repressor of the iron uptake gene, *ideR*.

This data collectively suggests that *Streptomyces* species may increase the iron uptake upon severe DNA-damage. As the excessive intracellular iron can lead to cell death, I hypothesize that *Streptomyces* may have designed an iron dependent mechanism to commit suicide when the DNA damage exceeds the capacity of the bacterium to repair it. It also indicates the presence of a

mechanism for the iron uptake that is independent of the extracellular or the intracellular iron concentration. How the DNA-damage is assessed by the cell and the key steps in the decision-making process ultimately leading to cell suicide remains to be determined.

Table 3.12 Fold difference in the expression of iron storage genes after MMC addition

		Expression after MMC addition		
Genes	**Function**	**20 min**	**40 min**	**80 min**
SVEN_1773	putative bacterioferritin	-2.4	-2.8	-9.3
SVEN_6912	Non-specific DNA-binding protein Dps or Iron-binding ferritin antioxidant protein or Ferroxidase	1.9	1.9	1.3

A similar increase in iron uptake as a result of the SOS response has been demonstrated earlier in *E. coli*. Davis and colleagues in their study used the hydroxyurea instead of MMC to induce the SOS-response in *E. coli*. They found up-regulation of iron uptake genes in response to the replication fork arrest by hydroxyurea. This ultimately led to the cell death in *E. coli*. The authors have proposed a mechanism of regulation of such iron-mediated death after the SOS-response, where the signal might be relayed by toxin-antitoxin pair *mazEF* and *relEB (Davies et al., 2009)*. However, a homology-based search for the presence of a toxin-antitoxin pair in *S. venezuelae* did not yield any significant hit. It is likely that *S. venezuelae* may undergo similar iron-mediated cell death in response to the DNA damage via a different iron uptake mechanism.

3.4 Materials and methods

3.4.1 Bacterial growth media

S. venezuelae was grown in MYM media with and without agar (Shepherd MD. *et al.*, 2010). The cultures were grown at 30^{o} C. Mitomycin C was used at various concentrations as indicated to induce the DNA damage.

3.4.2 Microscopy

Microscopy was performed as describe in chapter 2

3.4.3 Sample preparation for qRT-PCR and RNA-seq

Biological triplicates of *S. venezuelae* culture were used for the RNA extraction for qRT-PCR and RNA-seq. Briefly 1 µl of spore stock was inoculated in liquid MYM media in three separate flasks and incubated at 30^{o} C for 10-12 hours with agitation. After the incubation DNA damage was induced to three separately growing cultures with 0.25µg/ml MMC. 1 ml sample was collected from each flask before the MMC addition and at 20, 40 and 80 min after the MMC addition. Samples from each time point were pooled, harvested by spinning at 10,000 RPM, 4^{o} C for 1 min. The supernatant was discarded, and the pellet was flash frozen in liquid nitrogen. Five such samples for each time point were prepared of which two were used for the qRT-PCR, one for the RNA-seq and two were saved at -80^{o} C for future follow-up experimentation.

3.4.4 RNA Extraction, cDNA Synthesis and qPCR primers

Total RNA was isolated using the TRIzol Reagent (Invitrogen) and were purified using RNeasy Mini Kit (Qiagen). Briefly, the overnight cultures of *S. venezuelae* growing in liquid MYM

media were harvested as described above. For the RNA extraction 1 ml TRIzol was added to the frozen pellet and it was briefly sonicated. Total RNA was extracted by addition of 200µl of chloroform followed by centrifugation at 12,000 Xg for 15 min at 4^{o}C. The upper aqueous phase was separated and RNA was precipitated by the addition of ethanol. The samples were centrifuged at 12,000 Xg for 15 min, the supernatant was discarded and the pellet was air dried. The pellets were dissolved in RNase-free water and purified by RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions.

