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How to cite:

Akpe San Roman, Sandra (2006) *Characterisation of the esx1 locus of Streptomyces coelicolor A3 (2)..* thesis, Swansea University.
http://cronfa.swan.ac.uk/Record/cronfa42872

CHARACTERISATION OF THE *esx1* LOCUS OF *STREPTOMYCES COELICOLOR* A3 (2)

BY

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SEPTEMBER 2006

A THESIS SUBMITTED IN CANDIDATURE OF THE DEGREE OF
DOCTOR OF PHILOSOPHY OF THE UNIVERSITY OF SWANSEA

ProQuest Number: 10821262

ProQuest 10821262

ACKNOWLEDGMENTS

ABSTRACT

The *esxl* locus is important because it is associated with virulence in pathogenic mycobacterium including *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Mycobacterium marinum*, *Mycobacterium bovis*, *Corynebacterium diphtheria* etc. The presence of an orthologous locus in *Streptomyces coelicolor*, a Gram-positive non-pathogenic filamentous bacterium, is an exciting finding of comparative genomics (Pallen 2002). Clues to the function of the genes were elucidated from analyzing the genes in *S. coelicolor* by *in vitro* transposon mutagenesis. Reverse transcriptase experiments indicated that *esxB* (*SCO5724*) and *esxA* (*SCO5725*) are co-transcribed and encoded by a single mRNA species. That the products of these two genes can interact was confirmed by two-hybrid analysis using translational fusions of the two proteins. Phenotypic studies of the knockout mutants revealed a delay in spore chain maturation. In addition, the presence of *bldB* divergently transcribed away from the *esxAB* operon and evidence of *bldB* dependent regulation of the operon suggest that the genes may have a role in morphogenesis. Finally, data is presented indicating that EsxAB proteins are secreted via a dedicated system encoded by other genes of the *esxl* locus.

ABBREVIATIONS

ADP   Adenosine Diphosphate

APS   Ammonium persulphate

ATP   Adenosine Triphosphate

bp   Base pair(s)

C   Cytosine

CIP   Calf Intestinal Alkaline Phosphatase

DTT   Dithiothreitol

DNA   Deoxyribose nucleic acid

dNTP   Deoxynucleoside Triphosphate

EDTA   Ethylene Diamine Tetraacetic acid

g   Gram(s)

G   Guanine

h   Hour(s)

IPTG   Isopropyl-thiogalactoside

kb   Kilobase pair(s)

kDa   Kilodalton(s)

L   Litre

Mb   Megabase(s)

min   Minute(s)

ORF   Open Reading Frame

PAGE   Polyacrylamide gel electrophoresis

PCR   Polymerase Chain Reaction

PEG   Polyethylene Glycol

RBS   Ribosome Binding Site

RNA   Ribonucleic acid

r.p.m.   Revolutions per minute

sec	Second(s)
SDS	Sodium Dodecyl Sulphate
SSC	Standard Saline Citrate
TBE	Tris Borate EDTA
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
TES	N-tris(hydroxymethyl)-2-aminoethanesulphonic acid
Tris	Tris(hydroxymethyl)amino-methane
μ	Micro
UV	Ultraviolet light
V	Volt(s)
W	Watt(s)
X-gal	5-bromo-chloro-3-indolyl-β-D-galactoside

CONTENTS

CHAPTER I

INTRODUCTION

1.1 Classification.

1.1.1 Actinomycetes.

Actinomycetes are Gram-positive bacteria with a high guanine (G) plus cytosine(C) genome composition of 55 *per cent* or over. Organisms belonging to this genus are capable of unicellular or multi-cellular life styles. Actinomycetales are vigorous secondary metabolite producers responsible for the production of approximately 9000 different compounds. Remarkably the majority of the compounds are generated by the genus *Streptomyces* (Watve *et al.* 2001).

1.1.2 Streptomyces.

Initially regarded as intermediate between bacteria and fungi, the mystery of the genus attracted the pioneer in the field Dr David Hopwood. It was at Strangeways Laboratory that with the help of electron microscopy, Dr Hopwood established that Streptomycetes lack a nuclear membrane and thus belong to the bacteria Superkingdom (Hopwood 1999). Streptomycetes have a large linear chromosome with a high guanine (G) plus cytosine(C) DNA content of *circa* 70 *per cent*. Streptomycetes are multicellular organisms with a developmentally complex life cycle that consist of three differentiation stages: they emerge as filamentous vegetative growth, followed by the formation of aerial hyphae and the differentiation of these hyphae into dispersive unigenomic spores as reviewed by (Paradkar *et al.* 2003). These organisms have been studied for over 50 years because they produce valuable bioactive compounds. Streptomycetes are described as environmental organisms, with a core of essential genes and two arms containing conditionally adaptive genes that are not essential but that allow the bacteria to survive by metabolizing a great variety of substrates (Bentley *et al.* 2002). Streptomycetes were taxonomically classified by

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(Williams *et al.* 1983) using cytochemistry and morphological analysis. They ordered as many as 325 species and grouped them in two clusters: 19 major and 40 minor clusters.

1.1.3   Model organism *Streptomyces coelicolor* A3(2)

Embraced by Dr David Hopwood in 1954 because of its blue colour, this species was obtained from a collection of spores owned by Dr D. M. Webley that originally came from The Royal College of Technology in Glasgow (now Strathclyde University). *Streptomyces coelicolor* A3(2) produces four antibiotics: pigmented undecylprodigiosin (Red, mycelium pigment) and actinorhodin (pH dependent, secreted blue pigment), methylenomycin and calcium dependent antibiotic (Hopwood 1999). The genome sequence of *Streptomyces coelicolor* strain M145, a prototrophic derivative of *Streptomyces coelicolor* A3(2) lacking its two plasmids SCP1 and SCP2 was recently published (Bentley *et al.* 2002).

1.2   Ecology of Streptomycetes.

1.2.1   Natural habitat

Streptomycetes inhabit the oligotrophic soil environment. *Streptomyces* are saprophytes that perform a fundamental role in the degradation of biopolymers chitin and ligno-cellulose (Hodgson 2000). The soil is a constantly ever-changing environment where a large variety of organisms co-exist. Their genome composition reflects the unsteady nature of their surroundings acquiring a vast array of metabolic pathways to metabolize different types of substrates. They are readily isolated from soil, where they mostly exist as spores that can survive in soil for long periods. Their ability to form a mycelium network provides them with an advantage to grow on drier

soils. Germination is density dependent within spores of the same species. Studies confirmed that Streptomycetes colonise the plant rhizosphere and the marine sediment (Jensen *et al.* 1991). Although saprophytes, some species are also pathogenic having a choice of hosts. *S. scabies* and *S. acidiscabies* colonise the surface of potato tubers causing great economical loss. *S. somaliensis* is the causative agent of actinomycetoma in animals and humans (Proctor 1966; Bukhalid and Loria 1997; Bramwell *et al.* 1998; Toth *et al.* 2001).

1.3   Morphological differentiation of *Streptomyces coelicolor.*

Growth in *Streptomycetes* is divided in three stages each having a characteristic differentiated cell type. A spore sensing appropriate conditions germinates. Usually spores produce one or two germ tube/s that start to grow into vegetative mycelia. Vegetative growth expands into the agar or soil by tip extension of the cell wall forming a radial pattern of expansion (Flardh *et al.* 1999). The substrate hyphae contain multiple copies of the chromosome. When a septum is positioned on the substrate hyphae this hyphae stops growing until the next lateral branch emerges. Lateral branching allows the filaments to grow rapidly at a quasiexponential rate of development. As growth progresses the innermost parts of the colony accumulate excessive amounts of storage compounds: glycogen, lipids and polyphosphate. Then their growth stalls. It seems their growth stops because of the lack of one type of nutrient although on the other hand, other nutrients exist in excess (Chater 1993). These parts of the colony grow slowly. As the colony ages, the second type of tissue begin to develop and secondary metabolites start to be produced. Aerial mycelia emerge from the substrate mycelia into the air breaking the surface tension. These filaments form a new mesh of growth into the air, growing and branching

homogeneously throughout the whole colony posing the question of how nutrients are channeled from the bottom to the top of the colony (Miguelez *et al.* 1994). This growth is believed to be supported by a carefully regulated programmed cell death by which the substrate mycelia undergo a progressive disintegration. The cell wall remains intact to support the colony growth and perhaps the channeling of nutrients to the upper parts of the colony. Later, the aerial hyphae septate into chains of uninucleoid compartments, that eventually mature into dark-grey exospores. Aerial hyphae which do not differentiate into spores degenerate and die (Hardisson and Manzanal 1976; Hodgson 1992; Kelemen *et al.* 1998; Miguelez *et al.* 1999)

Figure 1.1 Lifecycle of *Streptomyces*.
Multicellularity starts from a unigenomic spore chain upon sensing ideal conditions for germination, one or two germ tubes are formed. They grow in a quasi-exponential rate to give rise to a mycelial terrestrial network that burrows into the food matrix. Signal accumulation stalls the primary growth to start a new phase of mycelial aerial growth into the air. About this time production of secondary metabolites takes place. Aerial hyphae curl and mature into the air then the apical compartment septates into genomic spore chains. The hydrophobic properties of the exospores allow their dispersal into the ecosystem allowing the opportunity to colonise new soils. The identity of the key genes involved at different stages of development is indicated. Modified from (Kieser 2000)

6
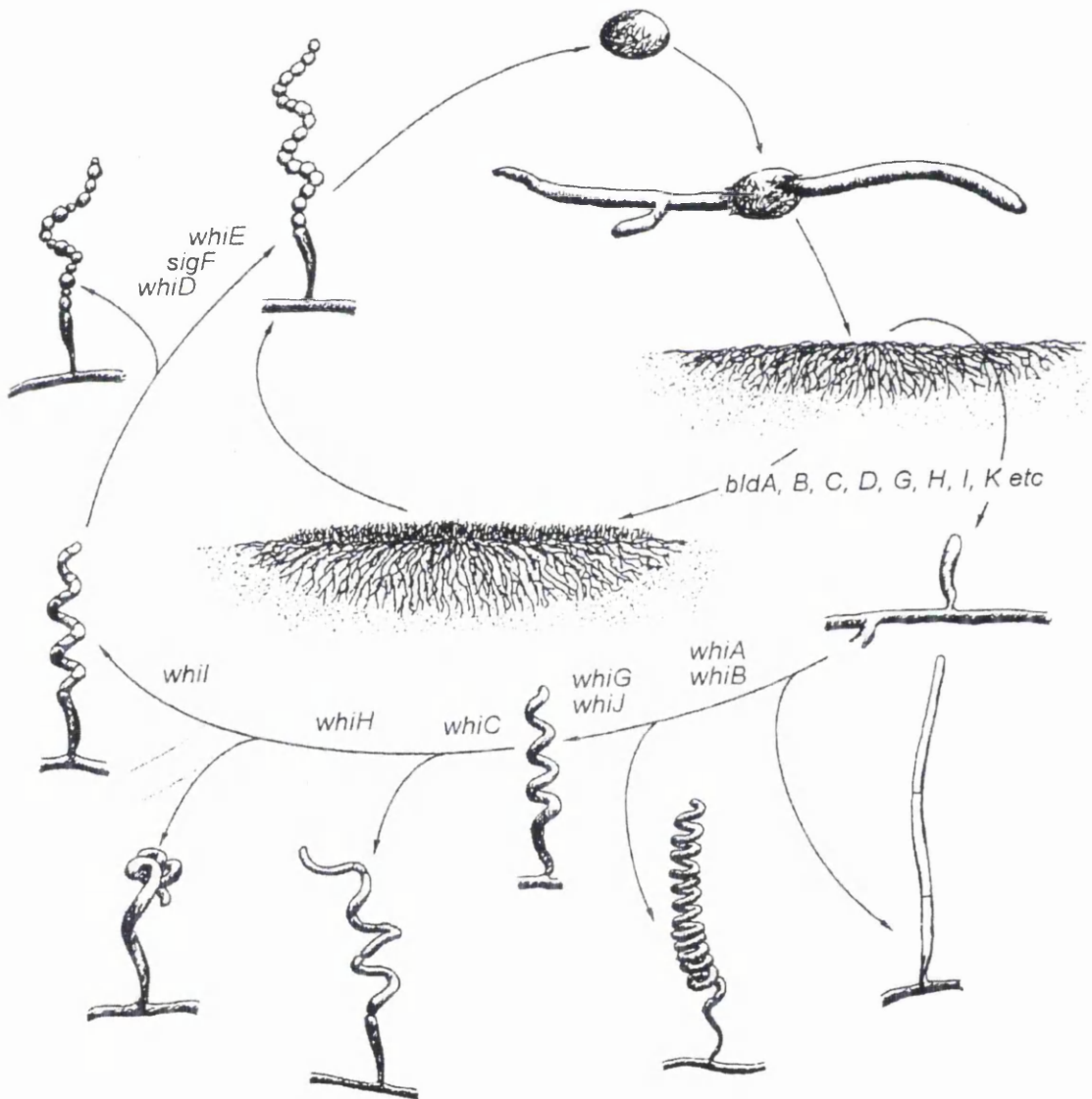
Figure 1.2 Electron micrographs of the distinct tissues of *Streptomyces coelicolor*.

From left to right, substrate mycelia, aerial hyphae and mature hyphae differenciated into spore chains. (Claessen *et al.* 2006)
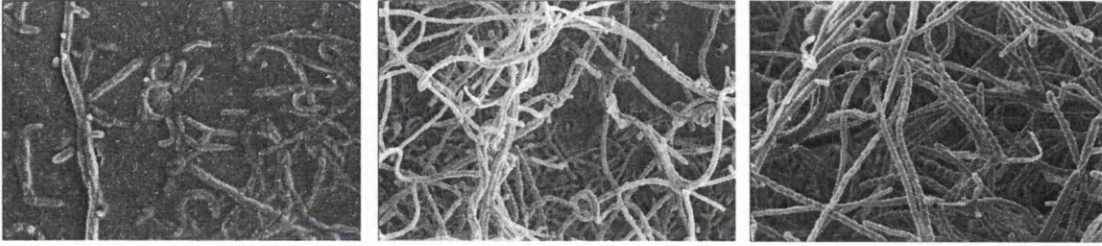
1.4   Molecular genetics of development in *Streptomyces coelicolor.*

Three main regulatory cascades control *Streptomyces* developmental cycle. The *bld* cascade, the *whi* cascade and the *sky* cascade. Pathways are interconnected permitting the colony to progress, sensing the external conditions and to precisely react to them by controlling gene expression. The *bld* cascade, (bald, unable to form aerial hyphae) ensures correct aerial hyphae formation by sensing the environment and signalling the onset of aerial formation. The *bld* mutants are blocked prior to aerial hyphae formation and are frequently defective in secondary metabolism because aerial growth and antibiotic production seem to be globally regulated. The *whi* cascade regulates sporulation, the *whi* mutants characterised because of a distinctive light-grey colour. They erect aerial mycelia which are unable to mature into spores, thus lack the distinctive polyketide grey pigment found on a mature spore surface. The *sky* cascade controls the expression of the chaplin and rodling genes (Claessen *et al.* 2006).

The *sky* pathway functions to signal between the *bld* and *whi* cascades. The *sky* pathway operates after aerial hyphae are emerging from the aqueous environment, it signals that the aerial hyphae have left the soil and it dictates the expression of the rodlin and chaplin genes. The *bld* cascade manages the initiation of aerial development controlling the steps that lead to the initiation of aerial growth.

1.4.1   *bld* mutants

The range of genes resulting in a bald phenotype (regulatory proteins, primary metabolism genes and genes encoding stress response sigma factors) indicates that the *bld* cascade allows the bacteria to assimilate hyphal density and energy status with external conditions (nutrient availability and stress). Each *bld* gene is involved directly or indirectly in the synthesis, perception of, or response to different

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extracellular signalling molecules. Each signal induces the production of the next signal leading to the production of SapB. SapB is a small diffusible peptide that is believed to coat the aerial hyphae before they break the surface tension to emerge into the air. The *ramS* gene encodes the SapB peptide of 42 amino acids that is post-translationally modified to adopt a lantibiotic like structure. The Ram cluster is composed of *ramS, ramR, ramAB* and *ramC. ramR* is a response regulator that activates *ramSCAB* operon transcription by binding to the promoter region. *ramR* is not transcribed in *bldA, bldB, bldH* and *bldD,* strongly suggesting that its transcription is triggered by the *bld* cascade. The C-terminus of *RamC* resembles that of lantibiotic modification enzymes. *RamAB* is an ABC transporter (Kodani *et al.* 2004; Kodani *et al.* 2005).

