Strathclyde Institute of Pharmacy and Biomedical Sciences

Elucidating the role of *SCO5753* in the synthesis of phosphatidylglycerol in *Streptomyces coelicolor* A3(2).

by

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Abbreviations

Standard Units

°C	Degree Celsius
g	gram
k	kilo
L	litre
M	Molar
Ω	ohms
Psi	pounds per square inch
V	volt
W	Watt

DNA Bases

A	Adenine
C	Cytosine
G	Guanine
T	Thymine
U	Uracil

Textual abbreviations

apr	apramycin
BLAST	Basic local alignment tool
Bp	base pairs
chl	chloramphenicol
CMP	cytidine monophosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonulease
dATP	deoxyadenisine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
dGTP	deoxyguanidine 5'-triphosphate
dNTP	dinucleoside 5'-triphosphate
EDTA	ethylendiaminotetraacetic acid
G/C	guanine/cytosine content
hyg	hygromycin
kb	kilobase
kan	kanamycin
L	litre
LB	Luria Broth
mL	millilitre
mM	millimolar
MS	Mannitol Soya
nal	naldixic acid
OD	optical density
PCR	Polymerase chain reaction
rpm	revolutions per minute
RNase	ribonuclease
SDS	sodium deodecyl sulphate
SDW	sterile distilled water

sp	species
ssDNA	single stranded deoxyribonucleic acid
tet	tetracycline
Tm	melting temperature
Tris	trishydroxymethylaminomethane
w/v	weight to volume ratio
YEME	Yeast Extract-Malt Extract Medium
x-gal	5-bromo-4chloro-3indoyl-□-D-galactoside

ABSTRACT

Phospholipids are distributed across the membrane of both prokaryotes and eukaryotes. Phospholipids also play an important role in cell division. Segregation and positioning of DNA before cell division is coordinated by phospholipids, mainly cardiolipin (CL). CL studies have been conducted in *Escherichia coli* and *Bacillus subtilis* and have shown that CL plays an important role in interacting with proteins, cell division and growth of the organism. They also provide the potential for drug targets in *Mycobacteria*. *Streptomyces*, being Gram-positive has a high lipid content in its membrane. In this project, we attempted to study the characteristics of *SCO5753* which is speculated to synthesize phosphatidylglycerol (PG), which in turn is dimerized to form CL by the action of cardiolipin synthase (*cls*).

In this study, we studied the gene *SCO5753* encoding phosphatidylglycerol and upstream genes and elucidated their essentiality in *S.coelicolor*. Cosmids containing the transposed genes were used to carry out recombination events in *S.coelicolor* and confirmed the strains by Southern blot analysis. Since the transposon Tn*5062* has a eGFP (enhanced green fluorescent protein) tagged in the gene cassette, the strains were microscopically observed for any fluorescence emitted. *SCO5752* and *SCO5753* could not be disrupted using transposed cosmids and were found to be essential in the growth and development of *Streptomcyes.*

CHAPTER 1

INTRODUCTION

1.1 Introduction to *Streptomyces*

Streptomyces is a genus of Gram-positive, aerobic, filamentous bacteria predominantly found in soil. Based on their morphology and cell wall properties, they are classified under the order *Actinomycetales* (Borodina *et al.* 2005), belonging to the family *Streptomycetaceae* (Anderson & Wellington, 2001). They exhibit mycelial growth during their life cycle. The filamentous nature of *Streptomyces* allows access to nutrients as they proliferate, whilst during depletion of nutrients, they sporulate. They are noted for the production of geosmin which gives an earthy odour to soil (Gust *et al.* 2003). *Streptomyces* produce a large number of compounds that are not required for their growth and are called secondary metabolites. The majority of secondary metabolites are biologically active and have an effect within the same species involving growth, development and differentiation and as an antibiotic (when they have a resistance gene) or toxin (on different species) (Kieser *et al.* 2000). *Streptomyces* are also known for producing industrially important enzymes such as proteases, amylases etc. *Streptomyces* have an excellent history of producing antibiotics and constitute up to 70% of antibiotics used commercially. Pigmented antibiotics like undecylprodigiosin and actinorhodin are produced by the model organism *Streptomyces coelicolor* (Hodgson, 2000). *S.coelicolor* is a well known model organism for heterologus protein expression and is the target of study to improve the productivity of proteins, enzymes and antibiotics products during fermentation (Champness, 2000). Molecular and physiological studies of this organism have been the topic of interest among various researchers.

S.coelicolor contains a large chromosome of 8 Mb in size. The G-C content of the chromosome is 72.1% and carries 7825 coding sequences (Bentley *et al.* 2002). It is believed that the *Streptomyces* genome is related to other actinomycetes, and has acquired the ability to replicate in a linear form. Studies have shown that the chromosome contains a centrally located origin of replication (*oriC*) that is present near *dnaA* and *gyrB* encoding the replication initiation protein and DNA gyrase respectively. The *oriC* region contains a slightly higher A-T content, which is a characteristic of bacterial replication origins and consists of 19 DnaA boxes (Paradkar *et al.* 2003). The chromosomal ends consist of inverted terminal repeats (TIR's) that range between 20-500bp in size (Lin Y S *et al.* 1993). However, the entire sequence is not conserved, with the exception of the first 200bp, which are palindromic. Chromosomal expansion has provided the organism with a huge variety of genes which allows the organism to bear a complex life cycle and also adapt to different environments (Bentley *et al.* 2002). The genomes of *Streptomyces* and *Mycobacteria* in spite of their different lifestyles reveal much similarity at the level of individual genes and also gene clusters (Bentley *et al.* 2002).

1.2 Life cycle of *Streptomcyes*

Streptomyces exhibit a complex life-cycle, progressing from dormant spores through vegetative mycelium, to aerial mycelium and then again into spore chains (Chater, 1999). The initial phase is the substrate mycelium, where growth occurs by cell wall extension at hyphal tips and appears as a mat on a solid medium. The next stage is involves the production of aerial mycelium by growing on substrate mycelium. During this phase, production of various secondary metabolites, enzymes and antibiotics is triggered. Studies have shown that aerial hyphae seem to reuse the materials accumulated in the primary stage, like DNA, proteins and other storage compounds. Thus, the aerial hyphae grow and the substrate mycelium is degraded (Miguelez *et al.* 1994). The growth of aerial hyphae stops eventually and the cell enters the sporulation phase. The spores are formed with cross-walls, organized as coiled chains. The spore wall is generally thick and contain a single chromosome. Germination of spores lead to the growth of primary mycelium and thus the life cycle continues (Wildermuth & Hopwood, 1970).

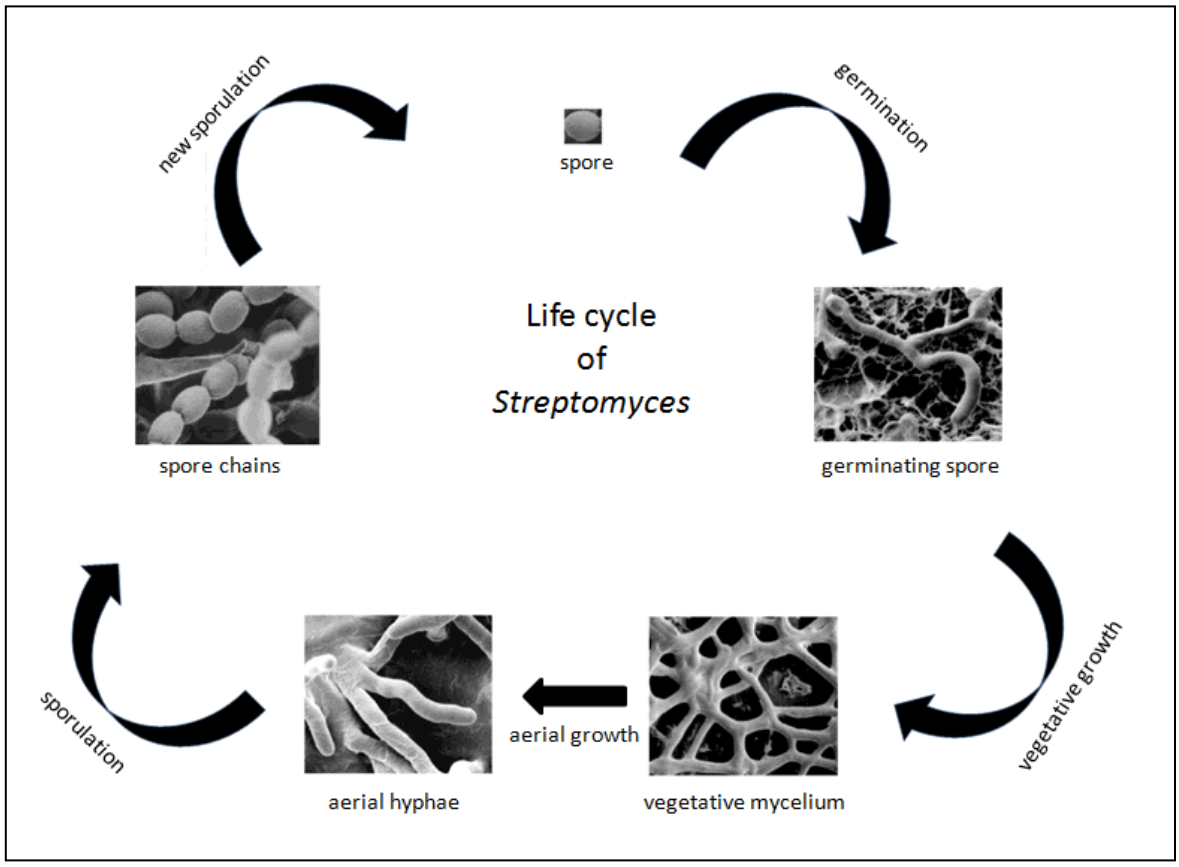


Figure. 1 The life cycle of *Streptomyces* show the spore chains which germinate to form the vegetative mycelium, followed by growth and development of aerial hyphae and once again the formation of spore chains and continuation of the life cycle.
(Image adapted from Kiesser *et al.* 2000)

1.3 Cell division and chromosome segregation

Streptomyces growth occurs by cell-wall extension at hyphal tips and hyphal branching that leads to the formation of vegetative mycelium (Prosser and Tough, 1991). The incorporation of fresh cell wall materials takes place only at the hyphal tips (Flardh, 2003). Growth by tip extension was also observed by introducing fluorescently labeled vancomycin to the peptidoglycan in the cell wall of *Bacillus subtilis*. This agrees with the hypothesis that, extension starts from the apex or the tip in a flexible form and slowly becomes rigid along the apex of the tip (Daniel *et al.* 2003). Recently, using time-lapse microscopy it has been reported that FtsZ follows the extending hyphal tip and is not required for branching, this contradicts the previous observation of emergence of new branch point with new hyphal tip enclosed between two cross walls (Chater & Losick, 1997, Jyothikumar *et al.* 2008).

Most of the cell division genes in *Streptomyces* are non essential unlike other bacteria. Most other bacteria divide by binary fission and have a single circular chromosome (Weiss, 2004). Chromosomal replication is initiated at a single origin, *oriC*, by the initiator protein DnaA, which specifically interacts with the DnaA boxes. In the case of *Streptomyces*, it is also observed that replication is bidirectional from the centrally located *oriC* towards the end of the chromosome (Musialowski *et al.* 1994).

Studies involving the interactions between DnaA and A-T rich regions indicated that *Escherichia coli* could unwind its DNA easily due to the presence of A-T rich regions upstream, whereas, in *Streptomcyes*, since the A-T rich region is not as evident as in other bacteria, accessory proteins such as DnaA could interact and help in unwinding the DNA (Smulczyk *et al.* 2006).

In *E.coli*, DnaA is activated in an ATP dependant fashion and once it recognizes a GATC sequence replication begins. Replication is regulated by inactivation of the ATP form of DnaA and newly synthesized DNA is hemi-methylated and bound by SeqA so that *ociC* is blocked (Smulczyk *et al.* 2006). Many species of *Streptomyces* that have been studied do not have a methylation system (MacNeil 1988).

It is speculated that ATP bound to DnaA in *Streptomyces* is inactivated by ATP hydrolysis since *Streptomyces* DnaA has ATPase activity similar to that of *E.coli* (Majka *et al.* 1997). The presence of DnaA boxes was confirmed by the sequence analysis of *S.coelicolor* genome (Smulczyk *et al.* 2006).

In bacteria, *ftsZ* codes for the Z ring which helps the chromosomal positioning of the cell division site. In *Streptomyces*, it is observed that FtsZ first polymerizes into spirals and then reorganized into regularly spaced rings, but the proteins that stabilize the ring formation and anchor it to the membrane are still unknown. Studies have also shown that *ftsZ* mutant of *Streptomyces* were viable but lacked cross walls and grew as a single consortium (Schwedock *et al.* 1997).

Two important genes which code for FtsW and FtsI required for cell division and cell elongation in *E.coli,* are also present in *Streptomyces* (Grantcharova et al. 2003) and findings by Bhavesh *et al.* (2008) showed that they are required for cell division in sporogenic aerial hyphae. *ftsW* and *ftsI* mutants are unable to assemble FtsZ spirals into Z rings in the aerial hyphae suggesting an early role for FtsW during septation. Studies have also shown that FtsQ is required for coordinating septal formation in association with the Z ring in aerial hyphae (Bhavesh *et al.* 2008, Del Sol *et al.* 2006).

In contrast to cell division, DNA replication takes place at similar rates along the hyphae (Flärdh 2003). It is postulated that the DNA replicates and move in relation to the cell envelope to enter into the extending tips and lateral branches. It is observed that the chromosome segregate from each other and a single chromosome ends up in a spore (Chater, 2001, Flärdh, 2003, Xiang *et al.* 1999).

1.4 Growth and development of *Streptomyces*

The search for differentiation factors in *S.coelicolor* showed several genes that are similar to those involved with cell division in *E.coli*. As explained earlier, colonial complexity increased during the growth of a colony from a spore (Hodgson, 1992). It was observed that the growth of colonies led to piling up of older mycelia and resulted in nutrient limitation and physiological stress (Chakraburtty & Bibb, 1997). *bld* genes are important in regulating the secondary growth in terms of increasing the production of extracelluar proteins, initiation of internal lysis of the substrate mycelium and also initiation of aerial mycelial growth. Mutants of *bldA* showed a lack of aerial growth and antibiotic production depending on the growth conditions (Chater, 1995). As the aerial hyphae grow, nutrients diffuse from the bottom to the tip. A category of genes consisting of white aerial mycelium *whi A, B, G, H, I* are found to regulate and play a role in the development of aerial hyphae, cell division at the tip, compartmentalization and formation of spores.

Studies on *SsgA* and *SsgB* mutants showed that these genes were also involved with the sporulation process, whilst their function is related to the formation of spore-wall peptidoglycan and cell division. Other *Ssg* genes like *SsgC*, *SsgD*, *SsgE*, *SsgF* are responsible for correct DNA segregation, spore wall synthesis and spore separation respectively (Noens *et al.* 2005).

During the growth of *Streptomyces*, formation of aerial hyphae, septation and differentiation into spores are observed. Recently, another pathway has been proposed that operates after aerial hyphae have started to grow in the air which is termed as the sky pathway, in which it is believed that the expression of a set of genes provides

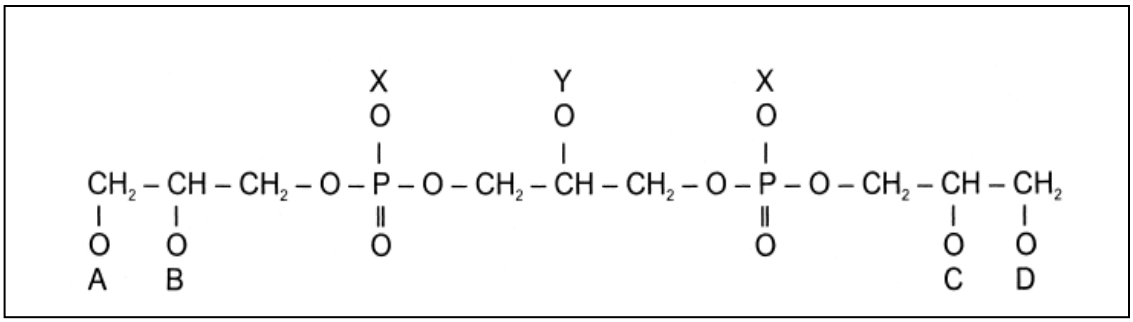
hydrophobicity to the aerial hyphae and the spores when they grow into air (Claessen *et al.* 2006).