Equal amount of total RNA from each sample were used for the cDNA synthesis and qPCR using two-step QuantiTech SYBR Green RT-PCR Kit (Qiagen) according to the manufacturer's instructions. CFX96 touch deep well™ real-time PCR detection system (Bio-Rad) was used for detection for the amplification and detection of the amplicon in qRT-PCR reaction. The specificity of the reaction was verified by melt curve analysis. To check for genomic DNA contamination, the similar reaction was performed with total RNA sample collected before cDNA synthesis. Oligonucleotide sequences used for qRT-PCR are listed in table 3.13.

Table 3.13 Oligonucleotide sequence used for analysing expression of known SOS-genes

Gene name	**Primer name**	**Primer sequence**	**Primer direction**
recA	recA_F	GAGTCCTCCGGTAAGACGAC	Forward
	recA_R	ACAGGATGAGGTTGTCGATG	Reverse
recN	recN_F	CGAGATCTCCATCCTGCTC	Forward
	recN_R	GATGTCCTCGCCGTACTTC	Reverse
umuC	umuC_F	ATCAGCGAGCAGGTGATG	Forward
	umuC_R	AAGGCCTCGTCGAGACTG	Reverse
hrdB	hrdB_F	CTCCACTGCGGTGTTATGTC	Forward
	hrdB_R	CGTCACCTCTTGAGTCATCG	Reverse

3.4.5 RNA-seq

The frozen sample of harvested *S. venezuelae* cultures as described above was used for the RNA-seq. The sequencing was done with biological triplicate. The frozen pellet was shipped to Ontogenetics co., GA for RNA extraction, rRNA depletion, library construction, sequencing and basic bioinformatics data analysis.

3.4.6 Tools used for the computational prediction of the putative SOS genes.

MEME stands for **Multiple EM (expectation maximization) for Motif Elicitation**. MEME suite is comprised of a group of tools for motif construction (MEME and GOMO), motif searches (MAST, FIMO, GLM2) and for the comparison of the new motifs generated to other known motifs in the database (TOMTOM). Motif construction is performed by searching pattern in a group of sequences that is represented as position-specific weight matrix (PSWM). In the case of a DNA motif, PSWM essentially describes probability of occurrence of a nucleotide at a particular position.

I used this program for *de-novo* discovery of a conserved motif (Bailey *et al.*, 1994). The upstream region of known DNA-damage inducible genes containing SOS-box and RecA-independent sequences were used to construct the motif for the RecA-dependent and RecA-independent SOS genes.

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SUMMARY AND CONCLUDING REMARKS

A typical bacterial cell reproduces by formation of cell envelope layers in between two newly replicated chromosomes followed by the separation of the daughter cells in two, a process known as the cell division. In this work, we inspected the regulation of bacterial cell division by two different vantage points: (1) by manipulation of the bacterial cell division using synthetic chemical molecules and (2) by examination its regulation by the bacterial cells itself, in a stressful condition.

Our work adds to the existing knowledge in the field of antibiotic discovery based on bacterial cell division machinery. We have established a novel screening method for antibiotics utilizing the visual cues provided by *Streptomyces* at various developmental stages along with dispensability of the cell division in this bacterium. The phenotypic manifestation of cell division inhibition in the streptomycetes provides a unique means of investigating the interactions of small molecules with the divisome and the factors that position and stabilize it. While cell division is relatively unimportant to the vegetative cells composing the *Streptomyces* substrate hyphae, it is essential to the normal completion of sporulation. Since successful sporulation is easily observed by colony pigmentation and is not a requirement for viability, we sought to determine whether the

observation of a white phenotype in *Streptomyces* could serve as an assay for identifying compounds that interfere with cell division.