Since the first study of the *bld* genes by (Merrick 1976), many *bld* genes have been characterised, every discovery provides a new clue for this complex regulatory puzzle. The *bldA* gene encodes a leucyl-tRNA essential for the translation of the infrequent codon UUA (TTA-DNA). The genome sequence of *S. coelicolor* (Bentley *et al.* 2002) reveals that only 1.85 per cent of the genes in the 8.7 Mb genome contain a TTA codon. The TTA-containing genes include regulators of morphological differentiation and antibiotic production hence the pleiotropic phenotype of the *bldA* mutants. Among them are pathway specific activators for the biosynthesis of actinorhodin and undecylprodigiosin production ActIIORF4 and RedZ, respectively. As a consequence of that a *bldA* mutant is unable to produce either of the pigmented antibiotics (Lawlor *et al.* 1987). Fernandez-Moreno *et al.* 1991 demonstrated that the regulatory gene is the sole and direct mean by which *bldA* exerts its effects on actinorhodin production. Thus, it appears that there is specific *bldA* governed regulators for secondary

metabolism and development. A TTA codon is also present in *bldH* is a homologue *of adpA* of *S. griseous,* that controls aerial mycelium formation (Takano *et al.* 2003). A regulatory target of *bldA* has been identified. *bldH* target encodes an extracellular protease inhibitor that could delay the digestion of the primary biomass until the colony is ready to progress to the aerial development phase (Chater 2006). *bldB* encodes a 99 amino acid peptide with a helix-turn-helix DNA binding motif. Structural and molecular analysis revealed that the product of the *bldB* gene has DNA binding activity. Eccleston *et al.* 2002 reported that BldB protein forms a higher order complex possibly a dimmer. The region comprising amino acids 20 to 78 are important for this interaction. Pope *et al.* 1998 indicated that tyrosine residue at position 21 was essential for BldB function; tyrosine residues may assist in protein – protein interactions or be phosphorylated. The *bldB* pleiotropic phenotype of the mutant indicates its role as a global regulator of morphogenesis, catabolite control and antibiotic production. Analysis of *bldB* expression using a reporter gene indicates that *bldB* is temporally regulated and controls its own activity. *bldB* is the most controversial of the *bld* genes not fitting into the hierarchical cascade proposed by Willey *et al.* 1993. Unlike most *bld* mutants its phenotype is not restored by growing in poor carbon sources, or by exogenous addition of SapB. *bldC* encodes a small DNA binding protein homologous to the DNA binding domains of the MerB family of transcriptional activators (Hunt *et al.* 2005). The gene is required for aerial mycelia formation, although the phenotype is partially rescued in rich media. *bldC* mutants have some defects in antibiotic production as they are unable to produce actinorhodin. The *bldC* gene is constitutively express and its transcription is independent of other *bld* genes. The MerB family members function as homodimers, they consist of an N-terminal highly conserved DNA binding domain and a specific C-terminal effector

recognition motif. Transcription activation is triggered by effector binding. *bldD* encodes a small DNA binding protein that represses developmental genes during vegetative growth. BldD regulates three targets: the developmental sigma factor genes, *whiG* and *bldN*, and the putative transcription factor *bdtA*. Electrophoretic mobility shift and DNase I footprinting assays were used to characterized and align the sequences bound by BldD suggesting AGTgA (n)m TCACc as a consensus BldD operator. (Elliot *et al.* 2001; Elliot *et al.* 2003) The *bldG* product is a putative anti-anti sigma factor. *bldG* is homologous to anti- anti sigma factors SpoIIAB and RsbW from *B. subtilis*. It has been suggested that BldG blocks aerial mycelium formation by preventing the activation of a sigma factor required for development. Such a sigma factor has not yet been found (Bignell *et al.* 2000). The *bldK* locus consists of five adjacent ORFs that specify homologues of the subunits of the oligopeptide-permease family of the ATP-binding cassette. *bldK* imports a signalling peptide of 655 Da encoded by the *bldJ* locus (Nodwell *et al.* 1996; Nodwell and Losick 1998). *bldM,* is a response regulator active in early and late stages of development (formally *whiK*) and was found to be an unconventional response regulator, with a putative helix – turn – helix DNA binding motif (Molle and Buttner 2000). *bldM* is grouped with *bldD* in the extracellular complementation cascade. *bldM*, like *bldN*, is transcribed at the time of aerial hyphae formation and during sporulation. *In vitro* transcription assays in conjunction with S1 mapping analysis confirmed that σ^{bldN} directs the transcription of *bldM. bldN* was initially defined as a *whi* mutant *(whiN)*. Nevertheless the construction of a null mutant gave rise to a *bld* phenotype. Transcription of *bldN* is dependent on both *bldG* and *bldH* and native transcription is initiated at the time of aerial hyphae formation. The protein has an unusual N-terminal extension which is

proteolytically processed at the time of aerial hyphae formation, possibly modulating

its activity (Bibb *et al.* 2000; Bibb and Buttner 2003).

Figure 1.3 Model of the hierarchical extracellular signalling complementation cascade in *S. coelicolor*.

Aerial hyphae development occurs as the culmination of *bld* genes signalling (Chater and Chandra 2006). Signal 1 consists of the uptake of the product of the *bldJ* locus by bldK, an oligopeptide permease this process activates the synthesis of *bldL/bldK* dependant signal 2. *BldA* influences development via *bldH* expression. Signal 3 triggers regulatory gene anti-anti sigma factor *bldG*, upon which signal 4 is dependant. *bldC* is a gene that influences bldD a DNA binding protein and bldM. Culminating in the production of morphogens that drives aerial mycelium formation. There are still factors that have been discovered that don't fit into the cascade as initiation of aerial mycelium formation is a complex signal transduction network.

13
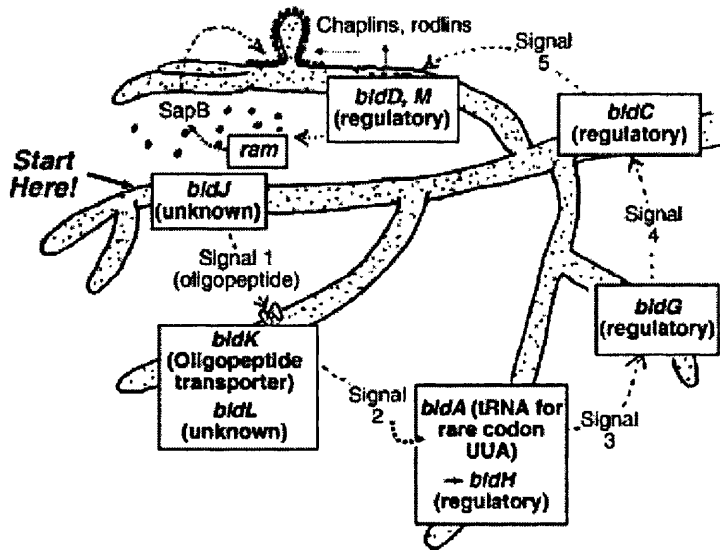
Table 1.1 Classical *bld* mutants.

Locus or loci	Gene product(s)	References
bldA	Leucyl tRNA required for the translation of unusual codon UUA	(Merrick 1976; Lawlor *et al.* 1987; Chater 2006)
bldB	DNA binding protein with a helix-turn-helix motif	(Pope *et al.* 1998; Eccleston *et al.* 2002)
bldC	Putative transcriptional activator of the MerR family	(Hunt *et al.* 2005)
bldD	DNA binding protein that binds to *whiG, bldN* and *sigH*	(Elliot *et al.* 2001; Elliot *et al.* 2003)
bldG	Putative anti- anti sigma factor	(Bignell *et al.* 2000)
bldJ	Putative first signal of *bld* cascade	(Nodwell and Losick 1998)
bldH	Homologue of *AdpA* contains an TTA codon target of BldA	(Takano *et al.* 2003; Chater 2006)
bldK	ABC family oligopeptide permease	(Nodwell *et al.* 1996; Nodwell and Losick 1998)
bldM	Response regulator	(Molle and Buttner 2000)
bldN	Extracytoplasmic function sigma factor	(Bibb *et al.* 2000; Bibb and Buttner 2003)

Table 1.2 Additional genes involved in aerial hyphae formation in *S. coelicolor*.
Modified from (Claessen *et al.* 2006).

Locus or loci	Gene product(s)	References
acoA	Aconitase citrate. Acidification of culture broth partially responsible for the phenotype	(Viollier *et al.* 2001)
amfC	Unknown; delayed and reduced aerial growth	(Yonekawa *et al.* 1999)
brgA	Unknown	(Shima *et al.* 1996)
catB	Developmentally regulated catalase. Mutants are hypersensitive to osmotic stress	(Cho *et al.* 2000)
chpA-H	Group of genes encoding the chaplin family of proteins. Structurally impaired in development when deleted.	(Claessen *et al.* 2003; Elliot *et al.* 2003; Claessen *et al.* 2004)
citA	Citrate synthase. Fails to form aerial hyphae due to reversible acidification of culture medium	(Viollier *et al.* 2001)
clpP1	Subunit of ATP-dependant protease	(de Crecy-Lagard *et al.* 1999)
cya	Adenylate cyclase. peptide permease. Fails to form aerial hyphae due to reversible acidification of culture medium	(Viollier *et al.* 2001)
osaAB	Response regulator	(Bishop *et al.* 2004)
ramCSABR	Gene cluster involved in synthesis, modification and putative transport of the SapB precursor RamR	(Ma and Kendall 1994; Nguyen *et al.* 2002; Kodani *et al.* 2004)
relA	(p)ppGpp synthetase, delay aerial growth	Sun 2001
rsuA	Anti-sigma factor	(Gehring *et al.* 2001)
sigB	RNA polymerase sigma factor (σ^B) controlling the expression of *catB*	(Cho *et al.* 2001)

1.4.2. The *whi* mutants.

By searching for colonies that lack the polyketide spore pigment many *whi* loci have been identify. Fourteen loci have been discovered and characterised so far. Transcription from the *whiEP1* and *whiEP2* promoters is developmentally regulated (Kelemen *et al.* 1996). Experimental evidence suggested that *whiEP2* is indirectly controlled by σF. Abolition of *whiEP2* results in a green spore phenotype, therefore *sigF* specifies the component of the spore pigment without directing its production. Early *whi* genes dictate the expression of the *whiEP1* and *whiEP2* promoters. The factors that trigger sporulation seem to be a complex cascade in which the lack of linearity of the path way is evident because of the many alleles that result from abolishment of individual *whi* genes. Two independent pathways lead to sporulation in *Streptomyces coelicolor*, one of the pathways is regulated by *whiG* and another is *whiG*-independent. *WhiG* is a sigma factor. Mutants of the gene cause the aerial hyphae to be infrequently septated and its over-expression results in premature sporulation. Sigma *whiG* specifically initiates the developmental program that leads to the production of spores. The importance of *bldD* acting as a repressor of σ^{whiG} provides the first link between the *bld* and the *whi* genes. When active, σ^{whiG} RNA polymerase holoenzyme transcribes early sporulation regulatory genes *whiH* and *whiI*. The products of these auto regulatory genes are proteins with a DNA binding domain and a signal sensing domain.

1.5 Physiology

1.5.1 Primary metabolism.

Research into primary metabolism in *Streptomyces* has been eclipsed by more extensive analysis of the secondary metabolites. Primary metabolism research

received more impetus with the completion of the genome sequence and the discovery of thousands of novel genes (Bentley *et al.* 2002). Findings correlate with the ecological habitat of the bacteria that feeds in the soil mainly on plant material. Plant-derived material is usually rich in carbon but deficient in phosphate and nitrogen and as a result considerable effort is invested by *Streptomyces* in multiple specific carbohydrate catabolic pathways (Hodgson 2000). In most *Streptomyces* the glycolysis pathway for glucose catabolism operates. The phosphoenolpyruvate: sugar phosphotransferase system (PTS) has also been reported in *Streptomyces lividans, S. coelicolor* and *S. griseofuscus* and is induced by fructose (Titgemeyer *et al.* 1995). Carbon catabolite repression (CCR) or the ability to metabolise a carbon source to prevent utilization of less efficient carbon sources have been documented in *Streptomyces. Streptomyces* CCR is very different from other bacterial systems, in that it lacks the cAMP catabolite repressor protein and has the ability to inhibit antibiotic production. Nitrogen uptake occurs via glutamate synthetase (GS) and glutamate (Fisher and Wray 1989). *Streptomyces* have two GSs: GSI that resembles that of prokaryotes and GSII similar to those found on eukaryotes. Amino acid catabolism pathways in *Streptomyces* are conserved with the exception of arginine. More than half of the pathways studied are constitutive reflecting the low abundance of amino acid in their environment. Amino acid production pathways are analogous to those of other bacteria. The only difference is the lack of regulation, with *Streptomyces* pathways expressed at basal levels.

Streptomyces antibioticus accumulates glycogen and trehalose in a characteristic way during growth on solid medium. Glycogen storage in the substrate mycelium takes place during development of the aerial mycelium. The concentration of nitrogen source in the culture medium influences the time at which accumulation starts as well

as the maximum levels of polysaccharide stored. Degradation of these glycogen reserves was observed near the beginning of sporulation. The onset of sporogenesis is always accompanied by a new accumulation of glycogen in sporulating hyphae (Brana *et al.* 1986).

1.5.2 Secondary metabolism.

Streptomyces have been a steady source of novel compounds for medical and pharmaceutical industries during the last fifty years since the discovery of streptomycin. The extensive repertoire of compounds that the genus produces includes: anti-parasitic, anti-viral and anti-cancer metabolites, immuno-suppresants, herbicides, insecticides and enzyme inhibitors. Many secondary metabolites produced by *Streptomyces* have a known biological function. Biotechnology advances have used *Streptomyces* as a host for the production of recombinant microbial products. *Streptomyces* were described by (Watve *et al.* 2001) as the largest antibiotic-producing genus in the microbial world discovered so far. Watve predicted that the number of antimicrobial compounds produce by this family is in the order of 100,000 compounds.

Normally, secondary metabolites begin to be produced at the end of the stationary phase of *Streptomyces* growth. Substrate mycelia expansion reaches an end and aerial mycelia begin to grow. Some hypotheses suggest that the role of secondary metabolites is to protect the nutrients of the disintegrating substrate mycelia. Other propositions indicate that the secondary metabolites are an accumulation of stalling compounds as a result of the growth of the colony.

Several investigations were conducted to document antibiosis in soil where antibiotics were detected in non- sterile amended, sterile amended and unamended soils. (Williams *et al.* 1983; Wellington *et al.* 1990)

1.6   *Streptomyces* genetics

The completion of the genome sequence of *S. coelicolor* A3(2), has provided *Streptomyces* research with a powerful tool. The total size of the chromosome is 8,667,507 bp being the largest genome of the bacterial super kingdom. Exhaustive investigation of the genome sequence using *in silico* techniques is taking place that in turn will provide us with valuable information. Transcriptome and proteome analysis will aid our understanding of microbial life in soil and facilitate the finding of new metabolites for further exploitation of the organism.

1.6.1   Genome structure

Streptomyces have a linear chromosome (Lin *et al.* 1993; Lezhava *et al.* 1995). During the long years that preceded the genome sequence project, many scientists studied its genetics still under the impression that *Streptomyces* had a circular genome. The first linkage map was introduced by Hopwood in 1958 (Hopwood 1999); a circular chromosome with six loci and two linkage groups. The origin of replication, *oriC*, and the *dnaA* gene are located 61Kb left of the centre. Around the origin there is a decrease in the GC percentage facilitating the unwinding of the chromosome and hence DNA replication. Coding density is homogeneous along the chromosome with a slight decrease in the distal regions. The chromosome appears to be composed of a central core of essential genes and two chromosome arms. The central core region of the chromosome extends from 1.5Mb to 6.4Mb and encodes

genes essential for life e.g. cell division, DNA replication, transcription and translation machinery as well as the genes responsible for amino acid biosynthesis. The arms, uneven in length (1.5 and 2.3Mb), contain contingency genes, such as hydrolytic exoenzymes and 'gas vesicle' proteins (Bentley *et al.*, 2002). Terminal inverted repeats (TIR) have been found on every *Streptomyces* species chromosome analysed. These TIRs can range from 24Kb in *S. griseus* to 550Kb in *S. rimosus* (Volff and Altenbuchner 1998). TIRs make up 21.6Kb in *S. coelicolor*. Initial comparisons with the complete genome sequence of *S. avermitilis* (Ikeda *et al.* 2003) revealed the conservation of an internal 6.5Mb region. A similar synteny was discovered by comparing *S. coelicolor* to two pathogenic Actinomycetes, *Mycobacterium tuberculosis* and *Corynebacterium diphtheriae*. The core region of the *S. coelicolor* chromosome, containing genes for primary metabolic functions, has a genetic arrangement that is conserved in comparison to the whole genome of both the pathogens; inferring that they share a common ancestor.