1.5 Phospholipids in *Streptomyces*

Though *Streptomyces* are Gram-positive bacteria, it has high lipid content in its membrane, including phosphatidic acid, diphosphatidylglycerol and phosphotidylinositiol. Studies have shown that these phospholipids not only help in the stability of the membrane but are also thought to play an important role in cell division (Hoischen *et al.* 1997, Mileykovskaya & Dowhan, 2005). In *E.coli* and *B.subtilis*, lipid-rich domains present in the centre and at the poles tend to dock certain cell division proteins responsible for identification of cell division sites. The Z ring, which is important in chromosomal segregation during cell division, may probably dock around the anionic phospholipids present in the cell membrane (Mileykovskaya & Dowhan, 2005).

This led to the study of interactions between lipid-protein at particular sites. Activation of DnaA by anionic phospholipid domains *in vitro* was suggested by Mizushima *et al.* (1996). The same interaction was studied in *E.coli* using regulated expression of *pgsA* *in vivo* and suggests that the heterogenous distribution of phospholipids throughout the membrane helps cell division and also in the process of invagination, realignment, and formation of lipid bilayers. Similar studies in *B.subtilis* have shown the presence of lipid spirals extending along the axis of cells. The spiral structures were absent in the cells that lacked the synthesis of phosphatidylglycerol which suggested an enrichment in

anionic phospholipids (Daniel *et al.* 2003). GFP fusions of MinD, which are involved in cell division, also formed spiral structures. Variability in lipid composition may be a general factor in shaping the interactions of membranes and proteins (Daniel *et al.* 2003). Hashimoto et al. in 2009 showed that *pgsA* encoding for phosphaidylglycerol synthase is essential in *B.subtils*. In *Schizosaccharomyces pombe*, it was observed that the cell underwent binary fission after the formation of septum which was mainly formed by lipid rafts (Mileykovskaya & Dowhan, 2005). The distribution of these lipid rafts were important for the positioning and function of actomyosin rings (Wachtler *et al.* 2003). Cardiolipin (CL) (Fig. 2) is a phopholipid, with a dimeric structure, with two phosphate groups linked by a glycerol molecule and bearing four acyl chains on either sides. CL is known for its unique ability to interact with many proteins. CL breaks down to form phosphatidic acid and PG upon hydrolysis by phospholipase D. It is plausible that cardiolipin engages in the formation of non-bilayer structures within biological membranes. Such structures may be involved in membrane dynamics, including membrane assembly and transmembrane movement of proteins (Shibata *et al.* 1994).



(Schlame *et al.* 2000)

Figure. 2 Structure of CL showing two phosphates groups bearing two acyl chains on either sides and linked by a glycerol molecule.

1.6 Biosynthesis of PG

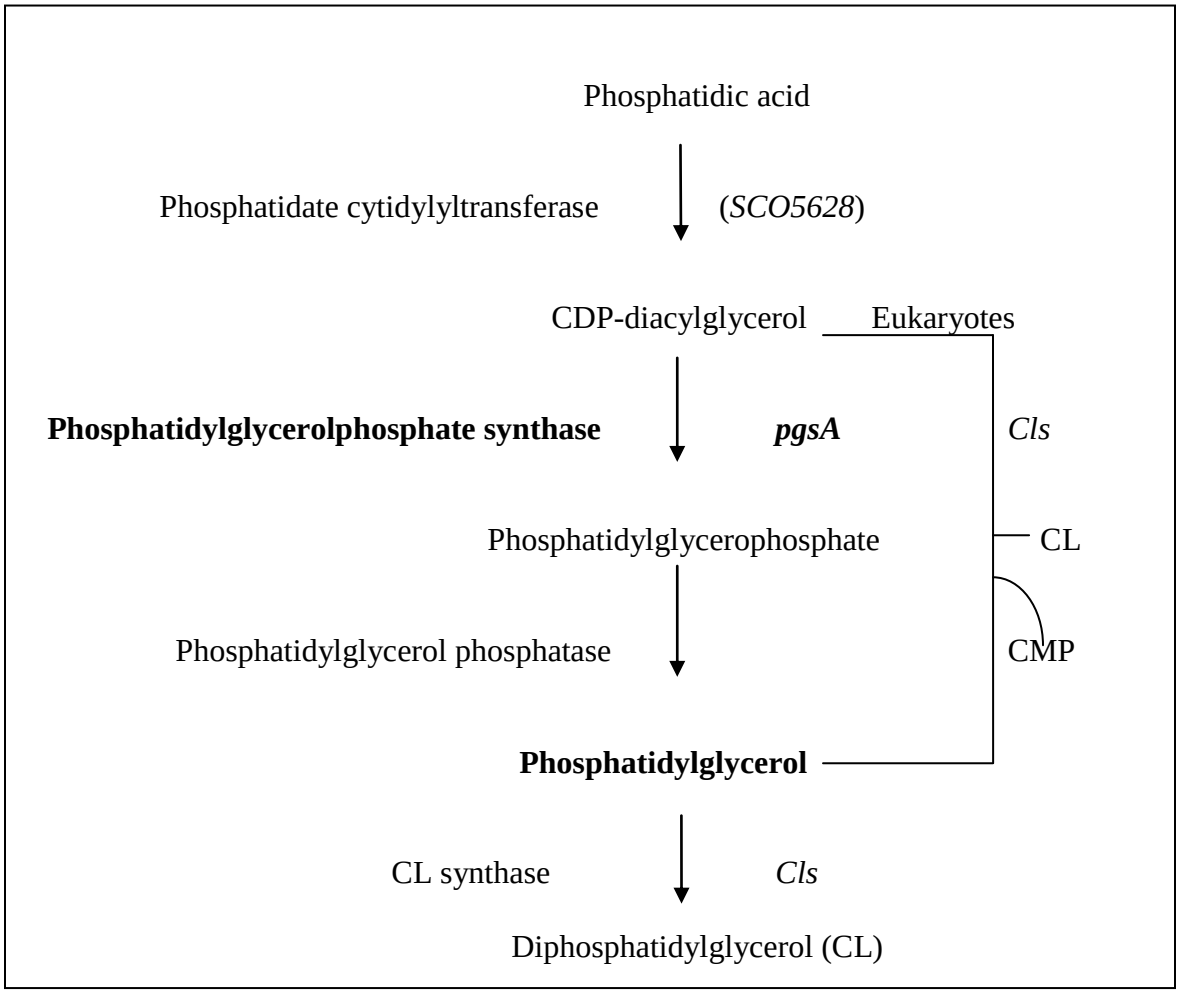


Figure 3. Biosynthesis of PG, prokaryotic and eukaryotic pathway

The pathway of PG is similar to other phospholipids (Fig. 3) and it passes through some of the common intermediates, phosphatidic acid and phosphatidyl-CMP with the help of the enzyme phosphatidate cytidylyltransferase (annotated as *SCO5628* in strepDB). The final step of CL synthesis is a unique reaction and is different in prokaryotes and eukaryotes. Prokaryotic CL synthase catalyzes a transesterification in which the phosphatidyl group of one phosphatidlyglycerol is transferred to the the free 3'-hydroxyl group of another phosphatidylglycerol. Whereas, eukaryotic CL synthase links an activated phosphatidyl moiety to phosphatidylglycerol. Since eukaryotic CL synthase is a mitochondrial enzyme and mitochondria are phylogenetic derivatives of ancient prokaryotes, it appears that the transition from bacterial to mitochondria CL also involved a transition from the prokaryotic to the eukaryotic reaction mechanism (Schlame *et al.* 2000).

Phospholipids such as phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG) and cardiolipin (CL) are abundant in bacteria. As explained earlier, the deficiency of some of these could affect the structural and functional organization of the organism. Phosphatidylglycerol and CL are the major anionic phospholipids in *E.coli*.

pgsA mutants in *E.coli* showed the accumulation of phosphatidic acid which is a precursor molecule for the synthesis of PG and CL (Matsumoto, 2001). Phosphatidylglycerolphosphate synthase (*pgsA*) is required for the synthesis of PG and CL (Xia & Dowhan, 1995).

CL was the most predominant phospholipid amongst lyso-cardiolipin (LCL), phosphatidylethanolamine (PE1 and PE2), lysophosphatidylethanolamine (LPE), phosphatidylinositolmannoside (PIM), phosphatidic acid (PA), dilyso-cardiolipin–phosphatidylinositol (DLCL-PI) when isolated and purified from *S hygroscopicus*. In the L-form (protoplast) strain of *S hygroscopicus* the phospholipid content was three-four fold higher (Hoischen *et al.* 1997).

10-*N*- nonyl acridine orange was used to stain CL which showed its presence at cell poles and at division sites or septa in *E.coli* and *B.subtilis*. However, phosphatidyethanolamine lacking mutants of *E.coli* and *B. subtilis* showed the accumulation of nucleoids towards the poles and at the potential cell division sites (Mileykovskaya & Dowhan, 2005). This pattern of localization suggests an important role in cell division and chromosomal segregation.

It is possible that anionic phospholipids play an important role in maintenance of proper membrane surface charge and maintaining membrane potential (Weiming & Dowhan, 1995, Sekimizu & Kornberg, 1988). One such anionic phospholipid, phosphatidylinositol (PI) is essential in *Mycobacterium tuberculosis* and the mutants could not survive devoid of PI. This showed that phosphatidylglycerolphosphate synthase in *Mycobacterium* is essential and could be a drug target in treating tuberculosis (Jackson *et al.* 2000).

In *E.coli*, removal of unsaturated fatty acid, oleic acid, halted the exchange of nucleotides and in turn pause the chromosome replication (Yat-Ming Yung *et al.* 1988). It is presumed that DnaA depends on membrane phospholipids and also the unsaturated

fatty acids for the initiation of replication and growth of the cell, and saturated fatty acids alone would not interact with DnaA. Restoring the cells with oleic acid restored the interaction between DnaA and phospholipids (Yat-Ming Yung *et al.* 1988). Studies conducted by Matsumoto (1999) showed that *pgsA* null mutants of *E.coli* could not grow on minimal media but grew almost normally in rich media but were not viable above 40ºC. A conditional mutant of *pgsA* was constructed with the help of a thermosensitive plasmid which expressed its wild type nature of growing normally at permissive conditions (30ºC) but failed to grow at 42ºC, the temperature at which the thermosensitive plasmid was unable to replicate. This worked paved way into further research on phospholipids, which were essential in *Mycobacterium* for its growth and pathogenicity. (Jackson *et al.* 2000).

The association of cytoplasmic proteins and the proteins that interact with nucleic acids are associated to phospholipids. By combining both, *in vivo* and *in vitro* studies, it was elucidated that phospholipids play a major role in chromosomal replication (Yat-Ming Yung *et al.* 1988). Recently expression of *SCO1389* (cardiolipin synthase) in CL deficient *Rhizobuim* mutant restored CL formation, showing that *cls* is responsible for CL formation and the orthologues of *SCO1389* in actinobacteria is believed to be similar to eukaryotic *cls* (Sandoval-Calderon *et al.* 2009).

The summary of above results not only supports the role of phospholipids in cell physiology & membrane integrity but also in membrane associated reactions and initiation of replication and cell viability (Dowhan & Heacock, 1987). The strepDB annotation of the *S.coelicolor* genome shows the close proximity of *SCO5753* and *SCO5750,* annotated as FtsK, which is responsible for septa formation during cell

division. Studies showed that *ftsK* mutant had segregational defects and anucleated spores denoting the importance and essentiality of the gene (Dedrick et al. 2009). This close arrangement of genes indicates some evidence that *pgsA* could probably be important in chromosome segregation and cell division.

CL is an important component of the mitochondrial cell membrane and constitutes about 20% of the total mitochondrial lipid content. It was also observed that CL strengthens the mitochondrial membrane at high temperatures. CL is found to be strongly associated with cytochrome c in the inner membrane of mitochondria (Mei Zhang *et al.* 2002). Epand *et al.* (2006) observed CL clusters promoted by mitochondrial creatine kinase. It helps in the formation of ADP by the conversion of creatine to phosphocreatine using up ATP (Clark, 1997).

CL and PE domains are observed in the bacterial cell membranes and on other specific membrane proteins like phospholipid synthases which helps in the formation of lipid domains in the cell (Matsumoto *et al.* 2006). Not only proteins, histones, DNA and RNA polymerases and topoisomerases but many other proteins interact with CL (Schlame *et al.* 2000). CL was first isolated from mammalian heart in 1942 (Pangborn, 1942). The biosynthesis and function of CL is well understood in yeast. It is found that the cells survive and respire in the absence of CL but only in the presence of phosphatidylglycerol, and at higher temperatures CL is absolutely essential.

The identification of yeast genes encoding the CL biosynthetic enzymes and subsequent generation of CL deficient strains has provided powerful molecular tools to understand the biosynthesis and function of CL (Schlame *et al.* 2000).

1.7 Osmoregulatory studies

Recent evidences have shown that the expression of cls depends on the osmotic conditions, suggesting that the levels of CL within the cell can be osmoregulated. It was also noticed that there was an increase in the PG content in cls deficient bacteria in response to osmotic stress. Romanstov *et al.* (2007) showed that the proportion of CL content of the *E.coli* membrane and the osmolarity required to activate the osmosensory transporter proteins increased during the stationary phase. It was speculated that CL could probably interact with osmosensory proteins such as ProP and regulate the maintenance of osmolytes in the cytoplasm (Bernal *et al.* 2007, Romantsov *et al.* 2007). On the contrary, *S. coelicolor* has a response regulator gene pair, *osaAB*, which is involved in osmoregulation (Bishop *et al.* 2000). This study has shown that *osaB* transcribes independently, and is essential for osmoadaptation. The *osaB* mutant cannot give rise to aerial hyphae and produces up to fivefold greater antibiotic yield than the wild-type strain. It has been found that *SCO1389* codes for cls, is essential in *S. coelicolor* and the strain lacking cls showed growth defects (Jyothikumar *et al.* unpublished data). With the above evidences and studies, it could be possible that CL plays a similar role in *Streptomyces* as well.

1.8 Detection of anionic phospholipids

Cells require a network of membrane lipid domains that interact with various membrane proteins (Bergelson, 1995). These lipid domains were earlier studied by indirect techniques like fluorescent depolarization, differential scanning colorimetry etc. Phospholipid distribution in *E.coli* membranes was demonstrated by fluorescence microscopy imaging by (Fishov & Woldringh, 1999). One such membrane lipid, CL was visualized by staining the living cells with the fluorescent dye 10-*N*-nonyl-acridine orange (NAO) (Mileykovskaya & Dowhan, 2000). NAO only binds to anionic phospholipids of the cells by interacting with the phospholipid and has high affinity for CL, by forming a dimer due to the presence of two phosphate groups (Mileykovskaya & Dowhan, 2000). Green fluorescent spots were observed along the cell at regular intervals and also at the poles.

In *B.subtilis*, fluorescence was observed at septal regions and poles and also during sporulation. However, the fluorescence was scarcely detectable in *clsA* disrupted mutants lacking CL (Kawai *et al.* 2004). Mutational studies were conducted in *E.coli* and *B.subtilis* in order to study the role and importance of CL in cell division and sporulation.

1.9 Mutagenesis in *Streptomyces*

The 8.7 Mb *S.coelicolor* genome was previously sequenced by using an ordered library of Supercos-1 clones (Bentley *et al.* 2002). Further to this, gene replacements were made using PCR targeting known as the PCR targeted mutagenesis (Redirect). The cloned *Streptomyces* genes were replaced with a cassette containing a selectable antibiotic resistance and *oriT* for transfer into *Streptomyces.* Upon conjugation, the clones readily undergo double crossover recombination and thus creating gene replacements (Gust *et al.* 2003).

In this study we adapt transposon mutagenesis which is carried out by the insertion of a transposon at a random point in a gene and thereby disrupting its function. Transposition is carried out by activity of the enzyme transposase.

Tn*5062* was constructed from the Tn*5* transposon and an *egfp* reporter gene, containing an apramycin resistance cassette, and *oriT* to allow the movement by intergenic conjugation (Dyson *et al.* 2005). Mutant cosmid DNA libraries were thus created.