We found that of 196 compounds previously shown to block spore-associated colony pigmentation in *S. coelicolor*, 19 also caused a filamentous phenotype in *B. subtilis*, consistent with a block in cell division. One of these compounds clearly did so through a DNA damage mediated SOS response. The remaining 18 acted independently of at least DNA damage and cell wall damage stress responses. Closer examination revealed that Fil-1 blocked an early step in cell division, impairing both the formation of septa and the segregation of the chromosomes. Fil-2 permitted normal chromosome segregation but caused the formation of misshapen and aberrantly localized membrane- and cell wall–containing structures in place of normal septa. Fil-3 permitted seemingly normal septation events but blocked cytokinesis. These data strongly support the idea that chemical inhibition of spore maturation in streptomycetes can be used as an enriching screen for inhibitors of diverse steps in the bacterial cell division process. In biochemical experiments, we found that none of Fil-1, Fil-2, or Fil-3 blocked either the GTPase activity or polymer formation by purified FtsZ protein, implying that they act via other divisomal constituents or that they compromise divisome placement or stabilization. The effect of Fil-1, Fil-2, and Fil-3 on endospore formation in *B. subtilis* is

also significant. It has been demonstrated previously that sporulation is impaired in the presence of cell division inhibitors. The fact that each Fil molecule impairs both endospore formation and vegetative cell division is also consistent with a molecular target that is either part of the divisome (although clearly not FtsZ) or in the apparatus that positions or stabilizes it.

This work suggests that the streptomycetes life cycle is a powerful tool for identifying chemical inhibitors of cell division. We believe that these bacteria could be employed to screen further molecules in conjunction with a screen for growth inhibition of *B. subtilis*. For example, a direct screen for compounds that block the expression of the *whiE* genes that generate the gray spore pigment might be an efficient screening regimen. Use of a luminescent reporter to monitor gene expression would be a good way to conduct such an assay in high throughput. Conversely, compounds that have been found to block growth in *B. subtilis* could be tested for effects on the sporulation cycle in a streptomycete as a means of narrowing in on divisome-targeting compounds. This approach would be particularly powerful when supplemented with genetic tests for the induction of stress responses to DNA or cell wall damage. This simple *in vivo* visual screening method may allow identification of inhibitors of the cell-division protein whose *in vitro* biochemical function is unknown. The compounds identified in this manner could be used as probes to

better understand the divisome or for the development of novel antibiotics against pathogenic bacteria, particularly those exhibiting resistance to existing antibacterial compounds.

In addition to the above-mentioned conclusions, the formation of white colonies after the addition of bleomycin, ascertained the intuitive phenotypic change in the colony morphology upon cell division inhibition by the DNA damage. To understand the cause of these changes, we inspected the changes in the gene expression after the DNA damage in *Streptomyces venezuelae*. Exploration of the transcriptomic changes by two different approaches; computational and mRNA-sequencing allowed us to gain better understanding of the DNA-damage response in *Streptomyces* and possible cause cell-division inhibition.

By taking the computational approach we constructed the RecA-dependent and independent promoter motifs and identified *Streptomyces* genes containing these promoter motifs as the potential SOS-genes. Functional analysis of the genes showed that a substantially larger number of the RecA-independent genes were involved in DNA repair or replication compared to the RecA-dependent genes falling into this category.

This indicates that the majority of SOS genes in *Streptomyces* might be regulated through RecA-independent mechanism.

Formation of white colonies instead of pigmented colonies was observed as the phenotypic effect of the DNA damage. The mRNA sequencing suggested this could be, at least partly, due to the decline in expression of cell division genes, specifically SsgB. Although, involvement of the developmental genes was ruled out by analyzing the transcriptomic changes, the presence of a dedicated cell division inhibitor gene, as seen in other bacteria, may not be precluded. We, therefore, attempted to identify the inhibitor founded on the characteristics of the similar proteins in other bacteria. Based on what is known in the close relatives of *Streptomyces* and the transcriptomic analysis we focused on 7 cell wall hydrolase genes and identified 2 as the potential cell division inhibitors induced by the DNA damage. Thus, this study examines the significant transcriptomic changes and sets the stage for a better understanding of cell division regulation in DNA-damaged *Streptomyces* cells.