Figure 1.4 Combined physical and genetic map of the *S. coelicolor* M145 chromosome

Outside of the circle are markers determined physically using the ordered cosmid library. Inside the circle are markers, which have mapped genetically and assigned by interpolation. *Ase*I and *Dra*I fragments are in Kb. ♦ indicates *oriC* (Redenbach M 1996).
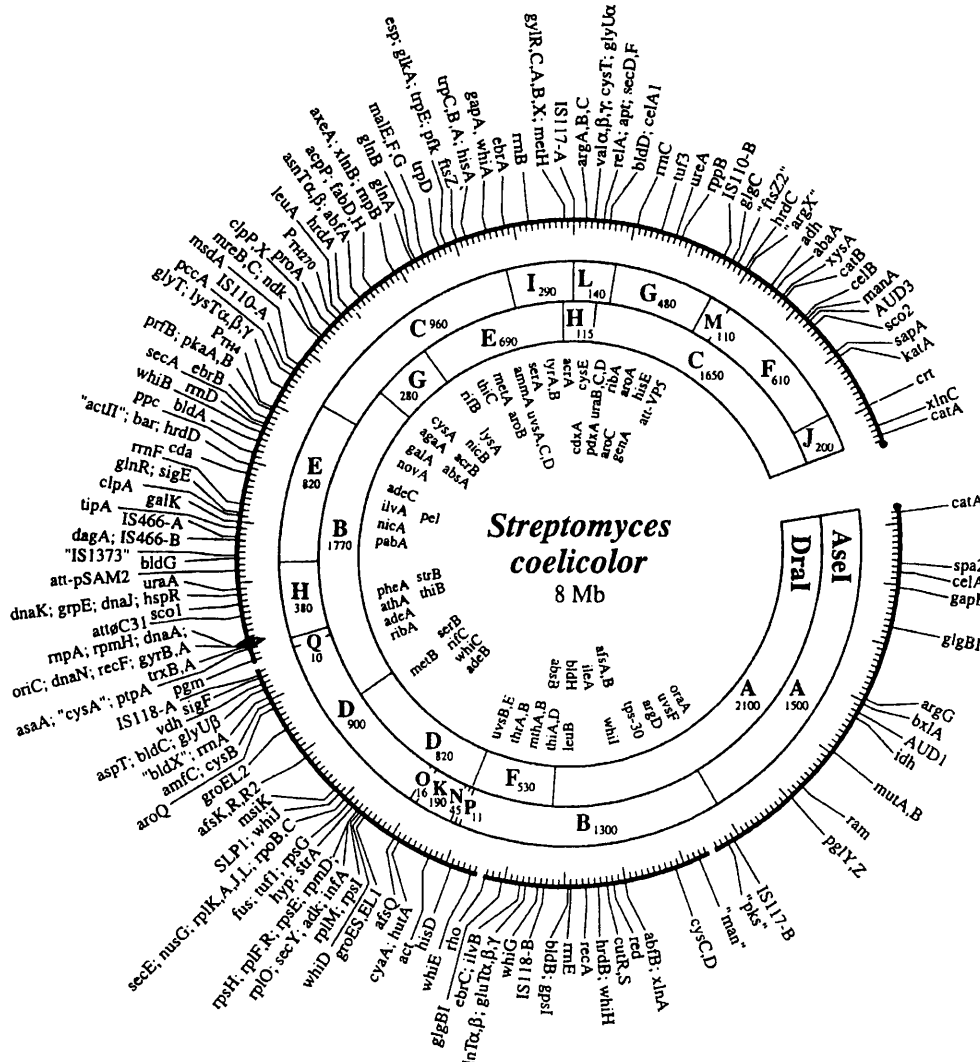
1.7 *Mycobacterium tuberculosis* and the ESAT- 6 family.

Tuberculosis is a threat to human life. The disease has been affecting mankind for over a century. However, exhaustive study of the bacteria has provided a vaccine for the disease and some treatments, but the *modus operandim* of the causative agent *M. tuberculosis* is still elusive. Tuberculosis was declared a global epidemic on 1993 by the World Health Organization (WHO). One third of the world population is infected with 8 million people suffering the active form of the disease and 2-3 million people dying every year. The scenario has been aggravated by the emergence of multidrug resistant strains and co-infection with HIV. In addition attenuated Bacille-Calmette-Guerin (BCG) derived from *M. bovis* that is the only available vaccine does not confer consistent protection against the disease. There are three main issues with BCG vaccination. Firstly it compromises diagnosis of the disease because it produces a delay type hypersensitivity response to protein purified derivative (PPD) that can not be distinguish from exposure to *M. tuberculosis*. The second disadvantage is that it is a live vaccine contraindicated to HIV patients and immuno-compromised individuals, and lastly BCG lacks the DNA segments present in pathogenic *M. tuberculosis* loosing some of its immunogenicity. Diagnosis is also problematic with the protein purified derivative (PPD) lacking specificity due to the sharing of some cross-reactive antigens with BCG and some environmental mycobacteria. The tuberculin test when negative does not rule out active TB, and a positive result can not distinguish between, a) active disease, b) BCG vaccination or c) exposure of healthy individuals to environmental mycobacteria. For all the reasons outlined above, an exhaustive search for new diagnostic tools, vaccines and an ultimate clue to the way the bacterium operates started.

Comparisons between virulent and avirulent mycobacteria identified region of difference 1, (RD1) as the main virulence determinant. RD1 is absent in all types of the attenuated *M. bovis* BCG vaccine strain and non-pathogenic mycobacteria. The *esx1* locus is located in the RD1 region. This encodes the ESAT-6 protein that was initially identified in the culture filtrate of *M. tuberculosis* hence the name Early Secreted Antigenic Target of molecular size 6 kDa. Another protein encoded by the *esx1* locus and identified in early experiments was Culture Filtrate Protein of 10 kDa (CFP-10). These two proteins reacted strongly with the T-cell component of tuberculosis patient's serum and therefore began to be studied in great detail (Berthet *et al.* 1998; Brodin *et al.*). The *esat-6* and *lhp* genes (as they were initially named before nomenclature changed to *esxA* and *esxB*, respectively) code for proteins that are secreted but lack any detectable secretion signals. ESAT-6 protein belongs to a family of 100-residue proteins abundant in *M. tuberculosis* characterised by the presence of a central WXG motif. The rest of the locus is composed of genes that code for cell-wall-associated serine proteases, putative ABC transporters, ATP-binding proteins and other membrane-associated proteins. Therefore the most logical explanation was that the genes coded for a novel secretion system of Gram positive bacteria. In *M. tuberculosis* the genes encoding these proteins lie in five large conserved clusters. The *esx1* locus is present in most mycobacteria strains and in *S. coelicolor* and *Corynebacterium diphtheria*.(Gey van Pittius *et al.* 2001; Pallen 2002). In *S. coelicolor,* there is one *esxAB* operon with the peculiarity that it is divergently transcribed from the important regulatory gene *bldB*. The locus in *S. coelicolor* resembles that of *M. tuberculosis* containing the genes that code for cell-wall-associated serine proteases, putative ABC transporters, ATP-binding proteins and other membrane-associated proteins indicating that the secretion apparatus could be

functional in non- pathogenic bacteria like *S. coelicolor* (Figure 1.5). Further analysis of the *M. tuberculosis esx1* locus revealed its importance for virulence and pathogenicity of Mycobacteria and also provided further information about which genes were essential for ESAT-6 and CFP-10 secretion.

In vivo Mycobacteria reside in the macrophages that are myeloid cells whose functions are to engulf and destroy foreign organisms. *M. tuberculosis* asserts a profound inhibitory influence on its host. The function of the ESAT-6 and CFP-10 proteins is unknown. Deletion of *esxA* and *esxB* resulted in a diminution of virulence (Wards *et al.* 2000). All BCG strain deletions encompass the *esx1* locus (Mahairas *et al.* 1996). The incorporation of the RD1 region into BCG restores virulence and immunogenicity of the bacteria as demonstrated by several studies (Pym *et al.* 2002; Lewis *et al.* 2003; Pym *et al.* 2003). Stanley, Raghavan *et al.* 2003 were the first to name the novel secretion system in bacteria. The system was called the Snm secretion system (for Secretion iN Mycobacteria). Stanley's study performed Western analysis to detect the proteins in the supernatant and cell pellets for the presence of ESAT-6 and CFP-10 and they identified three essential components of the secretion apparatus and the required substrates. The three essential components are *snm1 (Rv3870)* and *snm2 (Rv3871)* encoding amino-terminal transmembrane ATPases and *snm4 (Rv3877)* codes for an integral membrane protein that contains 11 transmembrane domains. Study of mutants defective in any essential component of the Snm system in vivo and *in vitro*, showed that each mutant displayed an attenuated phenotype similar to a mutant lacking the entire RD1 region (Guinn *et al.* 2004). Mutants were able to enter macrophages but then were not able to spread to uninfected macrophages resulting in reduced tissue invasiveness in lung tissue. In every case abolishment of

secretion of ESAT-6 and CFP-10 proteins was associated with a reduction in virulence and immunogenicity. A model of the Snm secretion system is illustrated in figure 1.6. All these evidence suggested that the clusters of *Streptomyces coelicolor* and *Mycobacterium tuberculosis* were analogous. Therefore if I could prove that the *S. coelicolor* cluster was functional it could constitute a good model to discover the function of the genes in *M. tuberculosis*.

The benefits of studying the *esx1* genes in *S. coelicolor* were that the organism was more tractable in terms of its genetics, containing a unique copy of the *esx1* cluster and also its culturability.

Figure 1.5 Schematic representations of the *S. coelicolor* and *M. tuberculosis esx1* locus.

The *esx1* locus in *S. coelicolor* genome contains seven genes homologous to the *M. tuberculosis* cluster. *SCO5721* is an amino-terminal transmembrane protein that contained an ATP/GTP binding site; *SCO5722* is a subtilisin like cell wall associated serine protease; *SCO5734* is a DNA segregation ATPase, ftsK-like chromosome partitioning protein (3x ATP/GTP binding sites) amino-terminal transmembrane protein.
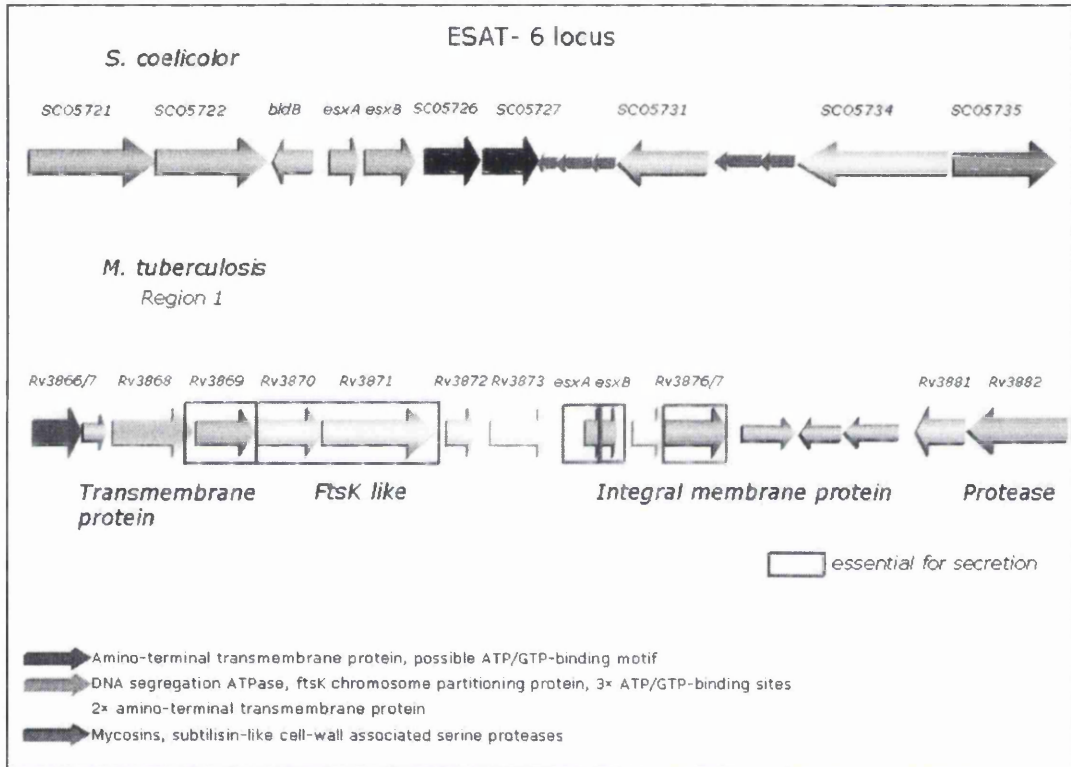
Figure 1.6 Diagram depicting a model of the *M. smegmatis* novel secretion Snm

pathway.

The pathway resembles that of *M. tuberculosis*. CM, cytoplasmic membrane; PG, peptidoglycan layer; mAG, mycolyl arabinogalactan layer. (Converse and Cox 2005)
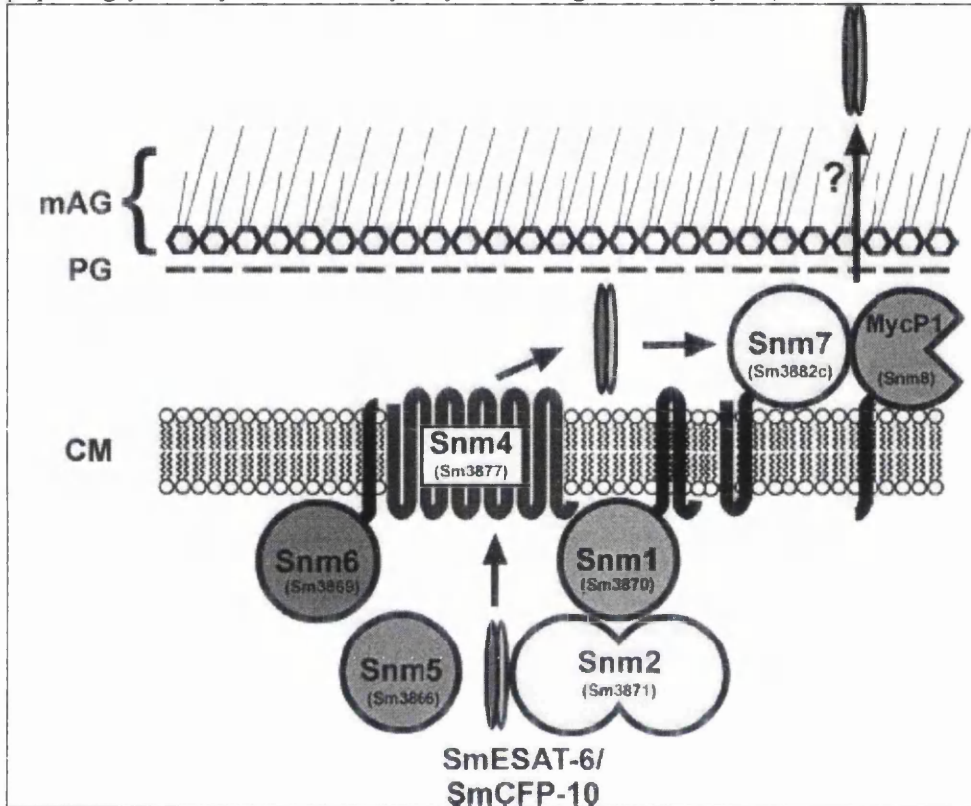
Information about the complex formed by CFP-10 and ESAT-6 was elucidated by (Renshaw *et al.* 2002; Renshaw *et al.* 2005). They postulated that the secretion of the proteins was an active process involving a membrane protein complex formed by the products of the flanking genes. They showed that CFP-10 and ESAT-6 form a tight 1:1 complex and that the C- terminus of CFP-10 is required for binding of the complex to the surface of macrophage and monocyte cells.

The core of the CFP-10:ESAT-6 complex consists of two similar helix–turn–helix hairpin structures formed from the individual proteins. The CFP-10:ESAT-6 complex has an extensive hydrophobic contact surface and the two proteins lie anti-parallel to each other to form a four-helix bundle (Figure 1.6). N- and particularly C-termini of both proteins (residues 2–5 and 86–100 in CFP-10 and 1–3 and 86–95 in ESAT-6) form long flexible arms at both ends of the four-helix bundle core. The two long helices in the hairpin structures are formed from residues Ala8–Gln40 and Ala47– Ala79 in CFP-10, and from Phe8–Trp43 and Glu49– Ala79 in ESAT-6. The helices in CFP-10 are completely α-helical, whereas in ESAT-6 both long helices terminate with a single turn of helix and ESAT-6 also contains a short helix close to the N-terminus (Gln4–Trp6) (Renshaw *et al.* 2005).

Analysis of the electrostatic surface of the complex suggest that the surface of the complex has a very uniform distribution of positive and negative charge, with no hint of a significant hydrophobic patch ruling out the possibility of the complex forming a membrane spanning pore. In addition, the complex does not seem to be involved in interactions with nucleic acids as the surface of the complex lacks any striking acidic or basic patches. clefts in the surface of the structure. the surface features of the CFP-

10:ESAT-6 complex seem most consistent with a function based on specific binding to target protein/s, perhaps playing a key role in pathogen–host cell signalling.

Figure 1.7 Schematic representations of the conformation adopted by the ESAT-6: CFP-10 heterodimer of *M. tuberculosis*.

Solution structure of the CFP-10:ESAT-6 complex. (A) A best-fit superposition of the protein backbone for the family of 28 converged structures obtained, with CFP-10 shown in red and ESAT-6 in blue. The long flexible C-terminal arms of both proteins are clearly visible, as is the propensity to helical structure in this region of CFP-10. (B) A ribbon representation of the backbone topology of the CFP-10:ESAT-6 complex based on the converged structure closest to the mean, which illustrates the two helix–turn–helix hairpin structures formed by the individual proteins. The orientation of the complex is identical to that shown in panel A, with CFP-10 in red and ESAT-6 in blue. **(Renshaw *et al.* 2005)**
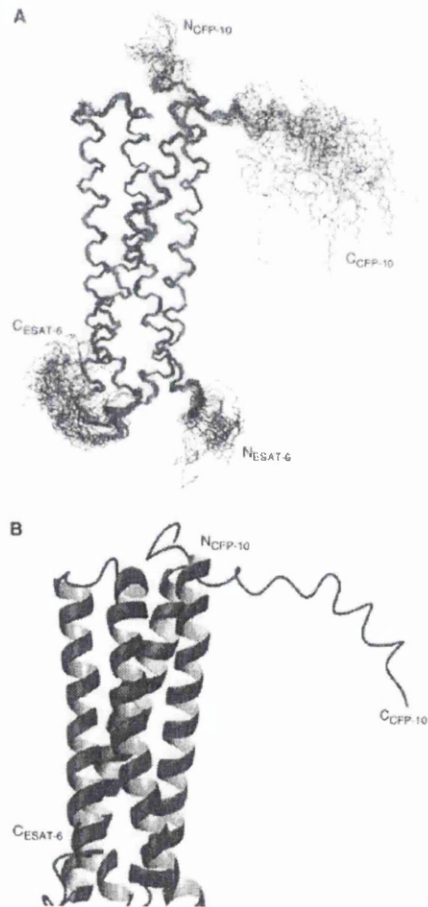
1.8   Protein secretion in bacteria.