St7C7, the cosmid in which the *S.coelicolor* genes were cloned were disrupted with the help of Tn*5062.*

1.10 Aim of the project

All these observations together suggest that anionic phospholipids may a role in cell growth and development and chromosome segregation in *S.coelicolor*. However, the role of *pgsA* is not known in *Streptomyces*, this project addresses mutational studies on *pgsA* that is thought to encode phosphatidylglycerolphosphate synthase, which would mediate the formation of phosphatidylglycerol which in turn is the substrate for cardiolipin synthase (*cls*) to dimerize to form CL.

To achieve this, the gene coding for *pgsA, SCO5753* and the genes upstream are to be mutated using the transposed cosmids and check for the type of recombination that occurs on introducing them into *Streptomyces*.

Once this is achieved, the mutant strains are screened and observed if there are any phenotypic changes and confirm through Southern blot analysis. This could lead us in knowing whether the gene is essential or not, in the synthesis of PG in *S.coelicolor*.

CHAPTER 2

MATERIALS & METHODS

Strains	**Characteristics**	**Reference**
S.coelicolor M145	Wild type, SCP1-,SCP2- prototrophic	Kieser 2000
S.coelicolor BR005	M145ΔSCO5751:Tn*5062*, apr^R Double crossover	This work
S.coelicolor BR006	M145ΔSCO5752:Tn*5062*apr^R, $kan^{R,}$ Single crossover	This work
S.coelicolor BR007	M145ΔSCO5753: Tn*5062* apr^R, $kan^{R,}$ Single crossover	This work
S.coelicolor BRMS001	M145: pLR102, hyg^R	This work
S.coelicolor BRD006	ΔSCO5752:pLR102,apr^R, hyg^R Double crossover	This work
S.coelicolor BRD007	ΔSCO5753:pLR102,apr^R, hyg^R Double crossover	This work
S.coelicolor BRMS06	BR006:pMS82, apr^R, $kan^{R,}$ hyg^R	This work
S.coelicolor BRMS07	BR007:pMS82, apr^R, $kan^{R,}$ hyg^R	This work
VJ101	Double crossover	Unpublished

2.1 Table. 1 *Streptomyces* strains

Strains	**Characteristics**	**Reference**
JM109	*recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, Δ(lac-proAB)/F' [traD36, proAB^{+}, lacI^{q}, lacZΔM15*	Yanish Perron *et al.* 1985
ET12567	*F- dam-13::Tn9,dcm-6,hsd M,hsd R,Rec F143, Tn10, gal T 22, ara14, lac Y 1, xy 1-5,leu B6, thi-1 ,tonA31, rpsL 136, hisG4, tsx-78, mtl-1, gln44, supE, hsd Δ5, thi Δ(lac pro AB)*	McNeil 1988

Table 2. *Escherichia coli* strains

Plasmid	Characteristics	Reference
pALTER1	*E.coli* cloning vector, tet^R	Promega Corporation
pMS82	Integrating vector, $attP_{\Phi BT1}$,hyg^R	Gregory *et al.* 2003
pUZ8002	*tra*,*RP4*, chl^R	Paget *et al.* 1999
pLR101	pALTER1 backbone with insert from SC7C7.F05, tet^R	This work
pLR102	pMS82 backbone with insert from pLR101, hyg^R	This work
pLR103	pUC19 backbone with insert from pLR 102, amp^R	This work
St7C7	Cosmid containing *SCO5751, SCO5752, SCO5752* ,kan^R, amp^R	Redenbach *et al.* 1996
SC7C7.F05	St7C7Δ *SCO5751*:Tn*5062*, apr^R, kan^R, amp^R	Bishop *et al.* 2004
SC7C7.B06	St7C7Δ *SCO5752*:Tn*5062*, apr^R, kan^R, amp^R	Bishop *et al.* 2004
SC7C7.B07	St7C7Δ *SCO5753*:Tn*5062*, apr^R, kan^R, amp^R	Bishop *et al.* 2004

Table 3. Vectors

2.2 Media, Antibiotics

Suppliers of various chemicals and ingredients for culture media are Sigma Chemical Company Ltd & Fisher Scientific UK and antibiotics were supplied by Sigma-Aldrich. UK

2.3 Enzymes and Kits

Restriction enzymes, polymerases and ligases were purchased from Promega, Invitrogen & New England Biolabs UK Ltd. Plasmid miniprep kits were purchased by Promega Corporation, USA and Gel Extraction kits were provided by Qiagen. Solutions were prepared with deionized water (Millipore), pH was measured using a Thermo Orion pH meter and a Thermo pH electrode stored in 3M KCl.

2.4 Table 4. Media & Buffers

1. LB- Broth (Luria Bertani broth) (1L)

Tryptone	10g
Yeast Extract	5g
Sodium Chloride	5g
pH	7.2

2. LB – Agar (1L)

Tryptone	10g
Yeast Extract	5g
Sodium Chloride	5g
Agar	15g
pH	7.2

3. SOC (1L)

Tryptone	10g
Yeast Extract	5g
Sodium Chloride	10ml
Potassium chloride	10ml
Magnesium Chloride (2M)	10ml
Magnesium Sulphate (2M)	10ml
Glucose (2M)	100ml
pH	7

4. MS agar (JIC) (1L)

Mannitol	16g
Soya Flour	16g
Agar	20g

Autoclave at 121°C for 40 minutes

5. 2XYT (1L)

Tryptone	16g
Yeast Extract	10g
Sodium Chloride	5g

6. Minimal Media (M9 media) (1L)

Magnesium Chloride (1M)	2ml
Calcium Chloride (1M)	0.1ml
Glucose (20%)	10ml
Sodium Chloride	0.5g
Ammonium Chloride	1g
Thiamine HCl	1ml

7. TFB II (100ml)

MOPS	100mM (filter sterilized)
Calcium Chloride	1M
Rubidium Chloride	1M
Glycerol	50%

8. TE buffer (10X) (1L)

Tris.cl	100mM (pH 8)
EDTA	1mM (pH 8)

9. TAE buffer (50X) (1L)

Tris	302.4g
Glacial Acetic acid	57.1ml
EDTA	0.5M 100ml

10. Agarose gel loading dye/Stopping buffer (10ml)

Bromophenol blue	0.5%
Xylene cyanol	0.05%
Ficoll	50%
SDS	1%
EDTA	100mM
pH	7.4

11. Stock solutions for DNA transfer (1L)

Depurination solution:	19ml of 13M Hydrochloric acid
Denaturation solution:	87g Sodium Chloride
	20g Sodium Hydroxide
Neutralization solution:	77.8g Ammonium acetate

12. Stock solution for pre-hybridization and hybridization (1L)

20X SSC:	173.5g Sodium chloride
	388.2g Sodium Citrate
SDS	10%
N-laurylsarcosine	10%

13. Maleic acid buffer: (pH 7.5) (1L)

Maleic acid	22.2g
Sodium Chloride	17.6g

14. Blocking solution (100ml)

Blocking reagent	10g
Meleic acid	90 ml

15. Solution for detection (Detection buffer) (300 ml)

Tris-Hcl	3.63g
Sodium Chloride	1.75g

16. Standard hybridization buffer (100 ml)

SSC	5X
N-laurylsarcosine	0.1% w/v
SDS	0.02%
Blocking solution	1%

17. Stringency buffer A (100 ml)

SSC	2X
SDS	0.1%

18. Stringency buffer B (100 ml)

SSC	0.2%
SDS	0.1%

19. Washing buffer (1L)

Maleic acid buffer	1X
Tween 20	0.3% w/v

Antibiotic	Concentration	Dissolved in	Storage temperature
Ampicillin	50mg/ml	Water	-20°C
Apramycin	100mg/ml	Water	-20°C
Kanamycin	25mg/ml	Water	-20°C
Chloramphenicol	25mg/ml	Ethanol	-20°C
Tetracycline	12.5mg/ml	Ethanol	-20°C
Hygromycin	50mg/ml	Water	4°C

2.4.1 Table 5. Antibiotics used

2.5.1 Bacterial growth

Liquid cultures of *E.coli* strains from which the plasmids were to be isolated, were grown in Luria broth (LB) with appropriate antibiotics. The amount of broth used was directly proportional to the amount of plasmid required. The cultures were inoculated overnight at 37°C and 250 rpm.

2.5.2 Bacterial storage

A single colony of the particular strain was inoculated overnight in LB with appropriate antibiotics. The following day, 625 µl of 80% (w/v) glycerol was taken and mixed with 325 µl of the culture in sterile cryotubes. The contents in the tube were evenly mixed and stored at -80°C until further use. The strains were revived by just scrapping over the frozen mixture and streaked for single colonies.

2.5.3 Preparation of *Streptomyces* spores

9ml of sterile water was added to the plate and the surface of the culture was gently scraped with gentle pressure with an inoculating loop. The spores were suspended in this process and then poured the suspension into the universal which contained the sterile water earlier. The suspension was filtered by passing it through non absorbent cotton wool using a syringe (previously sterilized). The filtered suspension was then centrifuged at 4500rpm for 10 minutes. The pellet was then vortexed briefly and the spores were suspended in the residual water. Sterile 20% w/v glycerol was added and the spores were collected in that and stored at -20°C for further use.

2.6 Isolation of DNA

2.6.1 Isolation of plasmid DNA by alkaline lysis (Birnboim & Doly, 1979)

Plasmid and Cosmid DNA were extracted from the bacterial cells using the alkaline lysis method

Solutions:

Solution I	**Solution II**	**Solution III**	
50mM Glucose	0.2N Sodiumhydroxide (4M)	5M PotassiumAcetate	60ml
25mM TrisCl (pH 8)	1% SDS (20%)	Glacial Acetic acid	11.5ml
10mM EDTA (pH 8)	(mix separately)	Water	28.5ml

Plasmids were isolated from 3-10 ml of overnight cultures of *E.coli*. The cells were pelleted by centrifuging at 10,000 rpm for 60 seconds and resuspended in 100 µl of ice cold Solution I and vortexed. Following this 200 µl of freshly prepared Solution II solution was added, mixed well and placed on ice for 5 minutes. Finally 150 µl of ice cold Solution III solution was added and mixed well and placed on ice for about 10 minutes. This was centrifuged at 10,000 rpm for 10 minutes and the supernatant was transferred to a fresh microcentrifuge tube.

2.6.1.1 Phenol Chloroform extraction

The supernatant obtained after lysis was then transferred to a fresh microcentrifuge tube and the DNA was extracted by adding an equal volume of phenol and chloroform.

2.6.1.2 Ethanol precipitation

The DNA was then precipitated with 2 volumes of ethanol and centrifuged at 12,000 rpm for 10 minutes. The ethanol was discarded and the micro centrifuge tube containing the DNA was spun in a vacuum rotor for 30 minutes. The DNA was then resuspended in 50 µl 1X TE buffer and 2 µl of DNase free RNase (20 µg/ml) was added and refrigerated.

2.6.1.3 Kits used for DNA isolation and purification

Wizard miniprep kit, Promega.

This was used for the isolation of DNA, by following the manufacturer's instructions.

QIAGEN QIAquick PCR purification kit.

This was used to purify the digested sample before setting up another restriction or a ligation reaction, by following the manufacturer's instruction.

QIAGEN QIAquick Gel extraction kit.

This was used to extract the DNA fragment from the agarose gel and purify it. This was mainly used for cloning purposes.

2.6.2 *Streptomyces* genomic DNA isolation

200 µl of S.coelicolor spore suspension was inoculated in 25 ml of YEME medium and incubated at 30°C for 72 hours. The culture was centrifuged at 10,000 rpm for 5 minutes and the pellet was resuspended in 3ml of 25mM Tris pH 8 containing lysozyme (2mg/ml) and RNase (10mg/ml) and incubated at 37°C for 30 minutes. This was followed by the addition of 5ml of 0.3M Nacl with 20% (w/v) SDS and mixed thoroughly. To this, 6 ml of phenol and chloroform was added and the solution obtained was an emulsion. After centrifugation at 10,000 rpm for 5 minutes, the supernatant was transferred to a new tube and again equal volumes of phenol – chloroform was added and centrifuged again. The aqueous phase was transferred to a new tube and about 10 ml f absolute ethanol was added along the sides. Then DNA was then visible and spooled out of the solution with the help of a Pasteur pipette and briefly dried and dissolved in 1X TE and stored at -20°C.

2.7 Introduction of DNA into E.coli

2.7.1 Preparation of competent cells- rubidium chloride method

The bacterium from stock (in this case it was JM109) was streaked for single colonies on M9 Minimal media plates and the single colony was inoculated overnight in LB – broth (Table 1). 1 ml of this was transferred to fresh LB – broth (100ml) and grown till the Optical density reached 0.4-0.6. The cells were incubated on ice for 15 minutes. The cells were then centrifuged at 2500 rpm for 10 minutes and resuspend in 75mM Calcium

Chloride solution (pre chilled). The cells were incubated on ice for 20 minutes and centrifuged at 2,500 rpm for 10 minutes and resuspended in 4ml of TFB II solution (Table 1). The cells were again incubated on ice for further 20 minutes. Finally, they were aliquoted into 100 µl lots and snap frozen in Liquid Nitrogen and stored at -80°C.

2.7.2 Transformation methods

2.7.2.1 Heat shock method

The competent cells prepared by rubidium chloride method were thawed on ice for 20 minutes to which the DNA of interest was added (about 1-3 µl) and incubated on ice for 3 minutes. This was then incubated at 45°C for 45 seconds (heat shock). The heat shocked sample was incubated on ice for 2 minutes and 1ml SOC was added and incubated at 37°C for 90 minutes and shaking at 200 rpm and finally plated on LB agar containing appropriate antibiotics.

2.7.2.2 Electroporation

Single colonies of the cells required for transformation were inoculated overnight in LB containing appropriate antibiotics and scaled up to 100ml the following day. Once the optical density of the culture reached to about 0.4-0.6 at 600nm, the cells were centrifuged at 2,500 rpm for 10 minutes and resuspended in 10ml ice cold 10% w/v glycerol. For ET124567/pUZ8002 this step was repeated three times in order to remove salts and antibiotics. This was centrifuged again and resupended in 5ml ice cold 10% Glycerol and aliquoted them into 100 µl lots. 1-3 µl of DNA sample was taken for a

100 µl aliquot of competent cells and electroporation was carried out in pre chilled electroporation cuvette using a BIORAD Gene Pulsar II, which was set to 200Ω, 25MF & 2.5KV. Once the buzzer beeped, immediately 1ml SOC was added to the electroporated cells and incubated at 37°C for 90 minutes and spread them on LB – agar plates containing appropriate antibiotics.

2.7.2.3 Introduction of DNA into *Streptomyces*

Overnight cultures (10 ml) of donor strains of bacteria were set up with appropriate antibiotics, and scaled up to 100ml the following day (1:100 volume of overnight and fresh culture). The culture was grown till the optical density at 600nm was between 0.4-0.6. The cells were centrifuged and washed with fresh LB – broth 3 times to remove all the antibiotics and further resuspended in 500 µl LB- broth. Meanwhile, 500 µl SOC media was added to 100 µl of the *S.coelicolor* spore suspension and heat shocked at 50°C for 10 minutes, following which, 500 µl of the donor strain of bacteria was added, mixed well and spread them on SFM media and incubated at 30°C for 16 hours. The following day, selective antibiotics were added for mutant selection and generally naladixic acid was used for deselection of the donor.

2.8 Screening methods

2.8.1 Blue/white selection.

X-gal (5-bromo-4-chloro-3-indolyl-ß- galactoside) at a concentration of 20µg/ml was used in addition to IPTG (Isopropyl β-D-1-thiogalactopyranoside) at a concentration of 50µg/ml to identify the strains of *E.coli* containing the plasmid with the insert. Clones containing the insert appeared white.

White colonies obtained from the LB plates with appropriate antibiotics were taken and inoculated into 10ml LB – broth (about 24 at a time) with the same antibiotics overnight. The following day, the DNA from those samples was isolated using the alkaline lysis protocol. Appropriate enzymes were chosen and restriction digests were set up for those samples at 37°C for 2 hours. After the incubation 2 µl of gel loading dye/stopping buffer was added and ran on a 0.8% Agarose gel (prepared with 1X TAE).

2.8.2 Screening for mutants

After the conjugation of *E.coli* (carrying the desired plasmid) and *S.coelicolor*. The colonies were patched on separate MS-agar plates containing specific antibiotics. The antibiotic markers in the plasmid made the selection process efficient. This way we could easily differentiate between single and double-crossovers when plated along with respective controls. Wild-type *S.coelicolor* strain and a control for both single and double crossovers were also patched to check the activity of the antibiotic.