Protein secretion is well documented in Gram negative bacteria. Most of the current knowledge of bacterial transport systems has derived from experiments carried out in Gram negative bacteria. There are three main transport routes to translocate substrates across the bacterial membrane:

Class 1 pathways translocate polypeptides and peptide subdomains across the cytoplasmic membrane. This pathway uses a different translocon for unfolded (Sec YEG/YidC) or for folded proteins (TAT ABC). Class 2 pathways are required for crossing the outer membrane from the periplasm, transporting proteins, in a folded state. Class 3 includes type I, type III, flagellar and the majority of Type IV systems moving 'unfolded' proteins, directly from the cytoplasm to the outside (or into a host cell cytoplasm). Outer membrane translocons are most likely derived from a single ancestor whereas inner membrane translocons are usually specific to each pathway (Holland 2004).

The Type I pathway is usually dedicated for pore forming toxins consist of an ATP-binding cassette (ABC) transporter. The family of repeat toxins (RTX toxins) are secreted this way. Gram negative bacteria use this system to transport virulence factors across the bacterial envelope. ABC transporters are used indistinctively by Gram negative, Gram positive bacteria and eukaryotes (from yeast to humans).

Type II substrates are transported in two stages, upon recognition of the signal peptide, sec machinery transports type II substrates across the plasma membrane. Then the folded substrates are the transported across the outer membrane.

Type III secretion systems are activated when the bacteria touches the host cell. Some bacteria sense the ion concentration in the cytoplasm of the host cells as a mechanism to trigger type III secretion. Salmonella uses this mechanism to enter epithelial cells and inject factors to activate polymerisation of actin. All Type III machines have an outer membrane structure called secretin. Secretin promotes the assembly of a needle structure that directs the channelling of the protein into the host cell cytoplasm.

Type IV transport systems are complex machines, transporters of proteins or nucleic acids that secrete substrates across the bacterial envelope or direct them into target cells. Two Gram negative pathogens, *Agrobacterium tumefaciens* and *A. rhizogenes*, infect specific plants to cause crown gall tumours, a weakening disease (Lai and Kado 1998; Zupan *et al.* 2000). Plant cells within the tumour are transformed by agrobacterial DNA, which is inserted into nuclear chromosomes. Expression of the bacterial genes (tumour or T-DNA) results in the production of opines (amino acids) that constitute a source of nutrient for the *Agrobacteria* (Zambryski *et al.* 1989). The bacterial virulence genes were found searching for genes that cause tumour production (Ward *et al.* 1988), several of which display homology to genes required for bacterial mating or conjugation. Conjugation requires physical contact with gram negative bacteria forming a pili that enable male (pili containing) bacteria to interact with female cells to proceed to the conjugation.

Pathways described above require a signal peptide that will target the substrate to the corresponding translocon. The lack of signal peptides for the secreted immunogens

CFP-10 and ESAT-6 indicates that these proteins are exported by a novel secretion system of Gram positive bacteria.

CHAPTER II

MATERIALS AND METHODS

Chapter 2. Materials and methods

2.1 Bacterial strains

E. coli JM109 was principally used to perform genetic manipulations. Mobilisation of plasmids and cosmids into *Streptomyces* by intergeneric conjugation utilised the non methylating *E. coli* host ET12567/ pUZ8002.

Table 2.1   Bacterial Strains

Bacterial Strain	Genotype	Source
S. coelicolor A3(2) M145	Prototrophic SCP1⁻ SCP2⁻ Pgl⁺	(Kieser 2000)
E. coli JM109	F' *traD36 proA+B+ lacIq Δ(lacZ)M15/ Δ(lac-proAB) glnV44 e14- gyrA96 recA1 relA1endA1 thi hsdR17*	Promega Corp. Yanisch-Perron *et al.*, (1985)
E. coli ET12567 (pUZ8002)	*Dam13*::Tn*9 dcm6 hsdM hsdR recF143* 16 *zjj201* ::Tn*10 galK2 galT22 ara14 lacY1 xyl5 leuB6 thi1 tonA31 rpsL136 hisG4 tsx78 mtli glnV44,* containing the nontransmissible *oriT* mobilizing plasmid, pUZ8002	(Flett *et al.* 1997)
E. coli XL10-Gold	Tetr Δ(*mcrA)183* Δ(*mcrCB-hsdSMR-mrr)173 endA1*	Stratagene

	supE44 thi-1 recA1 gyrA96 relA1 lac	
E. coli XL1-Blue MRF	Δ*(mcrA)183* Δ*(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F'proAB lacIqZ*Δ*M15* Tn*5 (Kanr)]*	Stratagene ,
BacterioMatch® II two-hybrid system reporter cells	Δ(*mcrA)183* Δ(*mcrCB-hsdSMR-mrr)173 endA1 hisB supE44 thi-1 recA1 gyrA96 relA1 lac* [F' *laqIq lacZ ampR* Kanr]	Stratagene

2.2 Plasmids

Plasmids and cosmids used in this study are listed in Table 2.2. The details regarding construction of plasmids originating from this study may be found in specific results chapters.

Table 2.2   Plasmids and cosmids

Plasmid/ Cosmid	Marker genes	Source
pBlueScript SK +	*bla*	Promega
pSA1	*bla*	This study
pSA2	*bla*	This study
pSF152	*aadA* derivative of pSET152	P. Herron
pSH152	*hyg* derivative of pSET152	R. del Sol
pME6	*kan bla*	P. Dyson

pALTER1	*bla tet*	Stratagene
pSA2mt1	*bla*	This study
pSA2m2	*bla*	This study
pBT	*cat*	Stratagene
pTRG	*tet*	Stratagene
pBTCFP	*cat*	This study
pTRGEST	*tet*	This study
pRlux86	*apr*	R. del Sol
pSAlux1	*apr*	This study
pSA152	*aadA*	This study
PSAXho1	*bla*	R. del Sol
pSAHIS1	*apr*	R. del Sol
pSAHIS2	*hyg*	This study
pSAHIS3	*hyg*	This study
pSA2633	*hyg*	This study
pSA23	*hyg*	This study
pQM5062	Tn*5062* in pMOD<MCS> (Epicentre)	P. Herron
pQM5066	Tn*5066* in pMOD<MCS> (Epicentre)	R. del Sol
pSX2633	*hyg*	This study
pSAN2	*apr tet*	This study
pSAN3	*kan bla*	This study
SC3C3.2.B06	SC3C3 containing Tn*5062* in SCO5721 *bla kan aac3(IV) oriT*(RK) *eGFP*	P. Herron
SC3C3.1.G11	SC3C3 containing Tn*5062* in SCO5722 *bla kan aac3(IV) oriT*(RK) *eGFP*	P. Herron
SC3C3.1.H11	SC3C3 containing Tn*5062* in SCO5724 *bla kan aac3(IV) oriT*(RK) *eGFP*	P. Herron
SC3C3.1.E07	SC3C3 containing Tn*5062* in SCO5725 *bla kan aac3(IV) oriT*(RK) *eGFP*	P. Herron
SC3C3.2.C01	SC3C3 containing Tn*5062*	P. Herron

	in SCO5725 *bla kan aac3(IV) oriT*$_{RK}$*) eGFP*	
SC3C3.1.G05	SC3C3 containing Tn*5062* in SCO5727 *bla kan aac3(IV) oriT*$_{RK}$*) eGFP*	P. Herron
SC3C3.2.F07	SC3C3 containing Tn*5062* in SCO5728 *bla kan aac3(IV) oriT*$_{RK}$*) eGFP*	P. Herron
SC3C3.2.C02	SC3C3 containing Tn*5062* in SCO5728 *bla kan aac3(IV) oriT*$_{RK}$*) eGFP*	P. Herron
SC3C3.2.F11	SC3C3 containing Tn*5062* in SCO5729 *bla kan aac3(IV) oriT*$_{RK}$*) eGFP*	P. Herron
SC3C3.2.F10	SC3C3 containing Tn*5062* in SCO5731 *bla kan aac3(IV) oriT*$_{RK}$*) eGFP*	P. Herron
SC3C3.2.D08	SC3C3 containing Tn*5062* in SCO5732 *bla kan aac3(IV) oriT*$_{RK}$*) eGFP*	P. Herron
SC3C3.2.C03	SC3C3 containing Tn*5062* in SCO5733 *bla kan aac3(IV) oriT*$_{RK}$*) eGFP*	P. Herron
SC3C3.2.B11	SC3C3 containing Tn*5062* in SCO5731 *bla kan aac3(IV) oriT*$_{RK}$*) eGFP*	P. Herron
SC3C3.2.F08	SC3C3 containing Tn*5062* in SCO5734 *bla kan aac3(IV) oriT*$_{RK}$*) eGFP*	P. Herron
SC3C3.2.C03	SC3C3 containing Tn*5062* in SCO5735 *bla kan aac3(IV) oriT*$_{RK}$*) eGFP*	P. Herron
pSAN3. 3.F11	pSAN3 containing Tn*5066* in SCO5723 *bla kan Hyg oriT*$_{RK}$*) eGFP*	S. Fielding
pSAN3. 3.F03	pSAN3 containing Tn*5062* in SCO5726 *bla kan Hyg oriT*$_{RK}$*) eGFP*	S. Fielding
pSA23	*hyg*	This study
pRlux86	*apr*	This study
pSAlux1	*apr*	This study

2.3 Chemicals and medias

Constituents of the reagents used and growth media can be found in Tables 2.3 and 2.4, respectively. Fisher Scientific Ltd., BDH Chemicals Ltd., and SIGMA Chemical Company supplied the majority of the chemicals. Bacterial growth media were purchased from DIFCO, Oxoid Ltd., Gibco BRL and Lab M (idg).

Solutions and media were routinely autoclaved at 121°C, 15 psi for 30 min or filter sterilised with a 0.2μm filter. pH was measured at room temperature. The preparation of solutions requiring de-ionised water was provided by the MILLI-Q water purification system, ddH_2O is ultra-pure deionised MILLI-Q water and dH_2O is MILLI-Q RO (reverse osmosis).

Table 2.3   Commonly used reagents

Reagents	Ingredients	Quantity per litre dH₂0 (unless indicated otherwise*)
10 x TBE	Tris	108g
	Boric Acid	55g
	EDTA	9.3g
20 x SSC	NaCl	175.5g
	tri- Sodium citrate	88.2g
Bromophenol Blue DNA loading dye*	Sucrose	40g
	Bromophenol Blue	60 mg
	1 x TBE	10 ml
	dH₂O	90 ml
Buffer I	Tris	12.1g
	NaCl	8.8g
Buffer II	Blocking reagent	0.25g
	Buffer I	100 ml
	Microwave to dissolve	
Denaturing Buffer	NaOH	20g
	NaCl	87.75g
Neutralising Buffer	Tris	121g
	NaCl	88g
	pH 7.5	
Prehybridisation solution*	20 x SSC	25 ml
	10% N-Lauryl sarcosine (w/v)	1 ml
	10% SDS (w/v)	200 µl
	Blocking reagent	5g
	Formamide	50 ml
	dH₂O	24 ml
Colour solution*	NBT/ BCIP Tablets (Roche)	1 tablet

	ddH$_2$O	10 ml
Stacking gel (x2)*	Acrylamide	650 µl
	dH$_2$O	3 ml
	1 M Tris pH6.8	1.25 ml
	10% SDS	50 µl
	10% TEMED	5 µl
	10% APS	25 µl
Resolving gel (x2)* 12% Polyacrylamide	Acrylamide	6 ml
	dH$_2$O	5 ml
	1.5 M Tris pH8.8	3 ml
	10% SDS	150 µl
	10% TEMED	15 µl
	10% APS	80 µl
Running buffer for SDS-PAGE	Glycine	14.4 g
	Tris	4 g
	SDS	0.1 g
PAGE Sample loading buffer*	1M Tris-HCl pH6.8	500µl
	1M Dithiothreitol (DTT)	1 ml
	10% SDS	2 ml
	1% Bromophenol blue	1 ml
	Glycerol	1 ml
	ddH$_2$O	4.5 ml
Coomassie blue*	Coomassie brilliant blue R250	0.25 g
	Methanol	45 ml
	dH$_2$O	45 ml
	Glacial acetic acid	10 ml
	Filter through Whatman N$^{o.}$1 filter	
Coomassie blue Destain solution*	Methanol	450 ml
	dH$_2$O	450 ml

	Glacial acetic acid	100 ml

Table 2.4   **Growth medium**

Media	Ingredients	Quantity per litre
2x YT	Tryptone Yeast Extract NaCl dH_2O adjust pH to 7.0 with NaOH	16 g 10 g 5 g up to 1L
Luria Bertani (LB) Broth	Tryptone Yeast Extract NaCl Glucose dH_2O adjust pH to 7.0 with NaOH	10 g 5 g 5 g 1 g up to 1L
Luria Bertani (LB) Agar	Tryptone Yeast Extract NaCl Glucose dH_2O adjust pH to 7.0 with NaOH then add agar	10 g 5 g 5 g 1 g up to 1L 10 g
Minimal Medium	L-asparagine K_2HPO_4 $MgSO_4.7H_2O$ $FeSO_4.7H_2O$ Agar dH_2O adjust to pH7.2 with NaOH If supplementing the media, add filter sterilised supplement after autoclaving. i.e. glucose (1%	0.5 g 0.5 g 0.2 g 0.01 g 10 g up to 1L

	w/v) or mannitol (0.5% w/v)	
Nutrient Agar	Oxoid Nutrient Broth	13 g
	Agar	12 g
	dH$_2$O	up to 1L
R2	Sucrose	103 g
	K$_2$SO$_4$	0.25 g
	MgCl$_2$.$_6$H$_2$O	10.12 g
	Glucose	10 g
	Casamino acids	0.1 g
	Agar	22 g
	dH$_2$O	up to 800 ml
	After autoclaving add (to 200 ml):	
	Trace element solution	0.5 ml
	5.73% TES Buffer pH7.2	25 ml
	0.5% KH$_2$PO$_4$	2.5 ml
	5M CaCl$_2$	1 ml
	20% Proline	3.75 ml
	1 N NaOH	1.25 ml
R2YE	Sucrose	103 g
	K$_2$SO4	0.25 g
	MgCl$_2$.$_6$H$_2$O	10.12 g
	Glucose	10 g
	Casamino acids	0.1 g
	Agar	22 g
	dH$_2$O	up to 800 ml
	After autoclaving add (to 200 ml):	
	Trace element solution	0.5 ml
	5.73% TES Buffer pH7.2	25 ml
	0.5% KH$_2$PO$_4$	2.5 ml
	5 M CaCl$_2$	1 ml

	20% Proline	3.75 ml
	1N NaOH	1.25 ml
	10% Yeast extract	12.5 ml
SFM (MS agar)	Soya Flour	20 g
	Mannitol	20 g
	Agar	20 g
	Tap water	up to 1L
SOC	Tryptone	20 g
	Yeast Extract	5 g
	1 M NaCl	10 ml
	1 M KCl	2.5 ml
	dH_2O	up to 1L
	after autoclaving add:	
	1 M $MgCl_2.6H_2O$	10 ml
	1 M $MgSO_4.7H_2O$	10 ml
	1 M Glucose	10ml
Tryptone Soy Broth	Tryptone Soy Broth from Lab M	30 g
	dH_2O	up to 1 L.

2.4 Antibiotic selection

All antibiotics were made up as stock concentrations (Table 2.5) and unless stated made up in ddH$_2$O and filter sterilised. Antibiotics were stored at -20°C with the exception of hygromycin that was stored at 4°C.

In some cases screening of colonies that result from cloning an insert that lacks a resistance marker is facilitated using blue/ white selection (if the plasmid contains a multiple cloning site within the *lacZ* gene). *E. coli* colonies containing plasmids with a disrupted *lacZ* gene may be identified because they are unable to produce β–galactosidase, which converts the chromogenic substrate X-gal into an insoluble blue dye and therefore the colonies will be white. On the other hand if the plasmids are intact the β–galactosidase enzyme will be active and the colony will be blue.

Table 2.5   Concentration of antibiotics

Antibiotic	Stock concentration (mg ml^{-1})	*E. coli* working concentration (μg ml^{-1})	*Streptomyces* working concentration (μg ml^{-1})
Ampicillin	50	50	-
Apramycin	25	100	25
Chloramphenicol[1]	10	10	-
Hygromycin	100	100	50
IPTG	20	25	-
Kanamycin	25	25	25
Nalidixic acid[2]	20	20	-
Spectinomycin	200	50	400
Streptomycin	100	25	100
Tetracycline[3]	10	10	-
X-gal[4]	20	25	-

To dissolve use: [1]100% Ethanol, [2]0.25M NaOH, [3]80% Ethanol, [4]DMSO

2.5 Culture conditions

2.5.1 Growth and storage of *E. coli*

E. coli strains were grown on plates of LB agar or as liquid cultures, in LB broth. Strains were cultivated at 37°C and stored in the short term at 4°C.

2.5.2 Growth and storage of *Streptomyces*

The standard medium we used to propagate *Streptomyces* cultures is SFM (MS) agar. When doing so it was necessary to spread the inoculum over the entire surface of the plate in the presence of appropriate antibiotics. *Streptomyces* were grown for approximately 5 days at 30°C until they had fully developed. *Streptomyces* cell stocks were prepared from a plate of sporulating cells incubated on SFM; spores were collected by flooding the surface with 10 ml sterile ddH_2O, and scraping off the surface with a sterile inoculating loop. The mixture was vortexed vigorously and then poured through a sterile 10 ml syringe with a cotton wool filter, to remove any pieces of agar and mycellia. Subsequently spores were pelleted at ca. 4000 rpm for 10 min and resuspended in 1 ml 20% glycerol. Spore suspensions were stored at -20°C. Solid *Streptomyces* cultures were kept at 4°C in the short term.

For certain experiments like protein isolation and luciferase experiments *Streptomyces* mycelium was grown in liquid culture. For this purpose cultures needed to be grown in sterile flasks containing stainless steel springs to enhance dispersed growth and on an orbital shaker for the cultures to be ventilated with sufficient space in the flask for

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respiration. To accommodate this 250 ml flasks with were used and 50 ml cultures were incubated at 30°C, 225 rpm.

2.6 Transformation

2.6.1   Preparation of electrocompetent *E. coli* JM109

A 1/100 dilution of a fresh overnight *E. coli* JM109 culture was inoculated into 500 ml LB broth. At an OD_{600} 0.5 – 0.7 the cells were chilled on ice for 20 min then harvested by centrifugation at 4000 rpm for 15 min at 4°C. The supernatant was decanted and the pelleted cells carefully resuspended in 500 ml ice cold 10% glycerol. The cells were then pelleted and resuspended in 250ml ice cold 10% glycerol. Finally cells were pelleted, resuspended in 20ml ice cold 10% glycerol pelleted again and resuspended into 2 ml ice cold 10% glycerol. The 2 ml suspension of electrocompetent JM109 cells was aliquoted out and stored at –70°C.

2.6.2   Transformation of electrocompetent *E. coli* JM109

A 40 µl aliquot of electro-competent cells (JM109) was thawed slowly on ice, 10 ng DNA was added and mixed gently by twirling the pipette tip. After a 2 min incubation on ice the mixture was transferred into a chilled electroporation cuvette then electroporated using a MicroPulser™ (BioRad). Immediately 1 ml SOC was added and the mixture was incubated in a Bijoux tube for 90 min at 37° C shaking at 225 rpm. The transformation mixture was plated onto LB agar with appropriate antibiotic selection.

2.6.3   Preparation of competent *E. coli* ET12567 for intergeneric conjugation

E. coli ET12567 / pUZ8002 possess the machinery for interspecies conjugal transfer. The plasmid confers kanamycin resistance and the *dam* mutation is maintained by selection with chloramphenicol. A ten fold dilution of a culture grown to stationary phase was inoculated into LB broth with 0.5 M $MgCl_2$. Cultures were grown to exponential phase shaking at 250 rpm, 37°C. During the following steps the cells were kept on ice and all equipment was chilled before use. After two hours at 37° C the cells were incubated on ice for 10 min and then pelleted at 6000 rpm for 5 min. The pellet was gently resuspended in 10 ml ice cold 50 mM $CaCl_2$, incubated for 30 min, then pelleted again. This $CaCl_2$ wash was repeated three times in total. After the final wash the pelleted cells were resuspended in 2 ml $CaCl_2$ and stored at 4°C overnight. Transformation efficiency is enhanced during the first 24 h. Cells can be stored at - 70° C but transformation efficiency diminishes with storage time.

2.6.4 Transformation of *E. coli* ET12567

200 µl aliquots of competent cells were transferred into pre-chilled sterile microfuge tubes containing 5 – 10ng plasmid DNA or up to 50ng of cosmid DNA. The transformation mix was incubated on ice for 15 min. Heat shocked for 5 min in a 37°C water bath. Then returned to ice for a further 15 min. 1 ml LB Broth and 20mM Glucose was added and the mixture incubated at 37°C for 90 min, inverting the tube to gently mix every 20 min. After incubation the transformation mix was plated onto LB agar containing the appropriate antibiotics and the plates were incubated overnight at 37°C.

2.6.5 Intergeneric conjugation

A transformed ET12567 competent cells colony was inoculated into 10 ml LB containing kanamycin, chloramphenicol and the antibiotic used to select for the oriT containing plasmid. The colony was grown at 37°C, 250 rpm until OD_{600} of 0.4 to 0.6. To reach that optical density usually the cultures would be incubated for 8-10 h. Exponentially growing cells were washed twice with an equal volume of LB and then gently resuspended in 1/10 volume of LB broth. During the washing steps care must be taken when resuspending the cells.

While washing the *E. coli* cells, for each conjugation approximately 10^8 *Streptomyces* spores were added to 500 μl 2 x YT Broth. *S. coelicolor* spores were germinated at 50°C for 10 min then cooled to room temperature. To the activated spores 500μl washed *E. coli* were added, the mixture was briefly spun down and most of the supernatant was decanted off. The pelleted cells were gently resuspended in the residual broth. This conjugation mixture was plated out onto SFM agar + 10mM $MgCl_2$. After incubation at 30°C for 16 h plates were overlaid with 1 ml sterile ddH_2O containing nalidixic acid (to inhibit *E. coli* growth) and the appropriate antibiotic selecting for the oriT containing plasmid. Plates were incubated at 30°C for a further 3 – 5 days until potential exconjugants could be picked and transferred to selective media containing nalidixic acid.

2.7 Isolation of DNA

2.7.1   Plasmid isolation from *E. coli*

E. coli plasmid DNA was isolated, using DNA binding filters, from overnight liquid cultures containing the appropriate antibiotics for plasmid selection. Mini preparations of

plasmid DNA utilised the WizardPlus SV Minipreps DNA purification system (Promega Corp.). Cells from an overnight culture were pelleted at 13000 rpm for 5 min, resuspended in 250 µl Resuspension solution and incubated for 5 min at room temperature with 250 µl Lysis solution. Gentle inversion mixed these solutions. After incubation, 350 µl Neutralisation solution was added, mixed by inversion and the resulting suspension was centrifuged at 13000 rpm for 5 min. The cell lysate was transferred to a spin column containing a DNA binding matrix. Centrifugation at 13000 rpm for 1 min allows DNA to be captured on the matrix, it is then washed twice with 750 and 250 µl Wash buffer, respectively, and the DNA was eluted in the final step in 100 µl sterile ddH$_2$O by centrifugation of the spin column, into a sterile microfuge tube, at 13000 rpm for 2min.

2.7.2   Genomic DNA isolation from *Streptomyces*

The FastDNA® SPIN Kit for Soil (BIO 101) is designed to rapidly isolate genomic DNA by lysing the cells using ceramic and silica particles. Lysing matrix tubes containing 978 µl Sodium Phosphate Buffer and 122 µl MT Buffer, were filled to 7/8 volume with mycelia from 24 h cultures grown on Nutrient agar plates. The tubes were fixed firmly into the FastPrep® Instrument and processed for 30 sec at speed 5.5. Then the cell debris and lysis matrix were pelleted by centrifugation at 13000 rpm for 30 sec. The supernatant containing genomic DNA was transferred to a fresh tube and mixed thoroughly with 250 µl PPS reagent. The mixture was centrifuged again at 13000 rpm for 5 min, after centrifugation the supernatant was transferred to a 15 ml universal tube and mixed with 1 ml Binding Matrix Suspension. The tube was placed on a rotator (or inverted by hand) for

2 min. Once the DNA was bound to the matrix it was left to settle for 3 min then 500 µl of the supernatant were decanted and the remaining solution was mixed and transferred into a spin filter, which captures the matrix and the bound DNA. The bound DNA was washed with 500 µl SEWS-M and eluted into a fresh catch tube with 50 µl DNase/ Pyrogen free water.

2.8   DNA manipulations

2.8.1   Enzyme reactions

Restriction enzymes, T4 DNA ligase, polymerase and calf intestinal alkaline phosphatases (CIP) were purchased from New England Biolabs Inc. and Promega Corp. The enzymes were used in agreement with the manufacturer's instructions.

2.8.1.1 Ligations

Prior to ligation, DNA fragments were purified by ethanol precipitation as described by (Sambrook *et al.* 1989) or by using a QIAquick PCR Purification Kit (Qiagen). The DNA fragments, in their enzyme reaction mixtures, were mixed with five volumes of Buffer PB and applied to QIAquick spin columns. Centrifugation at 13000 rpm for 1 min allows DNA to be captured on the matrix; it was then washed twice with 750 µl Buffer PE (wash buffer). After an additional centrifugation step at 13000 rpm for 1 min to dry the column, the DNA was eluted in the final step in 50 µl sterile ddH$_2$O by centrifugation of the spin column, into a sterile eppendorf, at 13000 rpm for 2 min. The ratio of vector to insert DNA was estimated, combined at a ratio of 3:1 or 5:1 and ligated overnight at 13°C.

2.8.2 Visualisation and quantification

2.8.2.1 Agarose gel electrophoresis

Agarose gel electrophoresis was used to analyse nucleic acids. The percentage of agarose in the gel depended on the size of the nucleic acid of interest. 0.8% gels are suitable to separate restriction digests of plasmid DNA (0.5 to 15Kb), 0.5% gels are used to visualise large pieces of DNA, for example, *Streptomyces* chromosomal DNA. To separate smaller PCR products or RNA 1 to 1.5% agarose gels provide better resolution. Standard 100 ml gels were routinely prepared as 0.8% (w/v) agarose in 1 x TBE buffer. Samples were generally in 20 µl volumes and were mixed with 3 µl Bromophenol blue DNA loading dye before being run for 45 – 60 min at 100V. After electrophoresis nucleic acids were visualised by staining the gel in ethidium bromide (0.5 µg ml^{-1}) for approximately 30 min and destaining in water for 20 min. Nucleic acids were observed using a Biorad transilluminator at 254nm.

2.8.2.2 DNA and RNA spectrophotometric quantification

The quality and quantity of nucleic acids may be assayed spectrophotometrically. The ratio of A_{260}/ A_{280} determines the purity of the nucleic acids; in 10mM Tris-Cl (pH 8.5) pure DNA has an A_{260}/ A_{280} ratio of 1.8 – 2 and in 10mM Tris-Cl (pH 7.5) pure RNA has an A_{260}/ A_{280} ratio of 1.9 – 2.3. The concentration of dsDNA, when $A_{260} = 1$, and the path length is 1cm, is 50 µg ml^{-1}. The concentration of RNA, when $A_{260} = 1$, and the path length is 1cm, is 40 µg ml^{-1}.

2.8.3   Southern hybridisation

2.8.3.1 Preparation of Digoxigenin labelled probes

The transposon specific probe and the λ*Hind*III probe were constructed using the Roche dig DNA labelling kit. 5 – 10 µg of Tn*5062* DNA was excised as a 3442bp fragment from pQM5062 using *Pvu*II and gel purified with a QIAquick Gel Extraction Kit (Qiagen). Random priming was then used to amplify labelled Tn*5062* DNA. DNA was denatured at 100°C for 10 min. It was transferred to ice before the other components of the reaction were added. The random priming reaction was incubated overnight at 37°C. The λ*Hind*III probe, which associates with the size ladder λ*Hind*III was also synthesised by random primed labelling.

Standard reaction mixture: 39 µl   Denatured DNA

   5 µl   Hexanucleotide mix

   5 µl   dig-dNTP labelling mix

   1 µl   Klenow polymerase

10ng ml^{-1} each probe was added to the Prehybridisation Solution and prior to use the probes were denatured by boiling for 10 min and transferred immediately to ice.

2.8.3.2 Blotting

The chromosomal DNA and the cosmid DNA use as a control were digested with appropriate restriction enzymes. After separation by agarose gel electrophoresis the DNA

was denatured before hybridisation. Firstly the gel was incubated in Denaturing buffer with gentle shaking twice for 15 min. Then the gel was washed three time in dH_2O and immersed in Neutralisation buffer twice for 20 min. Whilst neutralising the gel, a nitrocellulose membrane (Hybond-N, Amersham Pharmacia Biotech) was activated by soaking in dH_2O 10 min. Then the membrane and the Whatman paper were soaked in 10 x SSC for 20 min.

A Stratagene Posiblot Pressure blotter was used to transfer the DNA from the gel onto the treated nitrocellulose membrane. The pressure blotter was assembled with Whatman filter paper, soaked in 10 x SSC at the bottom, then the membrane (both 1cm bigger than the gel in width and height) and on top of this was a plastic mask. The plastic mask contained a hole in the centre, 0.5 cm smaller than the gel in width and height. Once positioned the gel lies on top of the mask sealing the assembly. Finally a sponge saturated with 10 x SSC was positioned over the gel and the apparatus was closed. With 75mmHg pressure applied for 90 min the DNA is effectively transferred on to the membrane. Once transfer was completed the DNA was fixed to the membrane by baking in an oven at 80°C for 60 min.

2.8.3.3 Hybridisation

Once the DNA was fixed to the membrane it was wetted in dH_2O and rolled inside a piece of nylon mesh. With the DNA facing innermost, it was placed into a hybridisation tube (Appligene) and washed with 2 x SSC. The following steps take place in a rotating hybridisation oven. The membrane was then prehybridised in 10 ml Prehybridisation

solution at 42°C. After 60 min the prehybridisation solution was decanted and the denatured probe was incubated with the membrane overnight at 42°C. 10 ng ml^{-1} of each probe was added to the Prehybridisation Solution and prior to use, denatured by boiling for 10 min and transferred immediately to ice. (After hybridisation the probe may be collected and stored at -20°C for reuse.) The following morning the probe was retrieved and the membrane was washed. With two five minute washes in Wash Solution I, then two stringent washes at 68°C in Wash Solution II, each for 15 min, the membrane was ready to be developed.

2.8.3.4 Immunological Detection

The membrane was rinsed for 1 min at room temperature in Buffer I, then for 30 min in Prehybridisation buffer. After this, it was briefly washed again in Buffer I before adding the antibody solution; 15 ml Buffer I with 3 µl Antidigoxygenin AP (the antibody conjugate). This was incubated for 15 min. To remove unbound antibody, the membrane was washed twice in Buffer I for 15 min. After these washes in the hybridisation tube, the membrane was transferred to a plastic bag where 10 ml of colour solution was added and the bag was sealed. To facilitate the colour reaction the membrane was placed in tray in the dark, at 37°C. After 1 – 3 h bands were detected and the filter was rinsed in dH$_2$O to stop colour reaction.

2.8.4   Polymerase Chain Reaction

Polymerase chain reactions (PCR) were performed by a PTC-200 DNA Engine (M.J. Research Inc.) using Vent^{TM} (Exo$^-$) DNA polymerase (New England Biolabs Inc.).

Oligonucleotides were designed using Primer3 <http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi> and purchased from MWG-Biotech. A list of primers used in this study is shown in Table 2.6. Annealing temperatures were optimised for each pair of oligonucleotides used.

Table 2.6   PCR primers

Oligonucleotide	Sequence
pBTRev	5′ (ggg tag cca gca gca tcc)3′
pTRGFor	5′ (cag cct gaa gtg aaa gaa)3′
pBTFor	5′ (tcc gtt gtg ggg aaa gtt atc)3′
pTRGRev	5′ (att cgt cgc ccg cca taa)3′

Standard PCR were carried out in a total volume of 25 μl in a 0.5 ml thin walled microfuge tube. A standard mixture added to a sterile 0.5 ml thin walled microfuge tube is as follows:

1 μM	Sense primer
1 μM	Antisense primer
4 μl	dNTP labelling mix (stock concentration 2.5mM)
2.5 μl	10 x Vent Buffer, magnesium free (NEB)
5 – 50 ng	Template DNA
1 mM	$MgSO_4$
0.5 μl	Vent^{TM} (Exo⁻) DNA polymerase
up to 25 μl	ddH_2O

Standard PCR conditions:

Denaturation   95°C for 60 sec

Annealing   variable for 60 sec

Extension   72°C for 60 sec

2.8.5   DNA sequencing

2.8.5.1 Automated sequencing

Sequencing is a fundamental tool for DNA manipulation, it was used routinely to verify the construction of plasmids, integrity of coding sequences and verification of mutagenesis reactions. Wizard DNA preparations were quantified spectrophotometrically to determine the appropriate amount of template DNA. The right amount of the template according to the plasmid length was mixed with ddH_2O denatured at 96° C for 1 min, cooled on ice and then mixed with DTCS quick start master mix (Beckman Coulter) and the desired sequencing primer. Sequencing reactions were amplified by thirty cycles of <96° C for 20 sec/ 50° C for 20 sec/ 60° C for 4 min> using a PTC-200 DNA Engine (M.J. Research Inc.). Sequencing reactions were separated using the Beckman CEQ 8000. Table 2.7 shows a list of most commonly used sequencing primers.

Table 2.7   Sequencing primers

Primer	Sequence
1224	5′ (cgc cag ggt ttt ccc agt cac gac)3′
1233	5′ (AGCGGATAACAATTTCACACAGGA)3′
T7 universal primer	5′ (TAATACGACTCACTATAGGG)3′

| LuxAB primer | 5' (CAGGTGGCTGATAAGTGAGAAGCA) 3' |

2.8.6   Site directed mutagenesis

The QuikChange®XL Site-Directed Mutagenesis kit (Stratagene) allows site specific

mutations to be introduced into plasmids. In this study it was used to introduce a

restriction site to the transcriptional start of *SCO5724* and *SCO5725*. Two

complementary oligonucleotides were designed containing the desired mutation flanked

by unmodified sequence for each gene (Table 2.8).