2.9 Restriction digestion

20 µl reaction mixtures were set up consisting of 15.5 µl of sterile distilled water, 2 µl of sample DNA, 2 µl of enzyme buffer and 0.5 µl of the restriction enzyme and incubated at specific temperature and time as instructed by the enzyme manufacturer. Following which, 2 µl of Gel loading dye/ stopping buffer was added.

2.9.1 DNA modifying enzymes

T_4DNA ligase was the modifying enzyme that was used to set up ligation reactions between the fragments of DNA cut with specific restriction enzymes. A 20 µl ligation reaction was set up which consisted of 10 µl sterile distilled water, 5 µl of the insert, 1 µl of the vector, 3 µl of Invitrogen 5X ligase buffer and 1 µl of T_4DNA ligase. Sticky end ligation was carried out at 16°C and blunt end ligation was carried out at 4°C overnight.

2.9.2 Agarose Gel Electrophoresis:

0.8% Agarose gels were prepared in 1X TAE. About 4 µl of ethidium bromide (10mg/ml) was added to 100ml of the gel. λ-Hind III marker was used as a molecular size marker and also for quantifying the DNA sample, by comparing the intensity of the bands in correspondence to dilutions.

Gel apparatus from Fisher Scientific and Biorad were used. Gels were caste on the gel tray by pouring the molten agarose gel into them and combs were inserted for well formation. Depending on the requirement electrophoresis was conducted with voltage ranging from 75-100V. Once the DNA had traveled the length of the gel, it was

visualized under a UV transilluminator. Photography of the gels were done using a Syngene Bioimaging IN Genius GelDoc system using the software Genesnap. The size (kb) of the DNA fragments was determined by comparison with a λ-Hind III marker.

2.10 Morphological analysis of mutants by microscopy

Once the mutant strains i.e. single crossovers, double crossovers and conditional double crossovers were obtained, they were grown on minimal media. Cover slips were sterilized by dipping them in ethanol and flaming them and introduced into the media at a 45° angle and 10µl of spore suspension of all the strains were inoculated in the interface of the cover slip and the agar. After 72 hours, these cover slips were fixed in methanol and placed on clean slides and mounted them.

These samples were observed under a Nikon TE2000 inverted confocal microscope (with a standard FITC filter). The images were captured under bright light as well as blue light (to excite eGFP) with the help of Hamatsu Orca-285 Firewire camera which would help us in observing the localization of the genes tagged with the green fluorescent protein.

2.11 Southern Hybridization

DIG-labelled (digoxygenin) DNA probes were made using the DIG-DNA labeling kit (ROCHE) along with Hybond-N^+ nitro-celluose membranes (Amersham Biosciences Ltd.)

DIG-labelling

Using Klenow enzyme, DIG-dNTP's were incorporated into the DNA fragment to be used as the probe. The set up for each DIG labeling reaction was done by taking 15 µl of 1 µg DNA (which was previously boiled for 10 minutes and placed on ice for 10 minutes), 2 µl hexanucleotide mix, 2 µl dNTP, 2 µl of DIG labeling mix and 1 µl of Klenow enzyme and was incubated at 37°C overnight.

Binding of DNA to nitro-celluose membrane

Genomic DNA was isolated for Southern analysis. The samples were concentrated by ethanol precipitation overnight. The DNA samples were run on 0.8% agarose gel overnight at 25V (Section 2.6.2). The nitrocellulose membrane to which the DNA binds was washed thoroughly with distilled water and then with 20X SSC and was transferred to the Vacugene XL blotting unit in the following order: filter paper, nitrocelluose membrane, mask and agarose gel.

The vacuum pump was connected to the blotting apparatus and vacuum pressure was set to 50-60 mbar. For the complete transfer of DNA to the membrane, depurination was carried out by adding 50ml of depurination solution on the agarose gel and maintained for 20 minutes and later removed gently by wiping off the surface of the gel. Following this, the denaturation process was carried out by adding 50ml of denaturation solution on

the agarose gel and maintained for 20 minutes and later removed gently by wiping off the surface of the gel.

Finally, neutralization was carried out by the addition of 60ml of neutralization solution on the agarose gel and maintained for 20 minutes and later removed gently by wiping off the surface of the gel. 20X SSC was added to cover the agarose gel (twice its thickness) and left for an hour. It was ensured that the gel was intact in its position throughout the transfer.

After 1 hour of transfer, the pump was turned off and the gel was carefully removed for photography, in order to ensure the complete transfer of DNA to the membrane. The membrane was carefully washed with 20X SSC to remove any traces of agarose from it.

Hybridization

The membrane was rolled and placed inside a hybridization tube. 20 ml of hybridization solution was added to membrane in the hybridization oven at 65°C for 1 hour. The solution was decanted and fresh hybridization solution with denatured probe (by boiling at 100°C for 10 minutes and cooling it on ice for 10 minutes) was added and hybridized overnight at 65°C.

Detection

About 30 ml of stringency buffer A was added to the hybridization tube and rinsed, following which 150 ml of stringency buffer A was again added and the membrane was washed for 15 minutes at 65°C. A rinse was again repeated with stringency buffer A. 30 ml of stringency buffer B was added and rinsed, following which the membrane was washed with stringency buffer B for 15 minutes at 65°C. The membrane was again rinsed with stringency buffer B.

The membrane was removed from the hybridization tube and rinsed in washing buffer for 30 seconds. It was then washed with freshly prepared blocking solution for 30 mins on a rocker. The membrane was then treated with antibody solution [3 µl of antidigoxygenin AP (antibody conjugate) in 30 ml of blocking solution] for 1 hour with gentle rocking. This was decanted and the membrane was washed in 100 ml of washing buffer for 15 minutes to remove unbound antibody. The membrane was then equilibrated in 20 ml detection buffer for 2 minutes. The membrane was finally sealed in a cling film and incubated in dark with 20 ml of freshly prepared colour substrate solution (2 NBT tablets dissolved in 20 ml of distilled water) till colour develops. To stop the reaction, the membrane was washed 5 – 10 times under running tap water and stored in the dark for further analysis.

2. 12 Softwares and Bioinformatics

StrepDB

StrepDB was the Streptomyces genomic database from which the position of various genes, operons, sequences and also the exact location of the transposon insertion were acquired. It also provided the gene number, size, orientation and numerous other details regarding the gene of our interest.

SciEd Clone manager

This program was used to work out the cloning strategies, enzyme selection and also to construct the maps of numerous plasmids and vectors. The sequences were read, edited and suited to the working style.

NCBI-BLAST

This program was used to perform the similarity search of the probable pgsA.

CHAPTER 3

RESULTS

3.1 Results of bioinformatic investigation

3.2.1Sequence information

The sequence of the putative *pgsA* gene was obtained from the StrepDB.

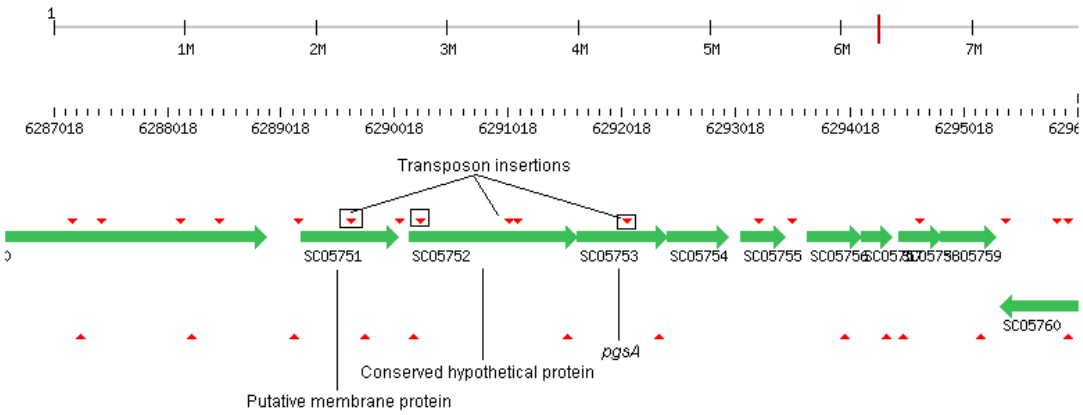


Figure. 4a Genome of *S.coelicolor* showing the organization of genes *SCO5751*- annotated as a putative membrane protein containing a possible hydrophobic membrane spanning region, *SCO5752*- conserved hypothetical protein and *SCO5753*- as a probable phosphatidylglycerolphosphate synthase on the StrepDB, The Streptomyces annotation server. The red arrows show the presence and direction of transposon insertion.

SCO5753 is a putative phosphatidylglycerophosphate synthase. It is made up of 263 amino acids and is an integral membrane protein and is a conserved motif, like CDP-diacylglycerol and glycerol-3-phosphate 3-phosphatidyltransferase. Based on homology studies, *SCO5753* is thought to encode phosphatidylglycerolphosphate synthase. *SCO5751* and *SCO5752* are annotated as a putative membrane protein and conserved hypothetical protein probably containing a hydrophobic membrane spanning region.

3.2.2 Basic Local Alignment Search Tool (BLAST)

BLAST allows comparing the nucleotide and amino acid sequences against NCBI or UNIPROT databases. In this case Uniprot Blast search was conducted. The analysis of probable pgsA of *S.coelicolor* from the Streptomcyes database server showed a fair sequence homology and similarities with the *pgsA* of *M.tuberculosis, B.subtilis and E.coli.* However, it was seen that *S.coelicolor* and *M.tuberculosis* shared 59% homology being actinomycetes and about 36-37% similar to *B.subtilis and E.coli.*

Function	Microorganism	E Value	Identity
phosphatidylglycerophosphate synthase	*Mycobacterium tuberculosis*	$2e^{-52}$	59%
phosphatidylglycerophosphate synthase	*Bacillus subtilis*	$5e^{-20}$	36%
phosphatidylglycerophosphate synthase	*Escherichia coli*	$7e^{-15}$	37%

Table. 6a Similarity search of pgsA (protein) among *M.tuberculosis, B.subtilis and E.coli* from BLAST analysis (sequences taken from NCBI)

Accession no	Protein	Microorganism	E Value	Identity
O86813	**phosphatidylglycerophosphate synthase**	*Streptomyces coelicolor*	$1e^{-143}$	100%
DE6Q38	**phosphatidylglycerophosphate synthase**	*Streptomyces lividans*	$1e^{-143}$	100%
O33288	**CDP-diacylglycerol-3-phosphate-3-phosphatidyltransferase**	*Mycobacterium tuberculosis*	$2e^{-52}$	59%

Table. 6b Similarity search of pgsA (protein) among *S.coelicolor*, *S.lividans* and *M.tuberculosis* from BLAST analysis (sequences taken from PDB)

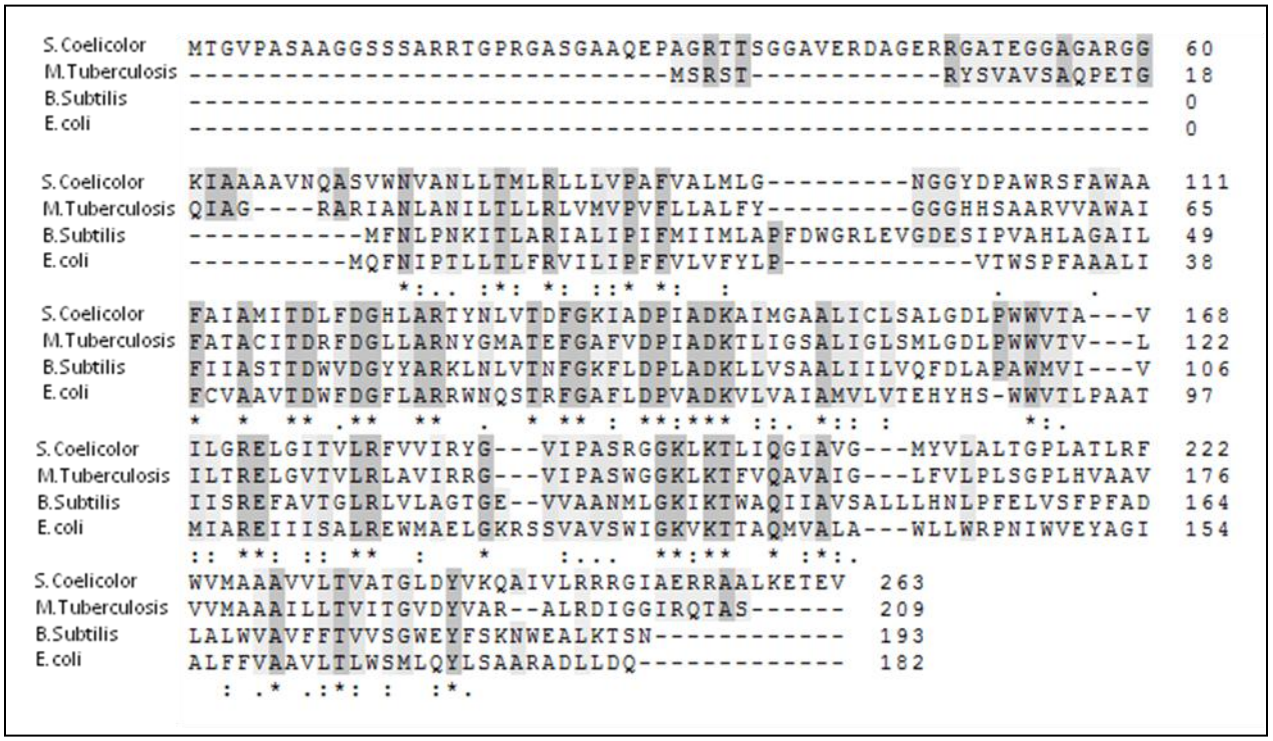


Figure. 4b Clustal alignment showing the homology between *SCO5753* and the other characterized *pgsA* from *M.tuberculosis, B.subtilis* and *E.coli.*

Few amino acids are found to be conserved in all the strains, where as majority of the similarities are observed between *S.coelicolor* and *M.tuberculosis* (highlighted in dark and light gray). From this search, the similarity in *pgsA* in actinobacteria is significant.

The genome of *S.coelicolor* A3(2) is available on StrepDB Streptomyces genome annotation server, and displays the location of Tn*5062* insertions (Figure. 3)

The gene operon in *S.coelicolor* shows the gene *SCO5751*, *SCO5752* and *SCO5753* being a part of a single operon and could be essential for the synthesis of phosphatidylglycerol which in turn would provide the substrate for the formation of CL.

Mutant libraries were constructed by using transposon. The library of *S.coelicolor* was constructed and the orientation of transposon can also be identified using the software – Transposon Express (Herron *et al.* 2004).

3.3 Cosmid verification

3.3.1 Transformation of *E.coli* JM109 with the cosmids

A strain of *E.coli*, JM109 cells were made competent using the chemical method. They were then transformed with the cosmids SC7C72.FO5, SC7C72.BO6 and SC7C72.BO7 (Table. 3). As mentioned in Table. 1, cosmids SC7C7.FO5, SC7C7.BO6 and SC7C7.BO7 have transposon insertions in the genes *SCO5751*, *SCO5752* and *SCO5753* (putative *pgsA*) respectively.