Sample reactions were set up as follows:

5 µl	10 x reaction buffer
10 ng	dsDNA template
125 ng	primer 1
125 ng	primer 2
1 µl	dNTP mix
3 µl	QuikSolution
1 µl	PfuTurbo DNA polymerase (2.5U/µl)
up to 50 µ l	ddH$_2$O

The new sequence was amplified in a thermal cycler:

95° C   1 min

} 20 cycles

95° C	50 sec
60° C	50 sec
68° C	1 min per kb plasmid length
68° C	7 min

After temperature cycling, the reaction was placed on ice for 2 min. 1 µl *Dpn*I was added to the reaction mixture and incubated at 37° C for 60 min to digest the parental (non mutated DNA). The newly mutated plasmid was transformed into XL10-Gold® Ultracompetent cells. The cells were thawed on ice and mixed with 2 µl β-mercaptoethanol for 10 min on ice. 2 µl *Dpn*I treated DNA was added to the cells and incubated on ice for 30 min. The cells were heat shocked at 42° C for 30 sec and then incubated with 0.5 ml LB Broth at 37° C, 225 rpm. After 60 min transformed cells were plated onto LB agar with appropriate antibiotic selection for the plasmid vector.

Table 2.8   Mutagenesis primers

Mutagenesis primer	Sequence
ESATMUT1	5′ (ccg tac gca acc gag gag aag gga tcc gtg agc gtc aat tac g) 3'
ESATMUT2	5′ (cgt aat tga cgc tca cgg atc cct tct cct cgg ttg cgt acg g) 3'
CFPMUT1	5′ (ccg ggc gga ccc gcc gtg gtg aca gcc acc ag) 3'
CFPMUT2	5′ (ctg gtg gct gtc acc acc cgg gcg ggt ccg ccc) 3'

2.9 RNA isolation

The first step before the RNA experiments was to remove all traces of ribonuclease (RNase) contamination. Glassware was baked at 240° C overnight. Disposable plastic ware was autoclaved twice before use and laboratory equipment and surfaces were dust free. RNA was isolated using Qiagen RNeasy® Midi Kits. *Streptomyces* spores were inoculated onto agar plates covered with sterile cellophane discs and at appropriate time points cells were harvested into 800 μl Bacterial RNA Protect (Qiagen) and incubated for 5 min. Mycelia were pelleted at 13000 rpm for 5 min and resuspended into 1 ml TE buffer containing 3 mg ml^{-1} lysozyme. 4 ml Buffer RLT was added and the bacterial lysate was centrifuged for 5 min at 4000 rpm to pellet cell debris. The supernatant was transferred to a fresh 15 ml tube and 2.8 ml Ethanol was added gently. The sample, including any precipitate, was applied to an RNeasy midi column and centrifuged for 5 min at 4000 rpm. Flow through was discarded and an optional on – column DNase step was incorporated. The silica matrix with RNA bound, was washed once with 2 ml Buffer RW1 then 160 μl Buffer RDD (containing 20 μl DNase I stock solution) was incubated on the column at room temperature for 15 min (RNase free DNase Kit, Qiagen). A second wash step, with 2 ml Buffer RW1 was then centrifuged through the column. Finally 2.5 ml Buffer RPE was applied to the column, centrifuged for 5 min at 4000 rpm, and the RNeasy column transferred to a fresh 15 ml collection tube. 250 μl RNase free water was applied to the column and incubated for 1 min at room temperature. The RNA was eluted by centrifugation then aliquoted and stored at -70° C.

2.10   Reverse- transcriptase RT-PCR experiments.

We used RETROscript® Kit from Ambion. The RT-PCR experiments were performed in two stages:

2.10.1 Stage 1.

For each cDNA required one reaction was set up by mixing together:

> 1–2 µg total RNA
>
> 0.1–2 µl of a 50 µM stock of the gene-specific first-strand primer
>
> to 12 µl Nuclease-free Water

These reaction components were mixed, spun briefly, and heated for 3 min at 70–85°C. The exact temperature was not critical, but 85°C was more appropriate for these targets that were high GC. The tubes were removed to ice, spun briefly and replaced back on ice. Then the remaining RT components were added:

> 2 µl 10X RT or 10X PCR Buffer
>
> 4 µl dNTP mix
>
> 1 µl RNase Inhibitor
>
> µl MMLV-RT
>
> 20 µl Final volume

These components were mixed gently and spun briefly. The reactions were then incubated at 42–44°C for 1 h. Followed by inactivation of Reverse Transcriptase by

incubating the tubes at 92°C for 10 min. The cDNA was ready to proceed to the second step.

2.10.2 Stage 2. Amplification of this cDNA molecule using different primers.

Two controls were introduced in this step; the first control was the RNA control from the previous step and the second was a minus-template PCR that had all the PCR components but water was used as template instead of an aliquot of the cDNA.

The reaction mixture was mixed on ice:

        1–5 µl RT reaction (from step 1)

        5 µl 10X PCR buffer

        2.5 µl dNTP mix

        to 50 µl dH$_2$O, nuclease-free water

        2.5 µl PCR primers (mixture with 5 µM of each primer)

        1–2 U Thermostable DNA Polymerase, mainly dynazyme ((Fynnzymes)

Denature: 94–95°C for 2–4 min

            94°C for 20–30 sec

            annealing temp. for 20–3   } 30 cycles

            72°C for 40 sec to 1 min

Final extension: 72°C for 5 min

Table 2.9   RT – PCR primers

Mutagenesis primer	Sequence

All2S1	5′ (cat gag cgt cga tta cag) 3'
All2SA1	5′ (cac atc tgg tcc atc tcc) 3'
All3S1	5′ (gat tac agc gac cag gac) 3'
All3SA1	5′ (ctg ctc agt tcc gtt gtc) 3'

2.11   Protein isolation.

2.11.1 Native protein isolation.

Spores were inoculated onto SFM agar plates covered with sterile cellophane discs. After incubation, cells were scraped off the cellophane disc and placed into 500 μl Lysis buffer containing 2 mg ml^{-1} lysozyme and protease inhibitors and incubated for 5 min at room temperature. Samples were sonicated on ice (condition vary but usually four 20 sec bursts at 7.5μm). The cell debris was pelleted by centrifugation at 13000 rpm at 4° C and the supernatant was transferred to a fresh microfuge tube and stored at -70° C.

2.11.2 Denaturing protein extraction

Spores were inoculated onto agar plates covered with sterile cellophane discs and at appropriate time points, cells were harvested into 5 ml Washing buffer. Cells were vortexed and pelleted at 4000 rpm, 4° C for 5 min. Pellets were resuspended in 400 μl Sonication buffer and sonicated at amplitude of 7.5μm for ten 2 sec bursts. During sonication samples were kept cool on ice. Once disrupted the debris was removed by centrifugation at 13000 rpm for 15 min at 4° C. The supernatant containing the whole

cell protein extract is stored in aliquots at -70° C. Prior to SDS-PAGE the samples were cleaned and quantified.

2.11.3 Trichloroacetic acid (TCA) protein precipitation from soluble fraction

Cultures were grown to exponential phase (OD_{600} 0.4 – 0.6) shaking at 250 rpm, 30°C. The cells were then pelleted (cells were kept to prepare intracellular fraction see section 2.11.4) and the supernatant collected in a 50 ml Falcon tube to which a tablet of complete inhibitor of protease activity (Roche) was added. TCA solution was added neat at a concentration of 1 in 10. The tubes were place on ice and kept overnight in the cold room at 4° C. Then the samples were centrifuged for 20 min and the supernatant decanted. The precipitate was washed twice with ice-cold acetone and left on the bench until all the acetone had evaporated. This pellet was resuspended in sonication buffer. Once the sample was resuspended sample loading buffer was added 1:1. Finally the sample was quantified with the 2D-Quant kit (Amersham Pharmacia Biotech). Before being analysed, the pH of the samples was adjusted in view of the fact that this procedure can sometimes produce an acidic pellet.

2.11.4 Denaturing protein extraction from liquid cultures

Cells were pelleted briefly at 4000 rpm to remove any remaining supernatant. Pellets were resuspended in equal volume of sonication buffer approximately 400 μl. Cells were sonicated at amplitude of 15μm for ten 20 sec bursts. During sonication samples were kept cool on ice. Once disrupted the debris was removed by centrifugation at 13000 rpm for 15 min at 4° C. The supernatant containing the whole cell protein extract was stored in aliquots at -70° C.

2.12   Protein quantification.

2.12.1 Quantification of protein extracts using the BioRad protein assay

The BioRad protein assay allows the rapid measurement of protein concentration in the absence of strong alkaline reagents or detergents. This method is based in the classic Bradford method. BSA was used as a standard; dilutions of BSA along with protein samples were assayed in volumes of 0.8 ml and mixed with 0.2 ml Dye Reagent concentrate. After 5 min (but no longer than 60 min) the optical density was measured at 595nm. A standard curve was plotted of BSA and the concentration of the unknowns was read off the standard curve.

2.12.2 Quantification of protein extracts using the 2- D Quant kit.

For protein samples obtained from TCA precipitation the 2D-Quant kit was used to determine the protein concentration. A standard curve was prepare according to Table 1 of the manufacturer's protocol using the 2 mg/ml Bovine serum albumin (BSA) standard solution provided with the kit. Two sets of tubes were prepared containing 2 and 10 µl of the samples to be assayed. The useful range of the assay is 0.5–50 µg. 500 µl of precipitant solution were added to each tube (including the standard samples). Tubes were vortexed briefly and incubated 2–3 min at room temperature. Then 500 µl of co-precipitant were added to each tube and mixed briefly by vortexing. The proteins were sedimented by centrifuging the tubes at 10 000 × g for 5 min. The tubes were retrieved from the microcentrifuge as soon as centrifugation was finished and the supernatant decanted (A small pellet should be visible after completion of this step). Without delay the tubes were inserted in the microcentrifuge as before, with the cap-hinge and pellet

facing outward, and centrifuged briefly to bring any remaining liquid to the bottom of the tube. A micropipette was used to remove any remaining supernatant until no visible liquid was left in the tubes. 100 µl of copper solution and 400 µl of distilled or de-ionized water were added to each tube and vortexed briefly to dissolve the precipitated protein. 1 ml of working colour reagent was incorporated rapidly to each tube to ensure instantaneous mixing. The mixture was inverted a few times and incubated at room temperature for 15–20 min. The absorbance of each sample and standard was measured at 480 nm using water as the reference. The absorbance should be read within 40 min of the addition of working colour reagent. A standard curve was generated by plotting the absorbance of the standards against the quantity of protein. This standard curve was used to determine the protein concentration of the samples.

2.12.3 SDS Polyacrylamide gel electrophoresis

Gels were cast following manufacturer recommendations (BioRad). We separated proteins according to size using polyacrylamide. We require a resolving gel; the percentage of acrylamide depicts the size of proteins of interest. For example, a 10% gel allows the resolution of proteins ranging from 16 – 68 kD. We used 12% gels for the majority of the experiments that provided better resolution for proteins of ca. 10 kDa. Proteins enter a stacking gel that ensures that all the samples are concentrated at the interface between the stacking and separating gels for an homogeneous run. Generally gels were poured and left to polymerise for 1 h or at 4° C overnight. Samples were mixed with sample loading buffer and denatured at 95° C for 10 min before loading into the stacking gel.

2.12.4 Visualisation of proteins

To visualise proteins separated by PAGE, two different staining methods were utilised. Coomassie blue detects up to 0.1 μg protein. After incubating the gel, gently shaking, for 4 h in Coomassie blue staining, the gel is destained for a further 4 – 8 h.   We also used Congo red if the proteins had been transferred to a nylon membrane.

2.12.5 Western blotting

2.12.5.1   Blotting

The protein samples were quantified and mixed with equal amount of sample loading buffer. 15- 30 ng of protein were loaded, taking care to use equivalent amounts of each sample. 10 ul of Molecular Weight dual marker (Bio-Rad) was also loaded in each experiment. The samples were denatured at 95 °C for 5 – 10 min before separation. After separation by PAGE the protein gel was soaked in ice-cold transfer buffer (transfer buffer was chilled before utilization) for 10- 15 min. The membrane (Hybond-P, Amersham Pharmacia Biotech) was activated by soaking in Methanol for 15 sec then, soaked in dH_2O for 5 min and finally for 15 min in transfer buffer. The rest of the components for the transfer were also soaked in transfer buffer. Once all the components were ready, the assembly was prepared placing the black part of the gel rig on the bench then sponge - filter paper - gel - membrane - filter paper and finally sponge. Care was taken to insert the assembly in the Mini-Transblot (Bio-Rad) with the correct orientation to ensure the transfer of the samples to the membrane. To maintain the transfer cool a cold pack was

inserted, finally the pre-chilled transfer buffer was poured until the tank was full. The power pack was set at 120 V for 1 h.

Once transfer was completed the membrane was incubated with gentle rocking overnight with blocking buffer (5% BSA, 10% milk in TBS/0.1TWEEN).

2.12.5.2   Hybridisation

After blocking the membrane was washed 5 times in TBS/Tween and then incubated in the primary antibody solution for 60–90 min. Most of the western experiments performed did not require incubation with a secondary antibody as the primary antibody was enzyme-linked. If the primary antibody was enzyme linked the membrane was washed 5 times in TBS/Tween after incubation and was then ready for the detection step.

If a secondary antibody was required, then the membrane was washed 5 times in TBS/Tween and the secondary antibody was added and the membrane incubated for 60 min. Finally the membrane was washed 5 times in TBS/Tween and was ready for detection.

2.12.5.3   Immunological Detection

The membrane was placed on a tray and 1ml of substrate solution was poured over the membrane uniformly taken care to cover the whole membrane surface. After one minute incubation, the emission of light would be detected exposing a photographic film to the membrane. The photographic film was developed using a Curier machine.

2.13   Bacteriomatch two hybrid system.

The Bacteriomatch two hybrid *Escherichia coli* based system was purchased from Stratagene and used according to the manufacturer's instructions. The first step in this experiment was to clone the genes in frame into the expression vectors pBT and pTRG; cloning was achieved by site directed mutagenesis (Section 2.7.6). Then the expression vectors were sequenced to assure that the coding sequences were intact. Then both resulting plasmids were co-transformed into an *E. coli* special strain bought from the kit manufacturer. Co-transformants were grown in plates selecting for the resistant marker of both plasmids. Rigorous controls were set up to avoid any possible faults in the test interaction.

2.14   Preparation of samples for light and fluorescent microscopy.

Cultures for light and fluorescence microscopy were set up by introducing a sterile coverslip at a 45 angle into the agar plate. Four μl of spore suspension was then inoculated in the acute angle along the glass surface (Chater 1972). Coverslips were removed after 2–4 d incubation at 30 C and cells on the coverslip surface were stained with propidium iodide for nucleoids, and/ or stained for the cell wall, with fluorescein-conjugated wheat germ agglutinin (Fluo-WGA) after fixation, treatment with lysozyme and blocking with BSA (Schwedock *et al.* 1997) fluorescent reagents were obtained from Molecular Probes. The samples were then washed five times with PBS (Sambrook *et al.* 1989), and mounted for microscopy in PBS containing 50% glycerol. Occasionally we grew the cultures normally and the collected the samples by taking surface impressions of two to five days old plates. Hyphae were fixed in methanol and stained with propidium iodide if the nucleic acid content was to be observed or fixed with glutaraldehyde and

stained with propidium iodide and fluorescent wheat germ agglutinin as described by (Flardh *et al.* 1999) and mounted in 40% glycerol (Kieser 2000). Samples were studied using a Nikon Eclipse E600 epifluorescent microscope with a standard FITC filter set and images were captured using a RS Photometrics digital camera. The same microscopic slide could be analysed with the red DNA filter to observed nucleoids or the green filter if we required to visualized the cell wall.

2.14.1 Measurement of septum compartments from micrographs.

A statistical analysis of the septum length was performed on cultures of *esxA* and *esxB* mutants. The program used to measured the compartment length was Scion scientific available from http://www.scioncorp.com/. Before the program was used the scale was set for one unit to be 15.1878 micrometers. In the ANALYSE tab we chose the perimeter length option. Each measurement was recorded when pressing CTRL + 1. When it was require to start a new set of results (a different hypha was been analysed) CTRL + 3 was depressed. The data was recorded in Excel spreadsheet and each column represented all the measurements taken from a single hypha. With the data obtained a histogram was constructed for each mutant and the wild-type strain.

2.15   Luciferase assays

2.15.1 Solid media

96 well culture plates (Greiner bio-one) were used to quantify *luxAB* expression assays. 300 µl agar was pipetted into each well and 2 µl spore suspension was aliquoted into each well. Cultures were then incubated at 30° C. At appropriate time intervals plates were

exposed to N-decanal; approximately 1 ml N-decanal was pipetted onto filter paper cut to the size of the plate. The saturated paper was laid on top of the plate for 1 min. This exposed the cultures to the volatile aldehyde and allowed the luciferase enzyme to undertake the oxidation of N-Decanal. The resulting luminescence (arbitrary units) was measured with Lucy1 Luminometer (LabTech). On each plate, samples were measured in triplicate and experiments were repeated at least twice to ensure reproducibility.

2.15.2 Liquid media

96 well culture plates (Corning) were used to quantify optical density and *luxAB* expression. At appropriate time intervals 1 ml of each flask was extracted in sterile conditions. The flask was then replaced in the 30 C shaker. To the 1 ml of the culture 7 µl of N-Decanal was added and the contents of tube mixed. 250 µl of this mixture would be inoculated into 96 well culture plates. The plate was then ready to be analysed; firstly the resulting luminescence (arbitrary units) was measured and then the optical density of the suspension at 610 n.m. to evaluate the growth rate. Both measurements were carried out with Lucy1 Luminometer (LabTech). On each plate, samples were measured in triplicate and experiments were repeated twice to ensure reproducibility.

CHAPTER III

PHENOTYPIC ANALYSIS OF *esxAB*

MUTANTS OF *STREPTOMYCES COELICOLOR*

Chapter III Phenotypic analysis of *esxAB* mutants of *S. coelicolor*.

3.1 Introduction.

My aim was to investigate the function of the *esx1* locus of *S. coelicolor*. This is orthologous with the *esx1* locus which is critical to the virulence of pathogenic mycobacterium in the host. The locus is the key to latency in *Mycobacterium tuberculosis*. The most important genes of the locus are *esxB* and *esxA*. The orthologues of these genes in *S. coelicolor* are *SCO5724* and *SCO5725*. This chapter explains the construction and characterization of mutants of the *esxAB* operon of *S. coelicolor*.

3.2 Disruption of *esxA* and *esxB* in *Streptomyces coelicolor*.

A complete archived mutant library is being constructed by *in vitro* transposon mutagenesis using the provision of an ordered cosmid library (Redenbach M 1996) and the information provided by the genome sequencing project of the 8,667,507 base pair genome sequence of *S. coelicolor* A3(2) (Bentley *et al*. 2002).

The first step of the procedure was to incubate cosmid SC3C3 with Tn*5062* and transposase. *E. coli* was electroporated with 1 μl of the *in vitro* transposition reaction. The subsequent steps have been automated: 192 apramycin-resistant clones were randomly picked and transferred to growth blocks; then cosmid isolation, DNA quantification and DNA sequencing were carried out by a robot and an automated DNA sequencer. The DNA sequence files were processed to identify the exact site of each Tn*5062* insertion using Transposon Express software, developed in our laboratory by Gareth Hughes (Herron *et al*. 2004). These data were then incorporated into ScoDB

at the John Innes Centre.

There is only one Tn*5062* insertion per mutated cosmid molecule. From 192 cosmid clones we obtained one insertion labelled as SC3C3.1.H11 and SC3C3.1.E07 in genes *SCO5724* and *SCO5725* respectively, in each of the desired genes.(Bishop *et al.* 2004) Intergeneric conjugations were performed to transfer the mutated cosmids into *S. coelicolor* A3(2) M145. The exconjugants were selected on the basis of the antibiotic resistant marker on the Tn*5062* transposon that contains an apramycin cassette. Double crossovers were selected because they lack the kanamycin resistance cassette of the cosmid backbone and were therefore kanamycin sensitive, apramycin resistant.

For *SCO5724*, five double exconjugants were obtained and the mutation was confirmed by Southern hybridization. From these five I worked with the three that were most phenotypically similar to each other. There were eight double crossover mutants isolated for *SCO5725* the mutation was confirmed by Southern hybridization for the six clones that had an identical phenotype. The mutants *DC5724* and *DC5725,* were called *esxA* and *esxB*, respectively.

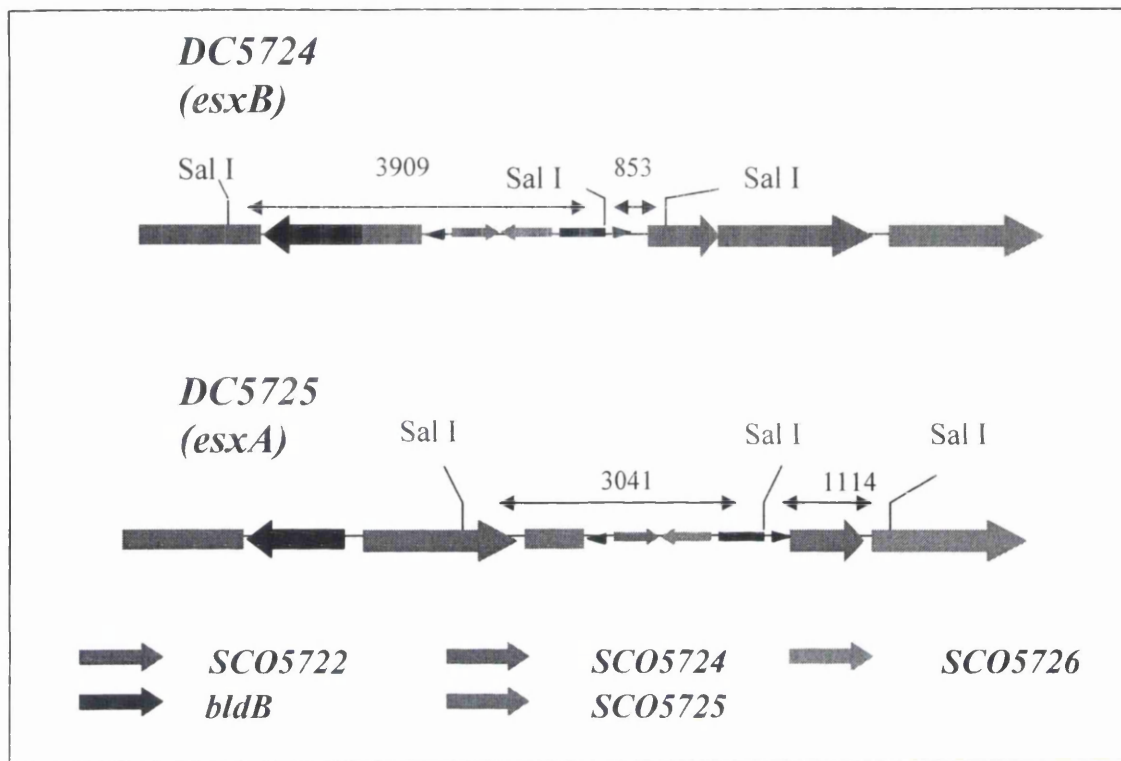**Figure 3.1 Diagrammatic representation of the Southern blot experiment.**

Figure 3.2 *DC5725* **Southern blot experiment.**

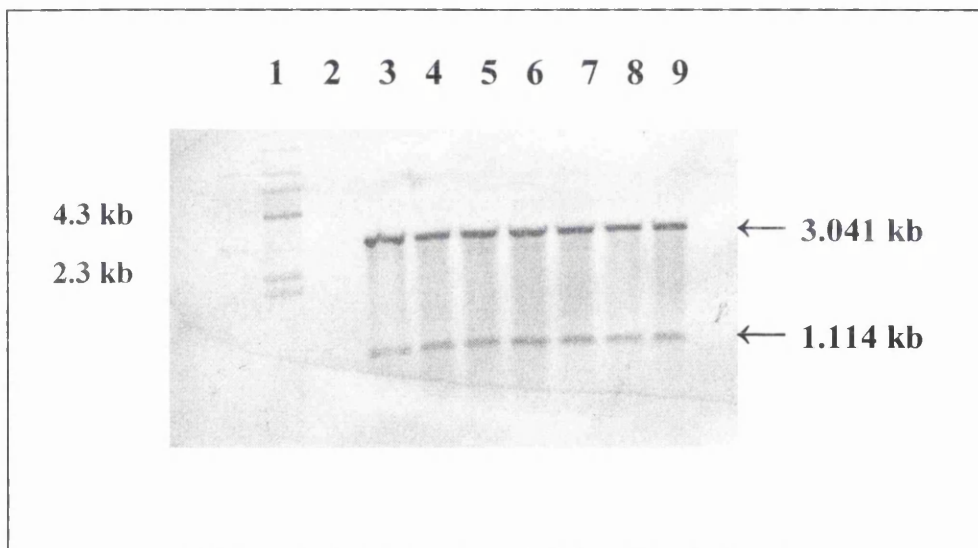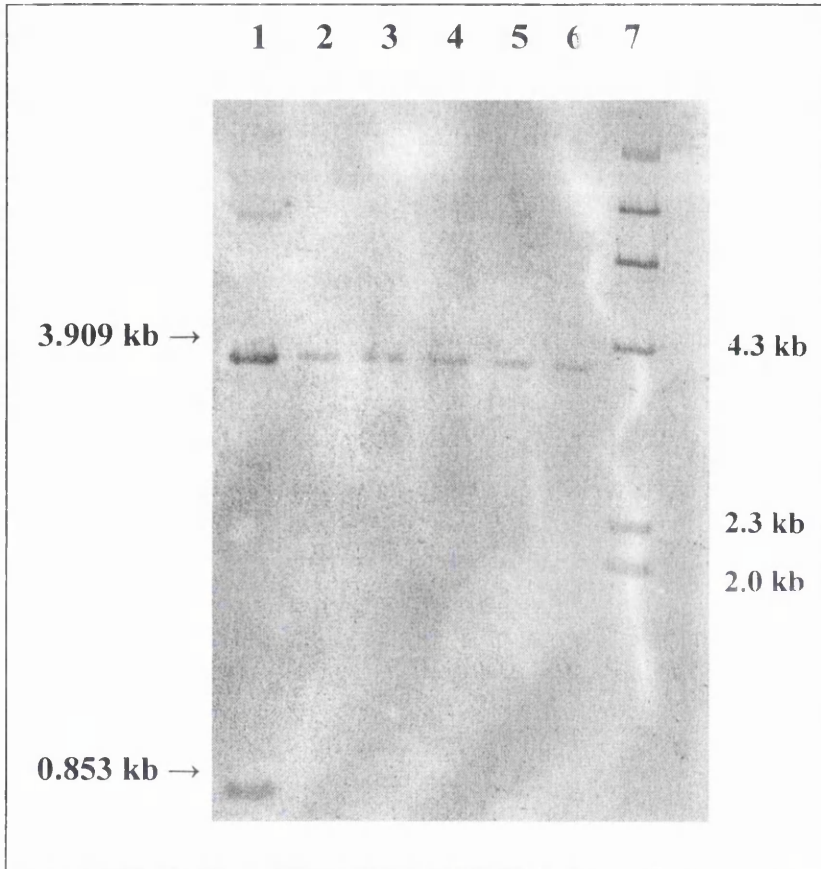Lane 1, lambda *Hind*III, Lane 3, cosmid DNA control, lanes 4-9, exconjugants.

Figure 3.3 *DC5724* **Southern blot experiment**

Lane 1, cosmid DNA control; Lanes 2-6, exconjugants; Lane 7, lambda *Hind*III molecular size marker.
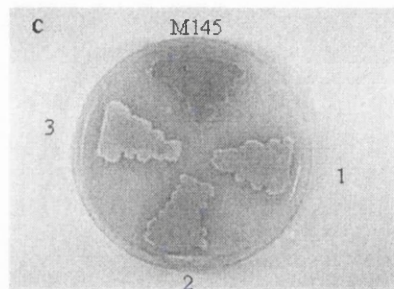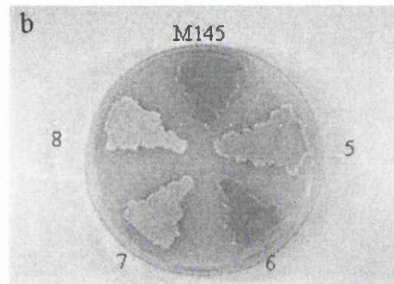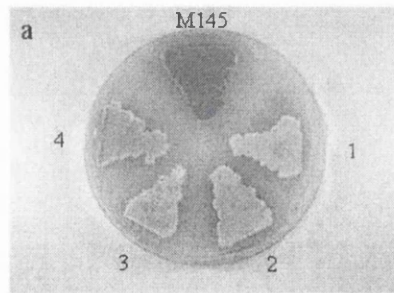
3.3 Phenotypic analysis of the *esxAB* mutants.

Intergeneric conjugation yielded several clones in which the allelic replacement had occurred. For the SCO5724 mutation, I isolated five independent clones of which three shared a similar phenotype. For the SCO5725 mutation, I isolated eight independent clones of which six shared a similar phenotype. Having isolated several independent clones, I then validated the allelic replacement by Southern hybridization. The clones were patched onto a nutrient agar plate and incubated overnight. 100 mg of mycelium was scraped off and DNA extracted using a Fast Prep Soil DNA kit (Q.BIOgene). The DNA was restricted with an enzyme predicted to cut once within Tn*5062* and at defined distances in either flanking sequence. As a positive control, the relevant cosmid DNA was restricted with the same enzyme. The samples were electrophoresed and blotted. The membrane was then hybridised with a digoxigenin labelled Tn*5062* 3442 bp *PvuII* fragment probe. The probe hybridised to the same two bands in the cosmid and genomic DNA lanes, as predicted (Figure 3.2; 3.3)

Colonies were patched onto MS agar without selection for two generations of growth before being phenotypically analysed (Figure 3.4). The mutants displayed a developmental phenotype. They develop slower that the parental strain. The aerial hyphae appears at the same time than in the wild –type but then there is a time lapse until those aerial hyphae reach maturity. *esxAB* mutants reached maturity after 7- 8 days incubation. They required up to 2-3 extra days more than the wild- type to develop. Even when they reached maturity they had a decreased number of spore chains as

demonstrated by visualizing microscopic slides at 40x -60x magnification and comparing

mutant fields to wild- type fields.

Figure 3.4. Photograph of the mutants after 48 h of growth on MS media without selection.

a, *esxA* mutant clones, top patch is M145 then clockwise clones 1-4 ;b, top patch is M145 then clockwise *esxA* mutant clones 5-8 ;c, top patch is M145 then clockwise *esxB* mutant clones 1-3.

## 3.4 Microscopic analysis of *S. coelicolor esxA* and *esxB* on solid media.

Cultures of the *esxA* and *esxB* mutants of *S. coelicolor* for light and fluorescence microscopy were set up by inserting a sterile coverslip at a 45 angle into MS agar and inoculating in the acute angle along the glass surface (Chater 1972). Coverslips were removed after 48 h incubation at 30°C and cells on the coverslip surface were stained with propidium iodide for nucleoids, and stained for the cell wall, with fluorescein-conjugated wheat germ agglutinin (Fluo-WGA) (Section 2.14). Cytological examination of the mutants revealed several aspects of their morphology. Firstly the most noticeable characteristic was the irregularity in the spore compartment sizes. In the wild- type mature hyphae there is the occurrence of some irregularities in the compartments but the frequency is very low. The norm is that the length of the compartment be between 1.5 – 1.7 μm. In the *esxAB* mutants the compartment size irregularities are much more frequent. The DNA of the apical compartment of the aerial hyphae needs to be segregated into unigenomic units in a process that is not clearly understood. There is limited knowledge about which step goes first. The DNA inside each compartment was visualized by the propidium iodide staining. In the wild- type the genetic material seems totally concentrated and segregated so that the DNA inside each cell appears as an oval-rounded sphere. The *esxAB* mutants DNA differ from the wild- type's in that it appears elongated as if the DNA is not completely segregated. In the *esxAB* mutants there were also a considerable number of ghost compartments devoid of DNA. To be able to analyse these differences quantitatively I decided to measure the interseptal distances (Section 3.5.1).

Figure 3.5 Micrographs of the M145 strain at 100 times magnification.
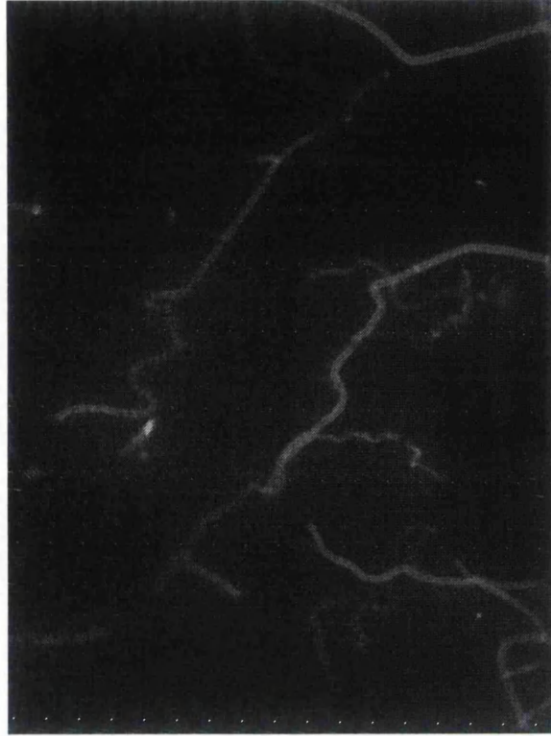
In these micrographs (a-f) the regularity in the septation of the parental strain is evident. The DNA is completely segregated and each spore compartment contains a single copy of the genome with all the compartments looking identical. Note that Fluo-WGA dye have the ability to only bind to nascent peptidoglycan unit and therefore mature spore chains may not appear in the green channel.