The transformants were carefully picked and inoculated overnight in LB broth with kanamycin and apramycin. The supercos contains two antibiotic markers kanamycin and ampicillin. The transposon Tn*5062* contains an apramycin resistance marker. Cosmids were isolated by alkaline lysis and restricition digestion was set up at 37º C using the enzymes *EcoRI*, *BamHI* and *XhoI*. (Figure. 5D)

Cosmid maps:

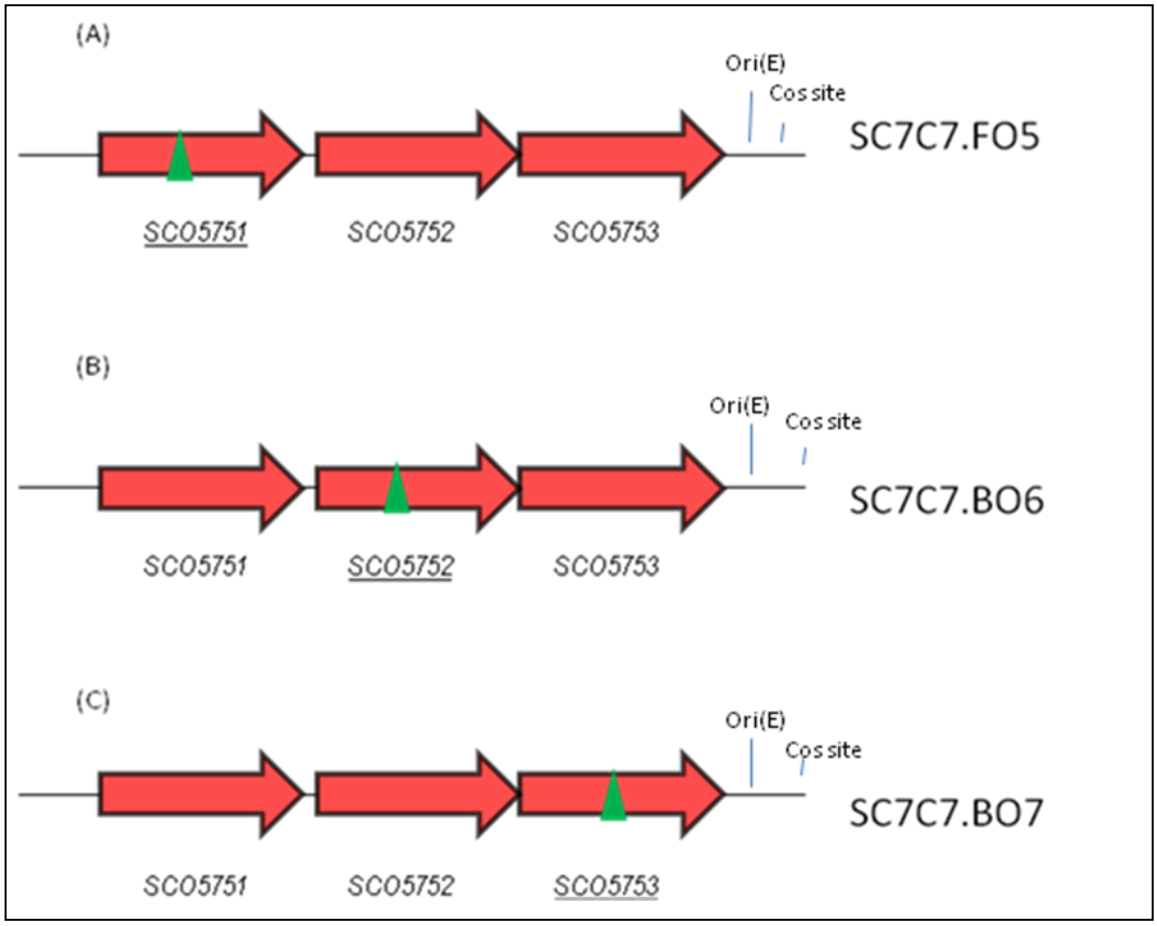


Figure. 5 (A) Tn*5062* insertion in *SCO5751* in the cosmid SC7C72.FO5
(B) Tn*5062* insertion in *SCO5752* in the cosmid SC7C72.BO6
(C) Tn*5062* insertion in *SCO5753* in the cosmid SC7C72.BO7

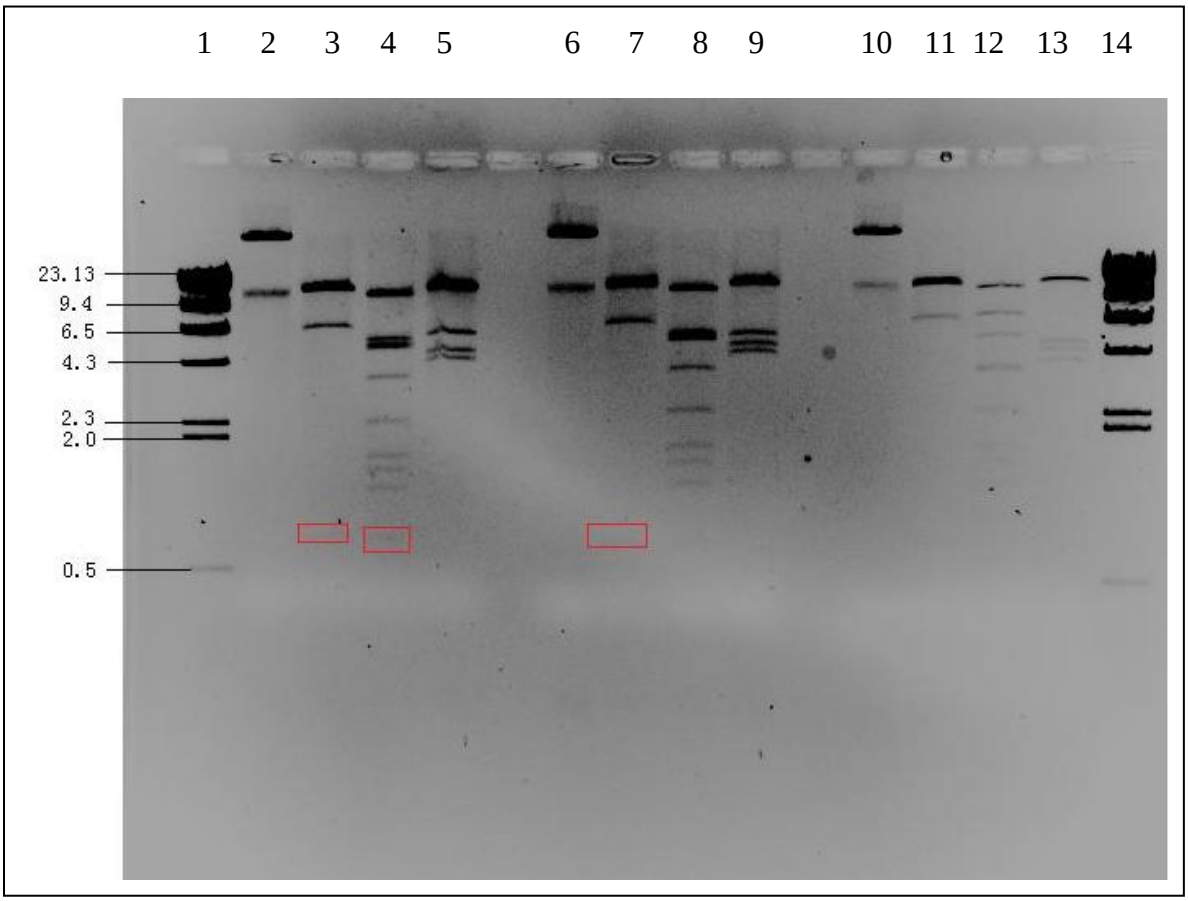


Figure. 5D Verification of cosmids SC7C7.FO5, SC7C7.BO6 & SC7C7.BO7 by restriction analysis using *EcoRI, BamHI* and *XhoI*. Lane 1 - λ *HindIII* Marker, lane 2- uncut SC7C7.FO5, lane 3 - *EcoRI*, lane 4- *BamHI*, lane 5- *XhoI*, lane 6 - uncut SC7C7.BO6, lane 7 – *EcoRI*, lane 8- *BamHI*, lane 9 - *XhoI*, lane 10 – uncut SC7C7.BO7, lane 11- *EcoRI*, lane 12 - *BamHI*, lane 13 - *XhoI*, lane 14 - λ *HindIII* Marker. The fragments of particular sizes were visualized as shown in Figure. 5D. *E.coli* ET12567/pUZ8002 was then transformed with the verified mutagenized cosmids. (Fragment sizes are predicted in Table A in appendix)

3.3.2 Allelic replacement with disrupted copies of *SCO5751, SCO5752* and *SCO5753*

ET12567/pUZ8002 was used to de-methylate the DNA (plasmid/cosmid) and then introduce it into *S.coelicolor*. The reason being the methyl specific restriction modification in *Streptomcyes* which does not accept or accommodate the introduction of methylated DNA. Competent cells of ET12567/pUZ8002 were prepared and then transformed with the cosmids and the transformants were plated on LB agar with kanamycin, apramycin, tetracycline and chloramphenicol. Once the transformants were obtained, colonies were carefully picked and conjugated with M145. After 72 hours of incubation, conjugants were observed on all the plates. At this point of time, single crossovers or double crossovers cannot be determined as the cosmid has both apramycin and kanamycin resistance in it. Hence the colonies were picked up and patched on SFM agar plates with apramycin and kanamycin separately for selection.

3.4 Mechanism of crossovers:

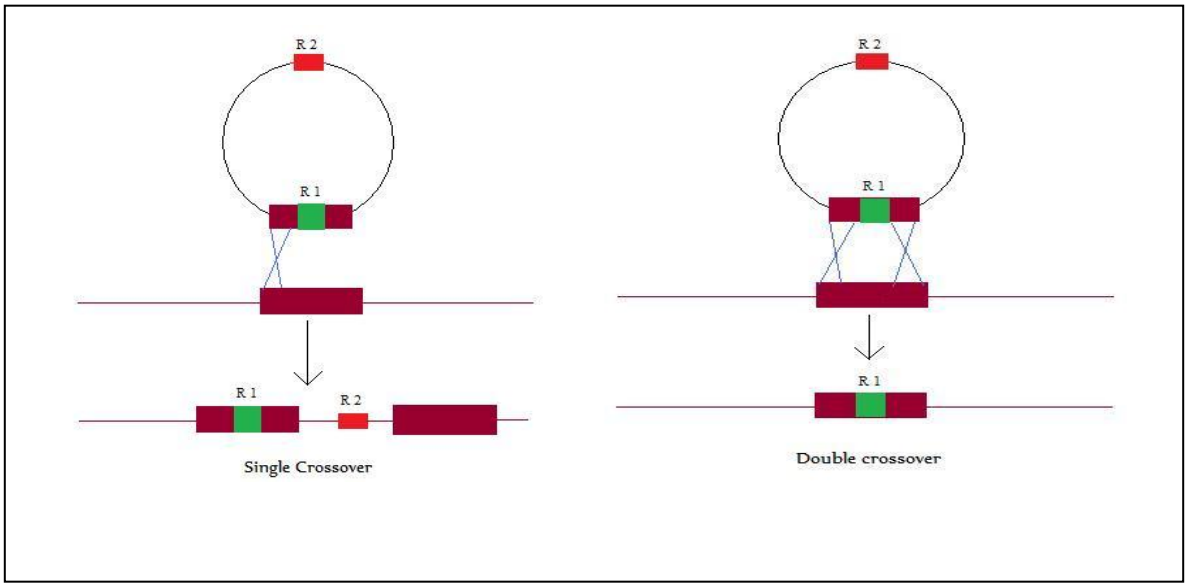


Figure. 6 showing the mechanism of Single and Double crossover

Single crossovers are obtained when there is a single homologus recombination and the entire plasmid/cosmid is integrated into the host genome, thereby providing resistance to both the antibiotic markers present on the cosmid. In this case, the single crossover samples will be resistant to kanamycin and apramycin. Whereas, in double crossovers, there will be a two homologus recombination event and the result will be the resistance offered to one antiobiotic, which is present in the gene as a result of crossover i.e. apramycin in this case.

3.4.1 Result of of allelic replacement

The colonies were patched on selective plates and incubated for 72 hours. Following which, the following result obtained are as follows

The cosmid overall had two antibiotic resistance markers, kanamycin and apramycin (in the Tn*5062* transposon) that can be used for selection in *Streptomyces.*

Once the conjugation was set up and the conjugants were plated over the media, after 24 hours of incubation at 30°C, the plates were overlaid with apramycin and naladixic acid. Apramycin would contribute for selection and naladixic acid would ward off the *E. coli.* ET12567 strains from growing further and let the *Streptomyces* spores germinate in the next 48 hours. The result of conjugation was the occurrence of colonies on all the plates containing appropriate antibiotics. Colonies from all of them were picked up and patched separately on apramycin and kanamycin plates along with proper controls (a double crossover strain VJ101 (unpublished data) and wt M145 strain). Colonies were observed on the plate containing apramycin and not kanamycin in the case of the cosmid SC7C7.FO5, indicating that they were double crossovers. The result of double crossover was a homologus recombination among the genes. However, colonies were observed on both apramycin and kanamycin plates containing the cosmids SC7C7.BO6 and SC7C7.BO7, indicating the presence of single crossovers. 1000 colonies were patched and there were no double crossovers in either of the cosmids. This result suggested that the genes *SCO5752* and *SCO5753* could be essential.

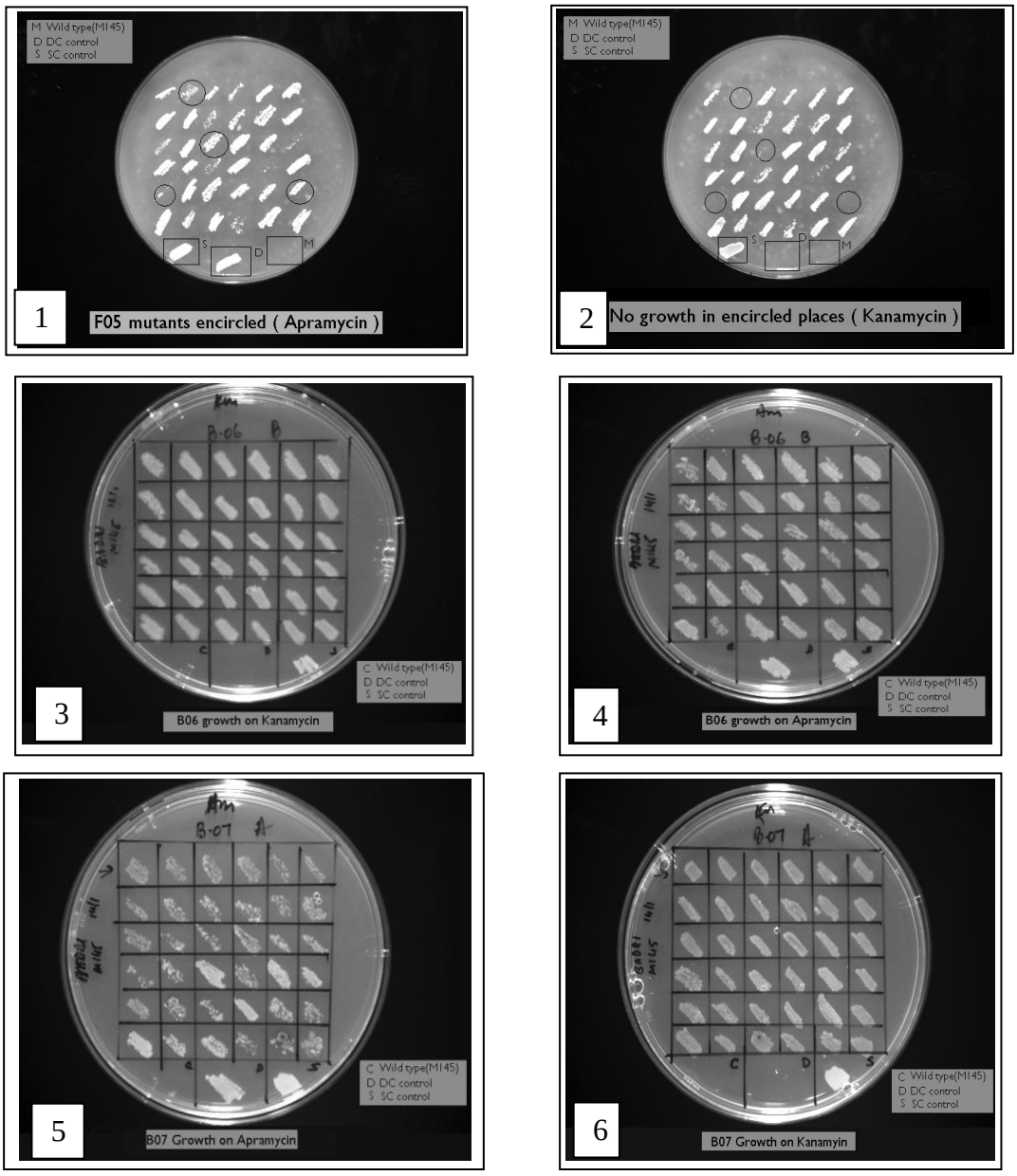


Figure. 7(1, 2) Integration of SC7C7.FO5 with *S.coelicolor* M145 giving rise to the mutant strain BR005 (double crossovers).Encircled places showing the presence of double crossovers (apr^R) growing on apramycin and not on kanamycin. Single crossover, double crossover and M145 (Table 1) spore samples were also patched to check the antibiotic throughout the experiment. Figure (3, 4) & 7(5, 6) Integration of SC7C7.BO6 & SC7C7.BO7 into *S.coelicolor* M145 resulting in the mutant BR006 and BR007. Single Crossovers were observed growing on both apramycin and kanamycin. Single crossover, double crossover and M145 spore samples were also patched to check the antibiotic throughout the experiment.

3.4.2 Numerical analysis of single and double crossovers

The result from the above table tells that 4 out of 36 colonies of SC7C7.FO5 conjugants patched on apramycin and kanamycin were double crossovers. Whereas, in the case of SC7C7.BO6 and SC7C7.BO7, there were no double crossovers in 1000 colonies that were patched on apramycin and kanamycin plates. This result was promising and indicated that the two genes could be important and essential for the growth and survival of the organism.

COSMID	SINGLE CROSSOVER	DOUBLE CROSSOVER	PLATE RESULT (no. of double crossover colonies)
SC7C7.FO5	Yes	Yes	4/36
SC7C7.BO6	Yes	No	0/1000
SC7C7.BO7	Yes	No	0/1000

Table. 7 Statistical analysis of crossovers

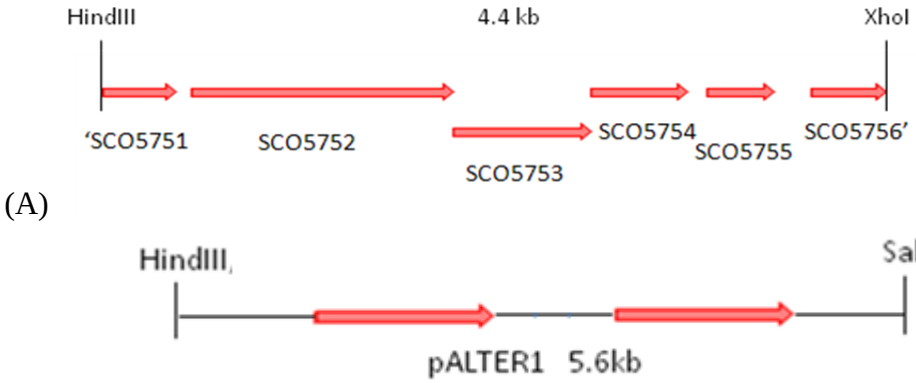
3.5 Cloning of the genes *SCO5752* and *SCO5753*

As double crossovers were obtained from SC7C7.FO5, it was concluded that the *SCO5751* is not essential. Double crossovers were not obtained in genes *SCO5752* and *SCO5753* indicating that the genes are essential. In order to obtain double crossovers with *SCO5752* and *SCO5753*, *S.coelicolor* was complemented with the functional copy of the genes before the introduction of the transposed genes. To achieve this, SC7C7.FO5 was digested with *HindIII* and *XhoI* and the resulting 4.4kb fragment was ligated with the cloning vector pALTER1 at *HindIII* and *SalI* sties (Figure. 8). The ligation mix was introduced into *E.coli* JM109 (Table. 2). The plasmid DNA was isolated on the following day and confirmed by restriction digestion and agarose gel electrophoresis.

The plasmid when digested with *HindIII* and *EcoRI* resulted in a band of 10.1kb, and when digested together gave two bands of sizes 5.7kb and 4.4kb.

SacI was used here as it has just one restriction site on the plasmid, but 4 sites within the inserted gene. Hence, 5 bands were seen. *PvuII* and *AflIII* were used as they have sites on the vector as well as the insert, which resulted in bands of sizes 4.1kb, 2.6kb, 2.2kb, 0.9kb, 0.4kb and 5.2kb and 4.9kb respectively.

3.5.1 Construction of pLR101



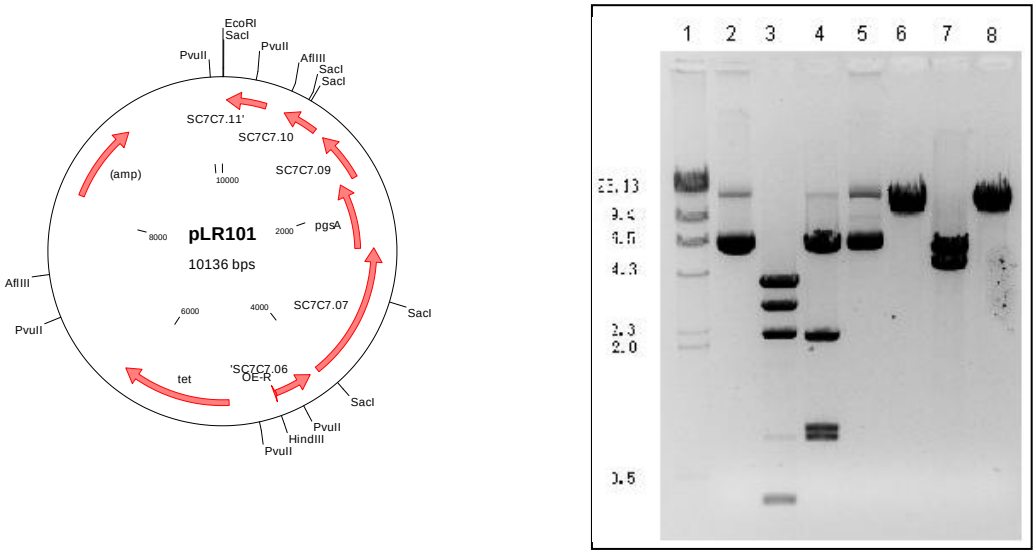


Figure. 8. (A). Fragment obtained from SC7C7.FO5 by using *HindIII* and *XhoI*. (B). Map of pALTER1 indicating the restriction sites and MCS. This is digested with *HindIII* and *SalI*. (C). Ligation of the 4.4kb fragment into pALTER1 resulting in pLR101. (D). Agarose gel ectrophoresis image of pLR101. Lane 1- λ *HindIII* Marker, lane 2 - uncut pLR101, lane 3 - *PvuII*, lane 4 - *SacI*, lane 5 - *AflIII*, lane 6 - *HindIII*, lane 7 - *HindIII* & *EcoRI*, lane 8 – *EcoRI* . The size of the bands obtained correspond to the values predicted in Table B (appendix) however, a 24bp band upon digestion with SacI was not visible on gel above.

3.5.2 Construction of pLR102

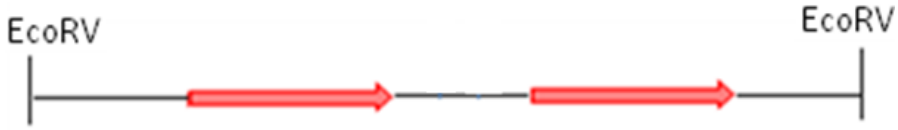
Once the 4.4kb fragment was cloned using the cloning vector pALTER1, the next step was to move it into the integrating vector pMS82 (Table 3).

pMS82 is a vector system used to deliver the DNA of interest into *S.coelicolor* by integration onto the genome. pMS82 is engineered to integrate with the *Strepromcyes* genome at a strong conserved phage attachment site Φ BT1 and also confers hygromycin resistance (Gregory *et al.* 2003).

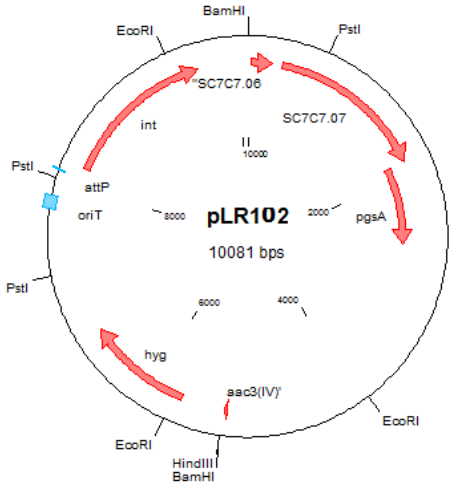
pALTER1 was cut with *PvuII* and a fragment of size 3.9kb (Figure. 9) was extracted from the gel using the Qiagen Gel Extraction Kit. This fragment was ligated into pMS82 which was cut with *EcoRV*. This was a blunt ended ligation and had to be carried out at 4C. Restriction digestion of the plasmid with *HindIII* and *EcoRI*, resulted in 10.1kb & 4.6kb, 3.5kb and 1.7kb bands respectively. *PstI* digested at a point in the insert resulting in a 5.4kb, 3.1kb and a 700bp band.

Figure. 9 Subcloning of pLR101

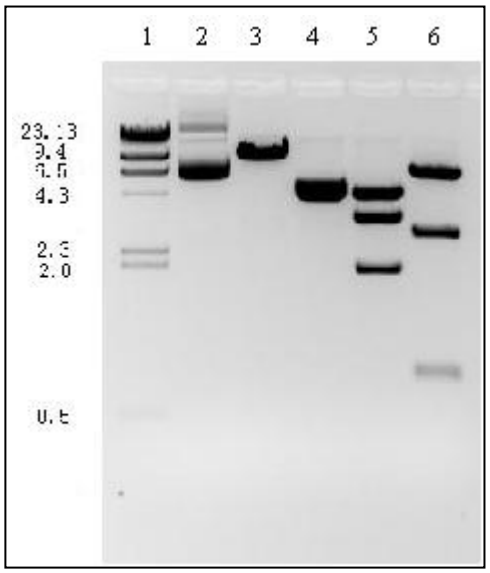
(A)



(B) pMS82 6.1kb



(C)



(D)

Figure. 9(A) 3.9kb fragment consisting of restriction *SCO5752 and SCO5753*(*pgsA*) obtained when pLR101 was digested with *PvuII*. (B) Map of pMs82 showing different restriction sites and MCS. (C) Integration of 3.9kb fragment from pLR101 into pMS82 resulting in pLR102. (D) Agarose gel ectrophoresis image of pLR102. Lane 1 - λ *HindIII* Marker, lane 2 - uncut pLR102, lane 3 - *HindIII*, lane 4 - *BamHI*, lane 5 – *EcoRI*, lane 6 – *PstI*. The bands obtained agree with the band sized predicted in Table C (Appendix)

3.6 Complementation of genes *SCO5752* and *SCO5753*

Once the genes were cloned in the integrating vector pMS82, they were ready for complementation.

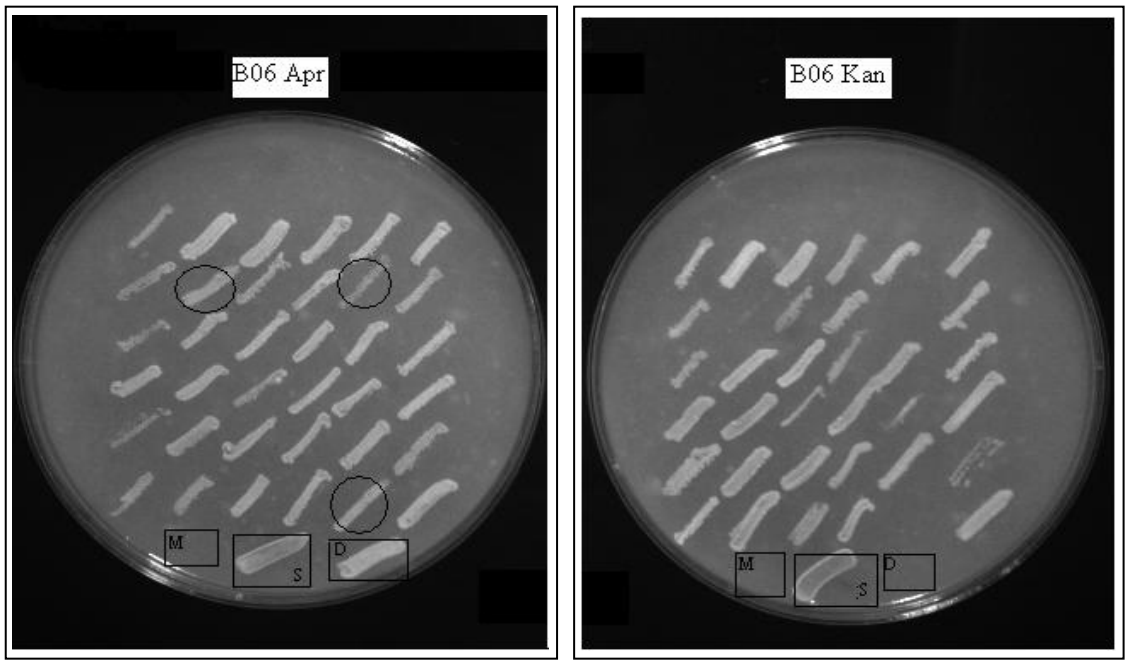
E.coli ET12567/pUZ8002 cells were transformed with pLR102 and plated on hygromycin, tetracycline, and chloramphenicol. The resulting colonies were picked and scaled up for conjugation with spores of *S.coelicolor* M145. After 72 hours, colonies were picked up and streaked on SFM plates to make spore suspensions of BRMS001.

Wild type and single crossover controls were also maintained which did not grow in the presence of hygromycin but the conditional double crossover strain did.

BRMS001 was the new strain of *S.coelicolor* M145 which had pLR102 integrated in its genome.

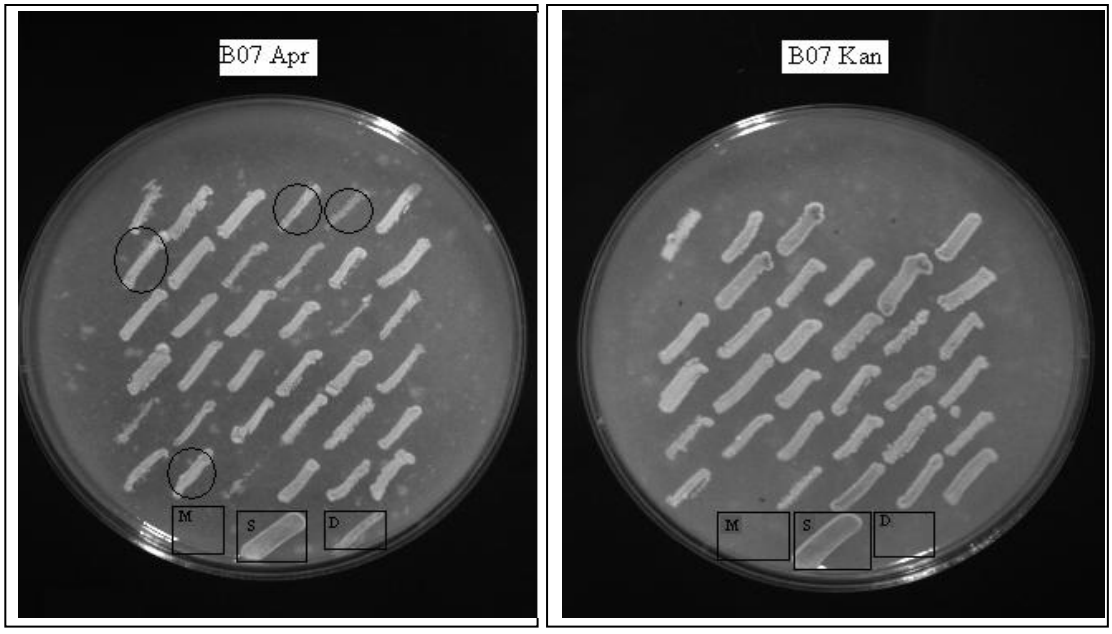
E.coli ET12567/pUZ8002 cells were transformed with the cosmid SC7C7.BO6 and SC7C7.BO7 separately and the same procedures were carried out during transformation and conjugation with BRMS001. Appropriate antibiotics were overlaid the following day and after 72 hours, the transconjugants were patched on apramycin-hygromycin and kanamycin-hygromycin plates along with the wild type, a single crossover and a double crossover control on each plate. Double crossovers should grow on apramycin-hygromycin combination and not on kanamycin-hygromycin combination.

3.6.1 Result of complementation studies:



(A) (B)

Figure. 10 (A) showing the three circled colonies growing on apramycin – hygromucin but not on the kanamycin-hygromycin plate (B) BR006. M, D and S indicate M145, Double crossover and Single crossover samples which were used as controls.



(A) (B)

Figure. 11 (A) showing the four circled colonies growing on apramycin - hygromycin but not on the kanamycin-hygromycin plate (B) BRD007.M, D and S indicate M145, Double crossover and Single crossover samples which were used as controls.

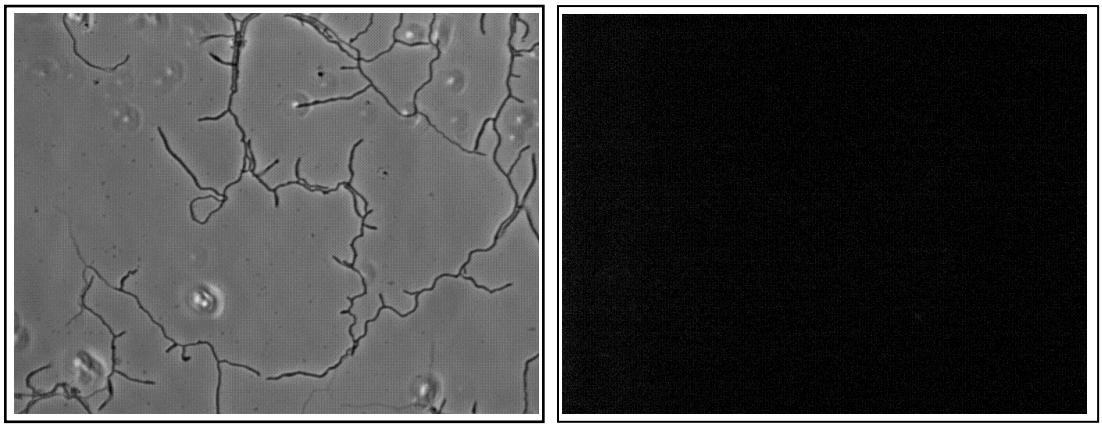
From the performed experiments, it was seen that *SCO5752* and *SCO5753* could not be disrupted with Tn*5062* transposed genes. Disruptions were obtained only by complementing the organism with the functional copy of the gene with the help of an integrating vector pMS82, i.e. pLR102.

Introduction of transposed cosmids into the pLR102 integrated *S.coelicolor* A3(2) M145 spores (BRMS102) resulted in a few colonies that were potentially double crossovers.

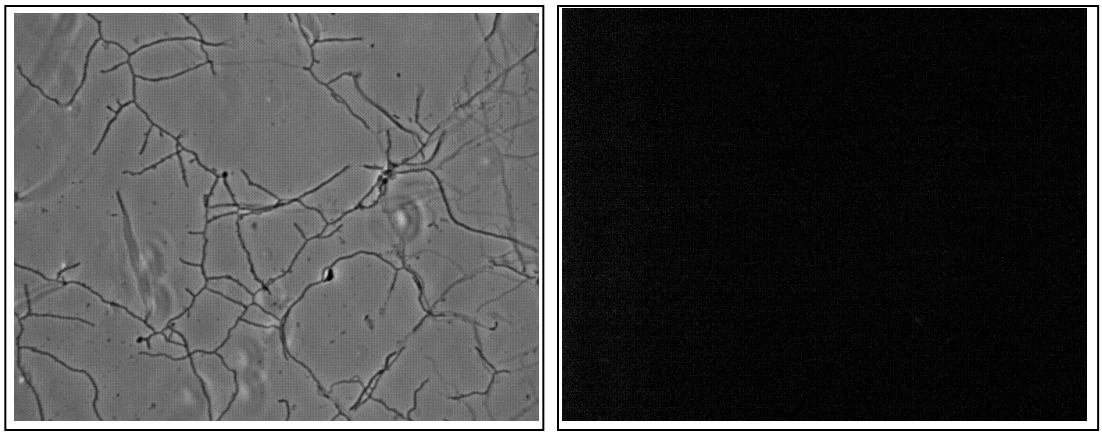
3.7 Morphological studies of the mutants by microscopy

The strains constructed during the course of this project i.e. wild type, single crossover, double crossover and conditional double crossover samples were observed using a Nikon TE200S inverted microscope at 100X objective. Captured images were processed using IPlabs 3.7 image processing software. All the strains were visualized under bright field, in order to check for any morphological changes that could have occurred after mutations and with blue light of wavelength (460-500) nm, to check for any fluorescence if emitted by the transcriptionally fused eGFP in Tn*5062*.

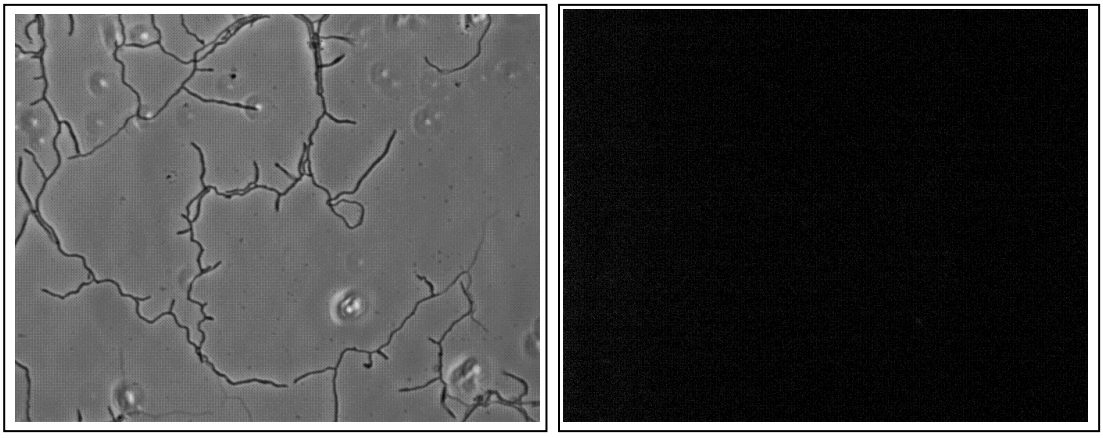
Bright Field FITC filter



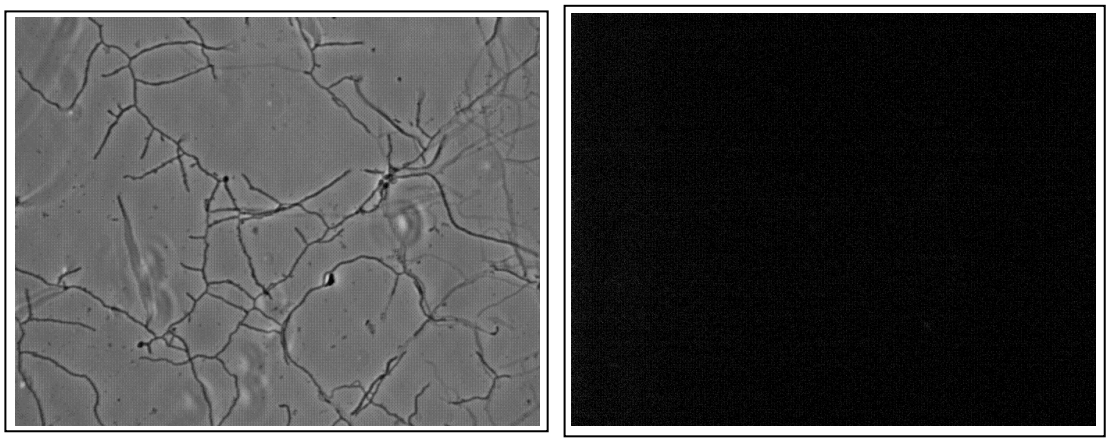
Wild type M145



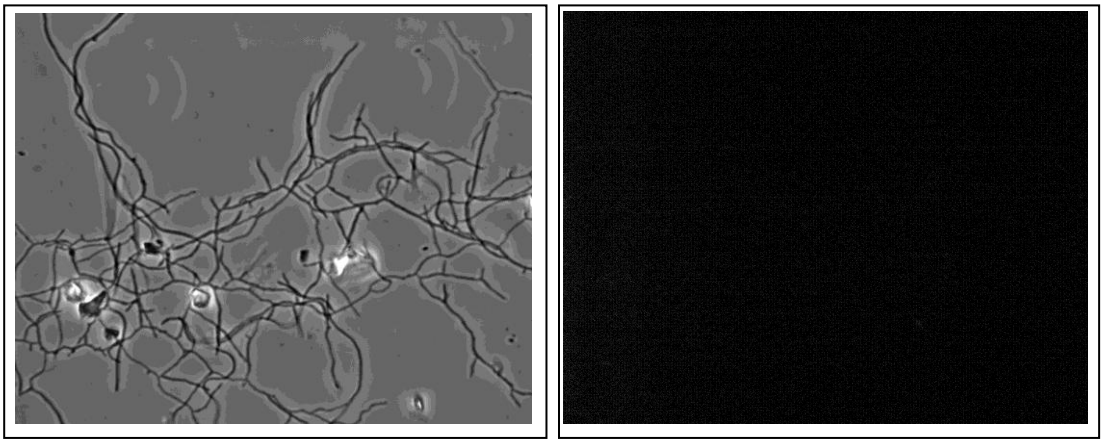
BR005



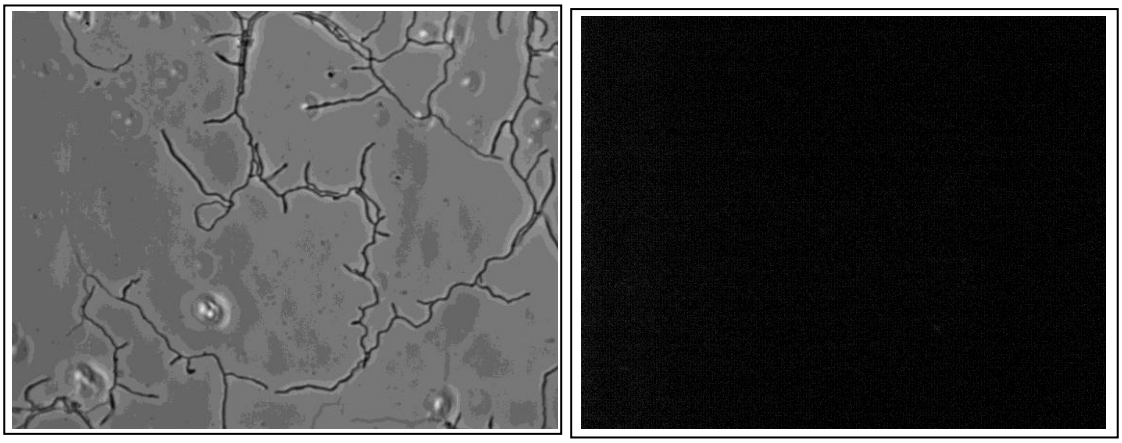
BR006 (single crossover)



BR007 (single crossover)



BRD006 (double crossover)



BRD007 (double crossover)

Figure 12. Analysis of mutant strains by phase contrast and fluorescent microscopy. Images of the left hand side are the bright field images and on the right hand side are the ones taken using a FITC filter and by emission of green light.

Results from preliminary microscopic studies conducted by scanning a few slides showed that there was no apparent morphological change observed in any of the mutants. GFP expression was not seen as e*gfp* were transcriptionally fused in Tn*5062* and would be transcribed at very low levels which makes it difficult to detect them.

K113 was the strain used as a control to check the expression of eGFP. FtsZ, an essential cell division protein was tagged with eGFP which is involved in the formation of ftsZ rings during cell division.

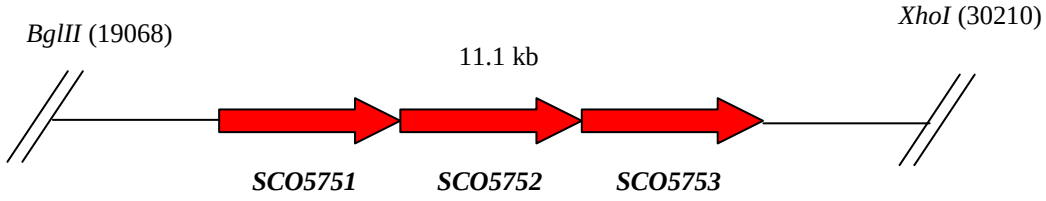
Figure 13. *eGFP* expression observed in *S.coelicolor* K113 (This is how *eGPF* should express when translationally fused) (Image taken from Jyothikumar *et al*. 2008)

3.7 Southern Hybridization analysis for allelic replacement

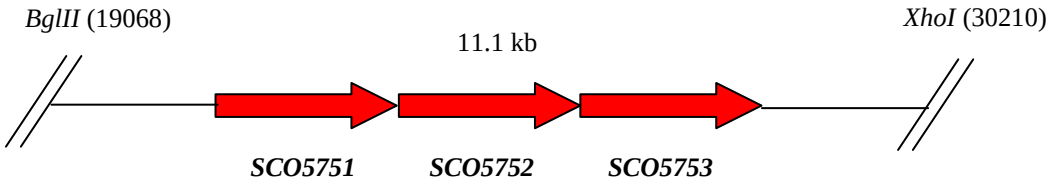
Southern blotting was performed to verify the presence of the desired gene integrated into the chromosome of *S.coelicolor*. A probe was constructed using the plasmid pLR101 (Table. 3)

Genomic DNA form all the strains, plasmids and the probe constructed in this study were digested with *BglII* and *XhoI* and ran an agarose gel. pLR101 was used here as a whole vector probe which contains the gene *pgsA* along with the upstream genes, *SCO5751* and *SCO5752*. Specific gene probes and apramycin probes were constructed earlier, but due to the poor visualization of the bands on the membrane pLR101 was chosen as the probe.

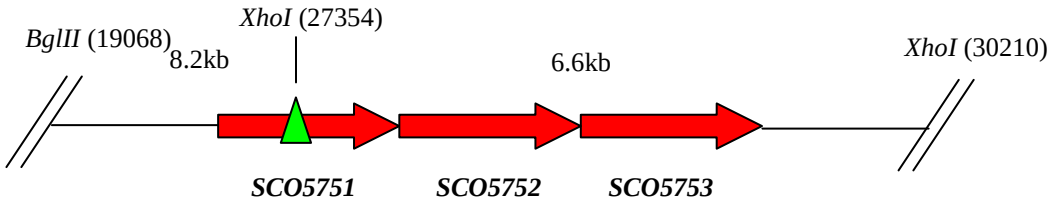
WT M145



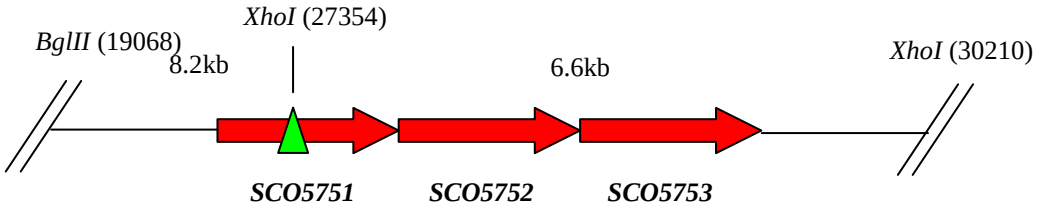
St7C7



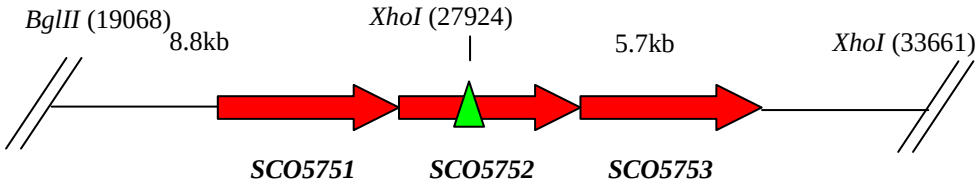
SC7C7.FO5



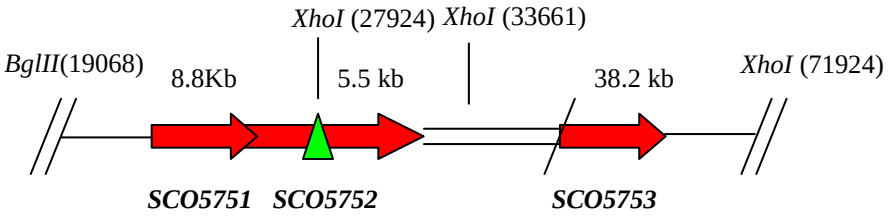
BR005



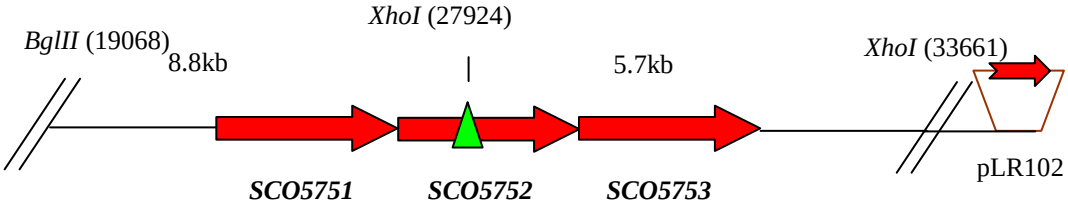
SC7C7.BO6



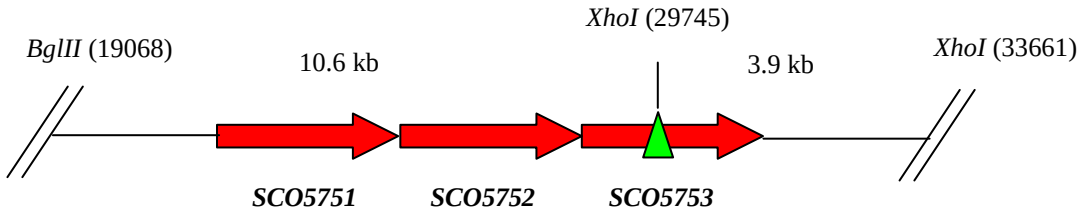
BR006



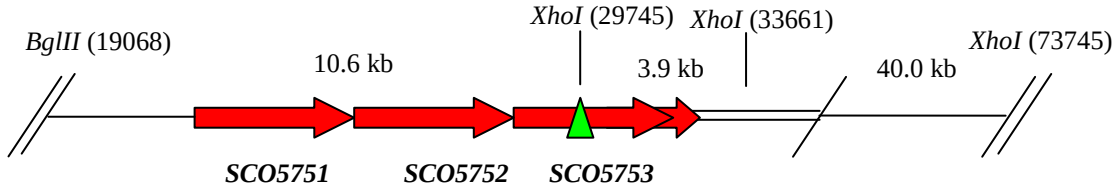
BRD006



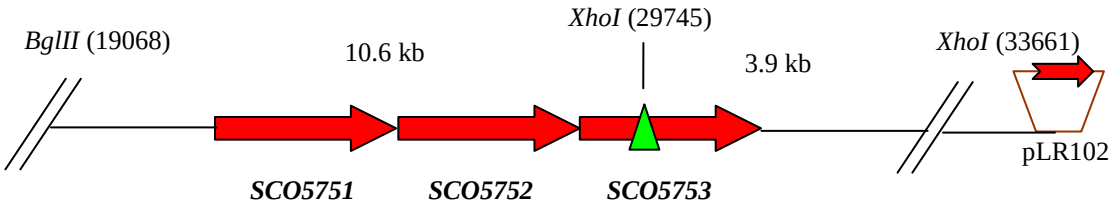
SC7C7.BO7



BR007



BRD007



BRMS102

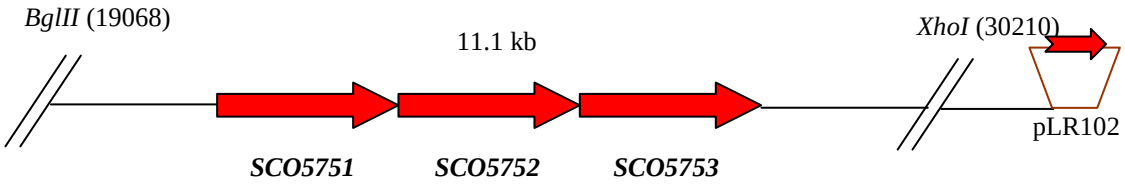


Figure. 14 Restriction site map of the integrated structures which resulted from single and double crossover events and digested with *BglII* and *XhoI*.

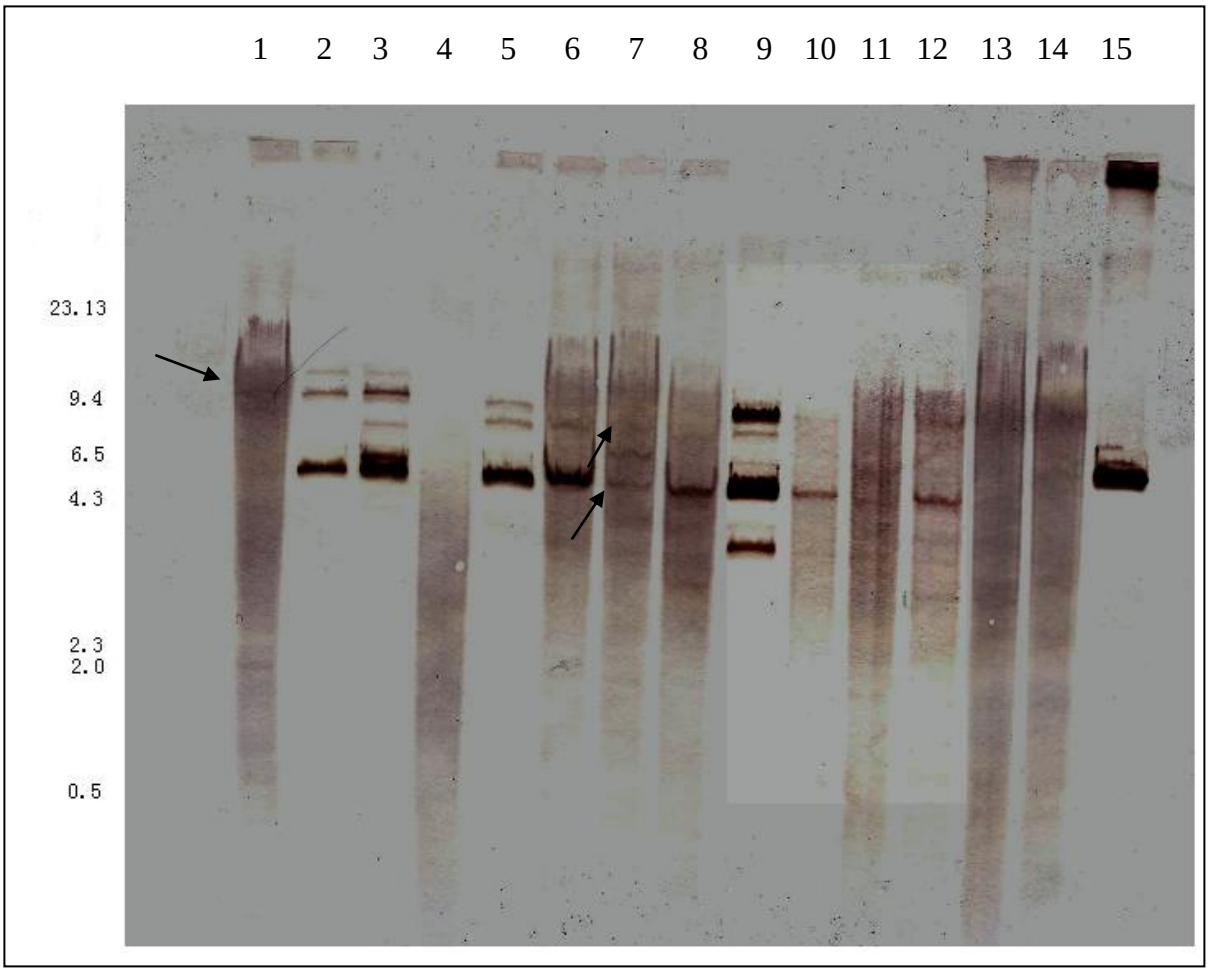


Figure 15. Different *Streptomyces* strains were cleaved with *XhoI* and *BglII* and probed with purified pLR101. Lane1 - Genomic DNA of *S.coelicolor* M145(arrow indicating 11.1kb), lane 2 - St7C7, Lane3 - SC7C7.FO5, lane 4 - BR005 (double crossover), lane 6 - BR006 (single crossover), lane7 – BRD006 (conditional double crossover, arrows indicating 8.8kb & 5.7 kb), lane 8 - BRMS06 (single crossover : pMS82), lane9 - SC7C7.BO7, lane10 - BR007(single crossover), lane11 - BRD007 (conditional double crossover), lane 12 BRMS07 (single crossover : pMS82, lane13 - WTMS82, lane14 - BRMS102, lane15 - pLR102 probed with pLR101.

Size markers in kilobases.

In lanes 4, 6, 8, 10, 11, 12 and 13 the band shift was seen. In lanes 2, 3, 5 and 9 multiple bands were observed. They did not match the predicted banding pattern. The Southern blot failed to confirm the mutants. We speculate that this could be a case of the poor quality of DNA used for the blot or may be a result of protein contamination we suspect partial digestion of DNA. Though the Southern blot was performed carefully, the bands could not be visualized clearly on the membrane.

CHAPTER 4

DISCUSSION & FUTURE WORK

4.1 Discussion and future work.

Phospholipids play an important role in the dynamic organization of bacterial and mitochondrial membranes (Mileykovskaya & Dowhan, 2009). Phospholipids such as phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylionositol (PI), phosphatidylglycerol (PG) and cardiolipin (CL) are abundant in bacteria.

In *B.subtilis*, it was reported that anionic lipid spirals form along septal region, where the divisome recruits cell division genes (Barak *et al.* 2008). Anionic phospholipid CL is found to perform multiple activities within the cell. In prokaryotes, CL is synthesized by condensation reaction between two molecules of PG. CL interact with supercomplex (e.g. cyt *c* and cyt *c* oxidase) found to be involved in oxidative phosphorylation and possibly play a role in ETC. It is observed that CL accumulates high curvature regions of the cristae and supports the synthesis of ATP. CL deficient systems did not show the high curvature like the former (Mileykovskaya & Dowhan. 2009). Recent evidences have shown that CL along with cytochrome *c* play an important role in apoptosis (Kalanxhi & Wallace, 2007). A structural rearrangement is observed when cyt *c* interacts with CL. This increases the peroxidise activity, CL gets oxidized and cyt *c* is released from the mitochondria and thus regulating apoptosis (Kagan *et al.* 2007).

However, eukaryotes and prokaryotes are thought to carry out different pathways to convert CDP-diacylglycerol to CL. In prokaryotes, phosphatidylglycerolphosphate is the intermediate, where as in eukaryotes; CDP-DAG is the donor of the phosphatidyl group which is transferred to a molecule of PG to form CL.

pgsA is found to encode phosphatidylglycerolphosphate synthase, which mediates the formation of PG. Similarity searches and based on the homology, it is speculated that *SCO5753* of *S.coelicolor* could potentially code for *pgsA*.

This study gives us a glimpse of the preliminary work done to decipher the importance of *pgsA* in *S.coelicolor*. The investigation of mutational study on the gene *SCO5753* (*pgsA*) showed that it is essential in *Streptomyces*. Using the transposed gene in the cosmid SC7C7.BO7 it was not possible to carry out the allelic transfers.

In order to disrupt the chromosomal copy of the gene, initially we have to complement the strain with an extra copy of gene *SCO5753* on a vector which has ΦBT integration site: pMS82 (Gergory *et al.* 2003). However, creating a conditional mutant suggests that this gene *SCO5753* has an essential role and is important for the cell to proliferate.

From the SCO database curated at JIC, we see that *SCO5752*, *SCO5753* and *SCO5754* belong to a single operon and may share a common promoter. This could be speculated as *SCO5752 SCO5753 and SCO5754* could be transcriptionally fused. This suggests that there may be a polar effect taking place because of their arrangement in the operon and this may be the reason for not being able to achieve a complete homologus recombination (double crossover) within the chromosome of *S.coelicolor*. However, double crossovers were obtained in *SCO5751*, indicating it is independent of the operon downstream. For genetic complementation, we decided to use the complete region of *SCO5752* and *SCO5753* with few hundred bases upstream (Figure. 5). Once the second copy of the gene was restored within the chromosome; it was easy to achieve the insertional conditional mutant. This showed that the gene was essential.

The mutant strains generated during the course of work could not be confirmed successfully by Southern blotting, as the band sized did not match the predicted scheme. The reason for this could be the poor quality of extracted DNA, or may be due to partial digestion which could have taken place due to protein contamination.

This project also focuses on generating the mutant of *pgsA* and localize within the hyphae using the transcriptional eGFP fusion that is present within Tn*5062* (Bishop *et al.* 2004). However, after microscopic studies it was not possible to localize pgsA within the hyphae. The reason could be that it is transcriptionally fused and there was not enough expression within hyphae. Null mutant of *pgsA* could not be achieved within the time frame of this project; also there is a good amount of work that can be carried out in future. These genes could be important and essential in other systems in the synthesis of PG, CL and also in maintaining the membrane stability and electrostatic poteintial.

Depletion strain of pgsA will give us more insight into the function of this gene under inducible promoter and by altering the levels of PG within the cell; we can document the growth characteristic in *S. coelicolor*. This is done by trans complementation of *pgsA* (Ali *et al.* 2002, Reynolds *et al.*, 2002). Overexpression studies can be carried out which would tell us the necessity of pgsA during the growth of the organism.

Subsequently, a translational fusion of this gene with *eGFP* and over expressing it could help us in studying the co-localization within the hyphae during growth, and also during cell division, branching and sporulation with the help of time lapse microscopy (Jyothikumar *et al.* 2008). Simultaneously DNA could also be stained to visualize the possible interaction with phospholipids.

CL is found to interact with numerous proteins such as DnaA, FtsW, FtsZ and possibly MinD (Barak *et al.* 2008). Interaction of various cell division proteins with CL can also be studied by differential staining and by fluorescent microscopy. They can also be studied in real time. RNA isolation at different time points and performing a RT-PCR would give us an idea of expression levels of pgsA during the growth cycle of *Streptomyces*. Osmolarity studies can be conducted with salt and sugar gradient plates and the growth can be observed in relation to the osmolyte concentration. This would support the theory of regulation of phospholoipds and cell development in relation to external osmolarity concentrations (Ali *et al.* 2002). Phospholipids can be isolated by extraction in chloroform, separated on a high performance silica gel thin layer chromatography plates with chloroform, methanol and acetic acid. CL could also be isolated and studied by this method. ESI-MS can be conducted on the pure form of PG and CL injected with acetonitrile and formic acid mix and the phospholipids can be characterized by determining their weights.

Furthermore, interactions between CL and cyt *c* can be studied by titrating CL against cyt *c* with the help of UV-Vis spectroscopy. The association and dissociation of this complex can be studied by Circular Dichroism and also by NMR. Not only in developmental biology, CL has its important place in biochemistry, drug development and attracts new research on its medical importance. Phosphotidylinositol (PI) is found to be an essential phospholipid in *Mycobacterium* (Jackson *et al.* 2000). It is also found that the cells cannot survive without PI. In conjunction with the results presented here, this suggests that PG and CL like phospholipids could have essential roles in polar growth and morphogenesis in many actinomycetes, not only those with hyphal growth.

Finally, this work shows that pgsA is essential in *S.coelicolor*. Furthermore, it was reported that cls is also essential in *S.coelicolor* (unpublished data). Genetic characterization and biochemical experiments for the proteins can be carried out along with the further experiments discussed above.

CHAPTER 5

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WORLD WIDE WEB ADDRESSES:

http://streptomyces.org.uk/

http://streptomyces.org.uk/cgi-bin/sco/dc2.pl?pri_tag=CDS&s_no=13241&start=6286623&end=6296623&width=900

http://blast.ncbi.nlm.nih.gov/Blast.cgi

http://www.ncbi.nlm.nih.gov/pubmed

Appendix:

Cosmids / Enzymes	SC7C7.F05	SC7C7.B06	SC7C7.B07
	Expected band sized in kb		
EcoRI	19019	19619	21440
	18052	17452	15631
	6793	6793	6793
	782	782	782
BamHI	16408	16408	16408
	5997	5918	7739
	5553	5553	5553
	5318	5297	3670
	3670	3670	3576
	2366	2366	2366
	1763	1763	1763
	1513	1513	1513
	1271	1271	1271
	787	787	787
XhoI	28943	29543	31364
	6337	5737	4927
	4927	4927	4439
	4439	4439	3916

Table A. List of enzymes used to verify the cosmids SC7C7.F05, SC7C7.B06 & SC7C7.B08 and the predicted sizes of the bands

Enzymes	Expected band sizes in kb for pLR101				
PvuII	3973	3047	2254	436	426
SacI	6240	2150	897	835	24
AflIII	6725	3411			
HindIII	10136				
EcoRI	10136				

Table B. list of enzymes used to verify the plasmid pLR101 and the predicted band sizes

Enzymes	Expected band sizes in kb for pLR102		
HindIII	10081		
BamHI	5268	4813	
EcoRI	4685	3651	1745
PstI	6561	2159	802

Table C. list of enzymes used to verify the plasmid pLR102 and the predicted band sizes