a

b
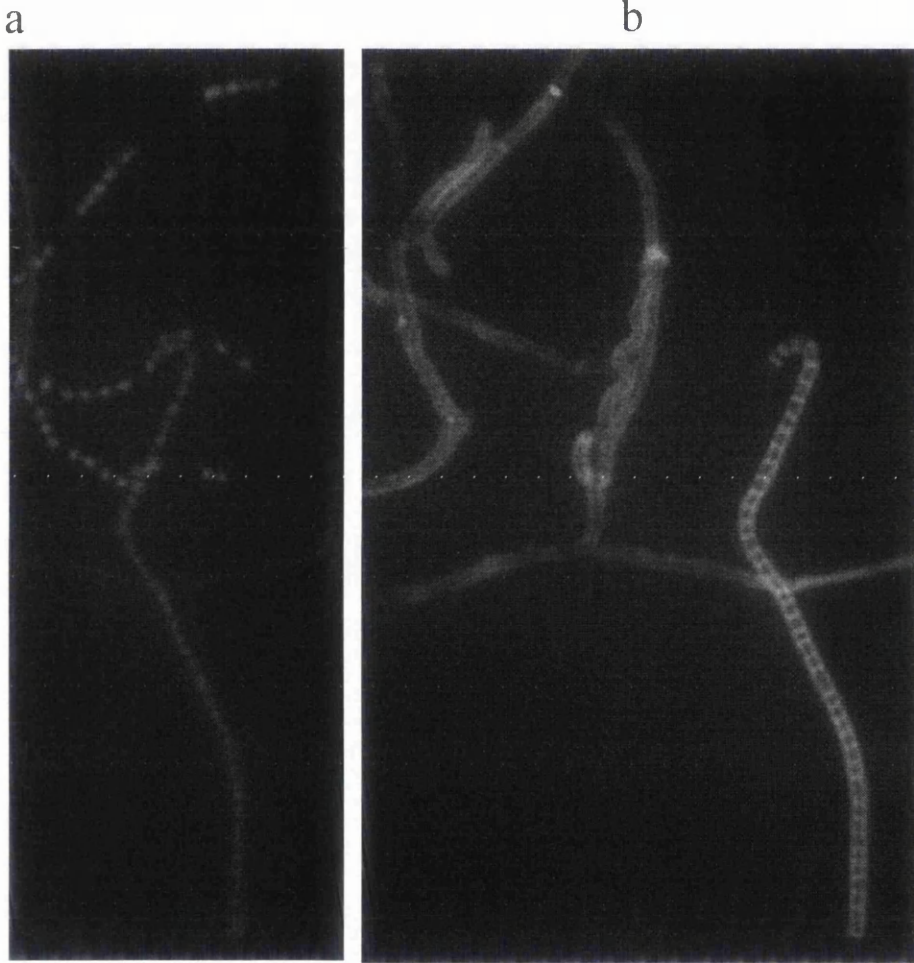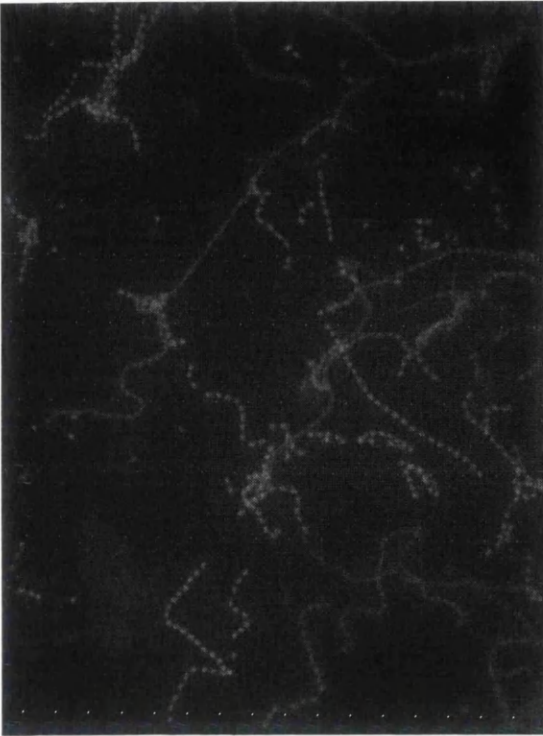
c   d
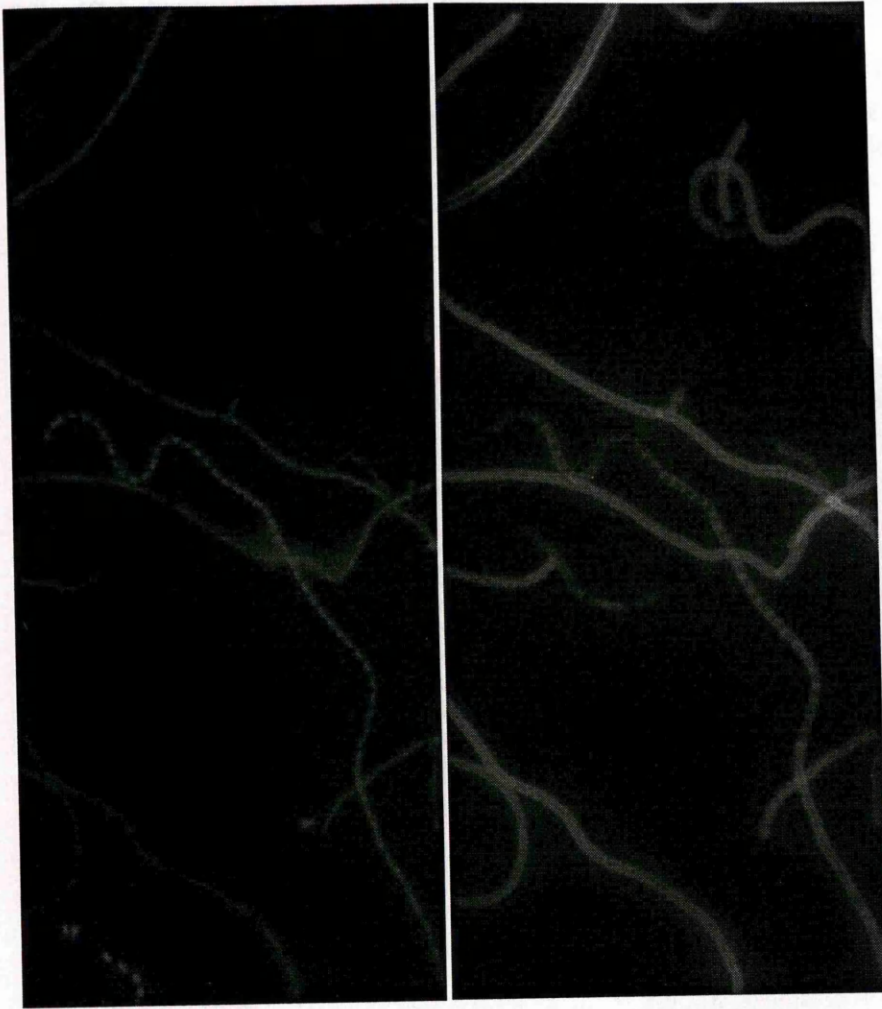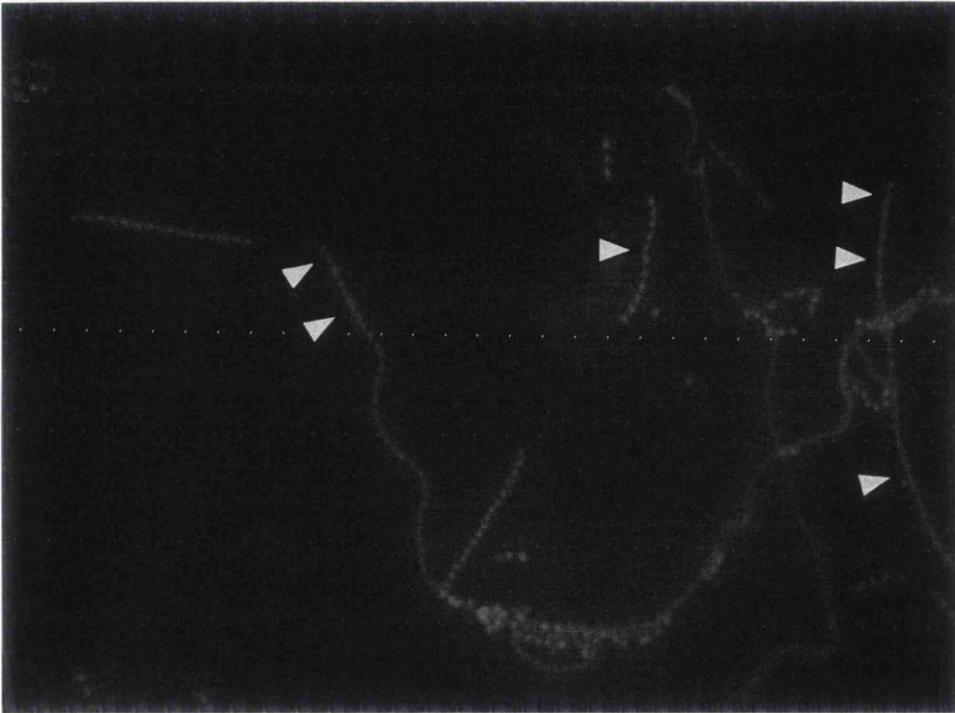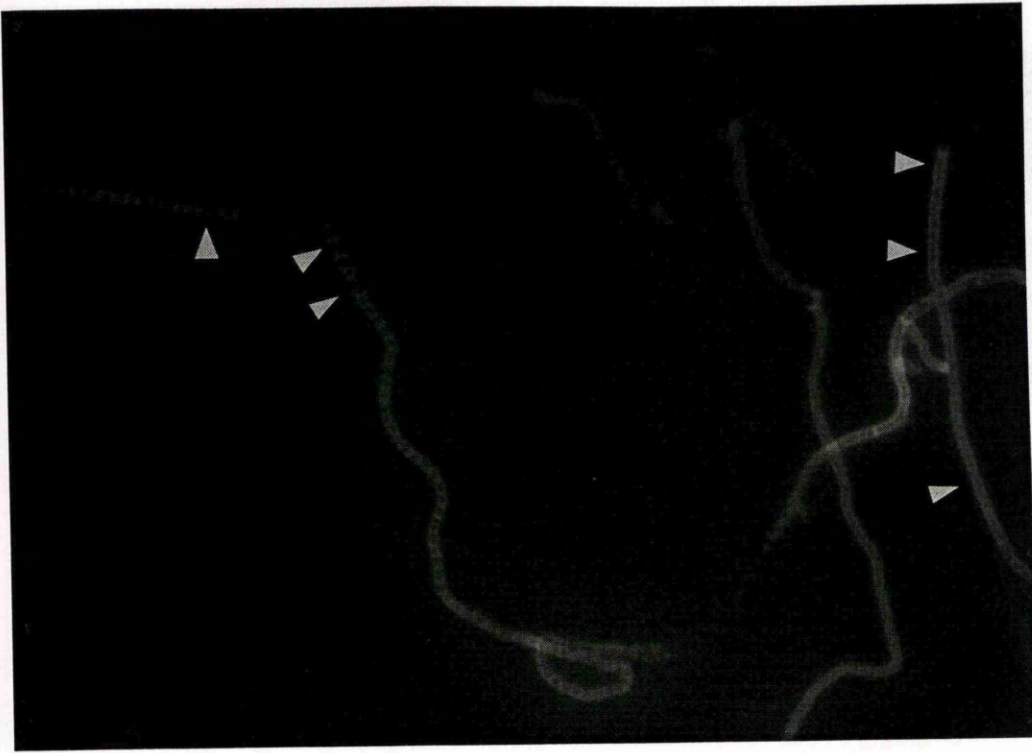
e   f

Figure 3.6 Micrographs of the *esxA* mutant at 48 h time point viewed at 100 times magnification.

These micrographs (a-d) clearly show irregularities in the septation of the *esxA* mutant. The DNA is segregated. Each spore compartment does not seem identical however you look at the Fluo-WGA staining of the cell wall or the propidium iodide DNA staining. The arrows indicate irregularities.

a

b

c

d

Figure 3.7 Micrographs of the *esxB* mutant strain at 48 h of growth viewed at 100 times magnification.

These micrographs (a-d) clearly show irregularities in the septation of the *esxB* mutant. The DNA is segregated. Each spore compartment does not seem identical however you look at the Fluo-WGA staining of the cell wall or the propidium iodide DNA staining. The arrows indicate irregularities.
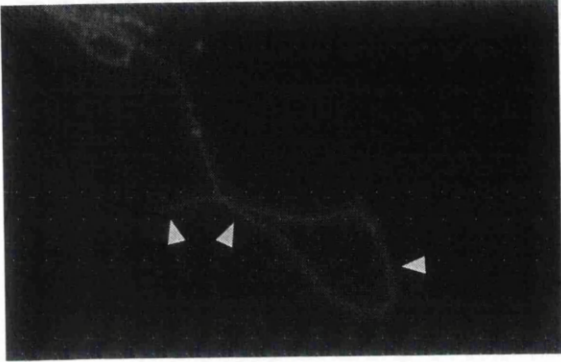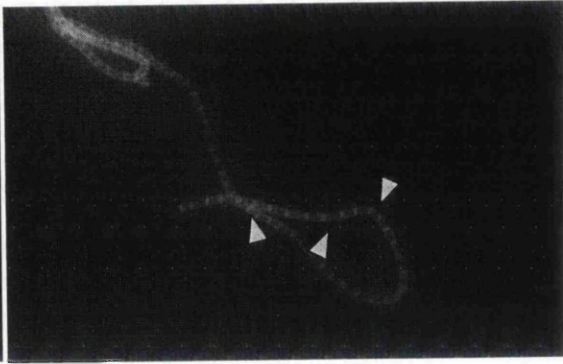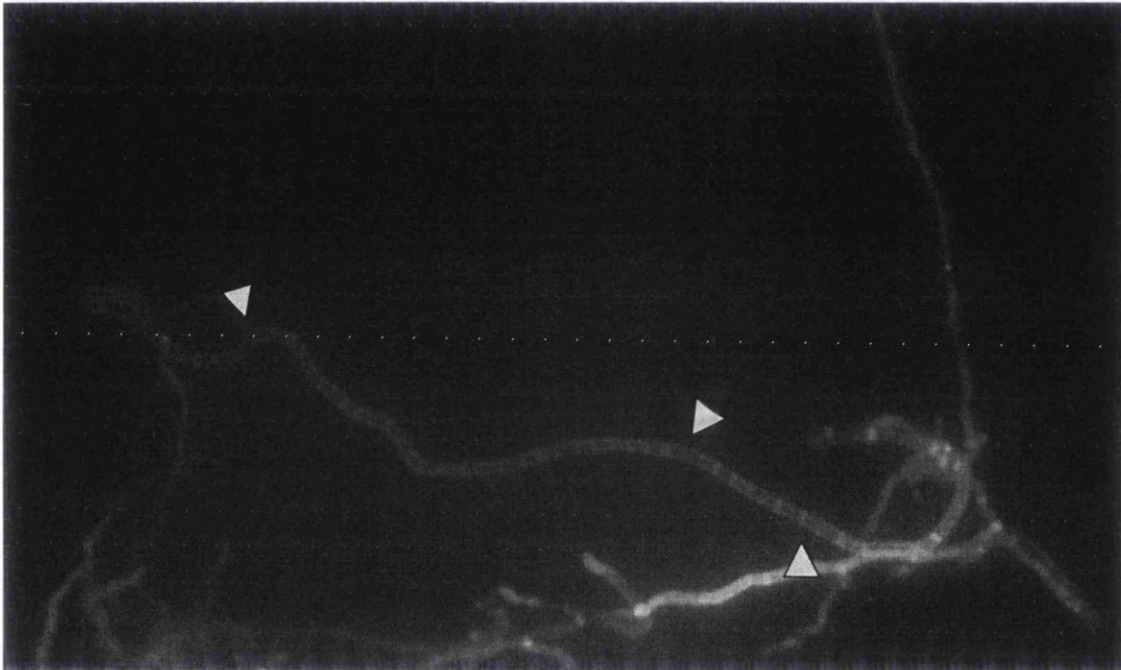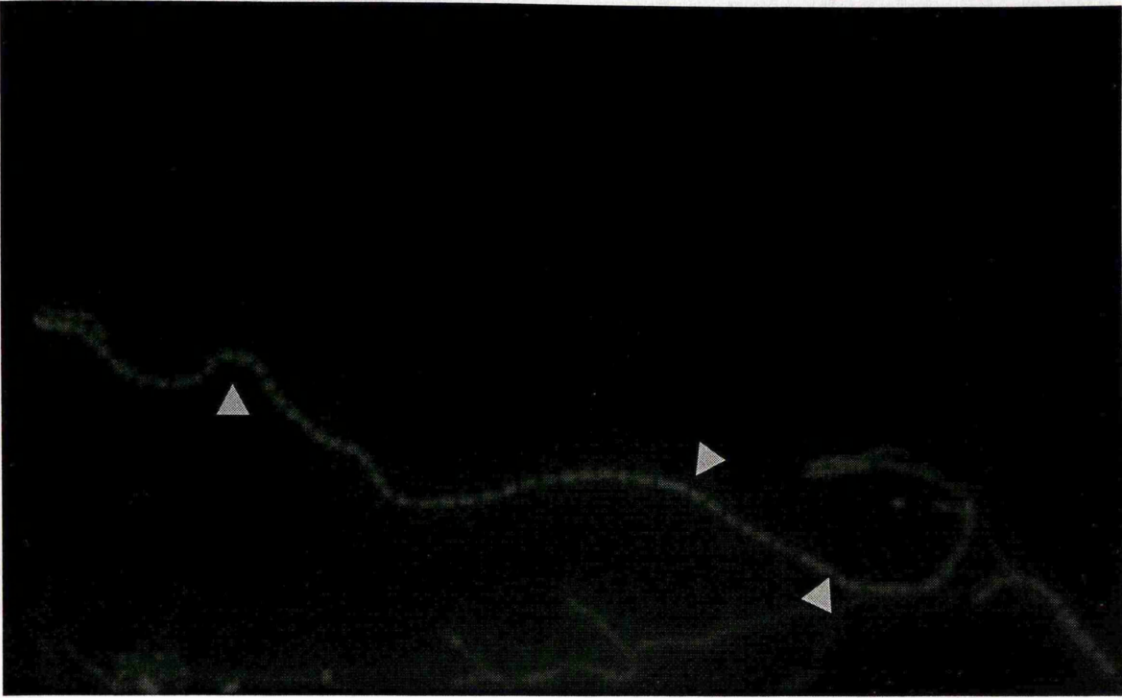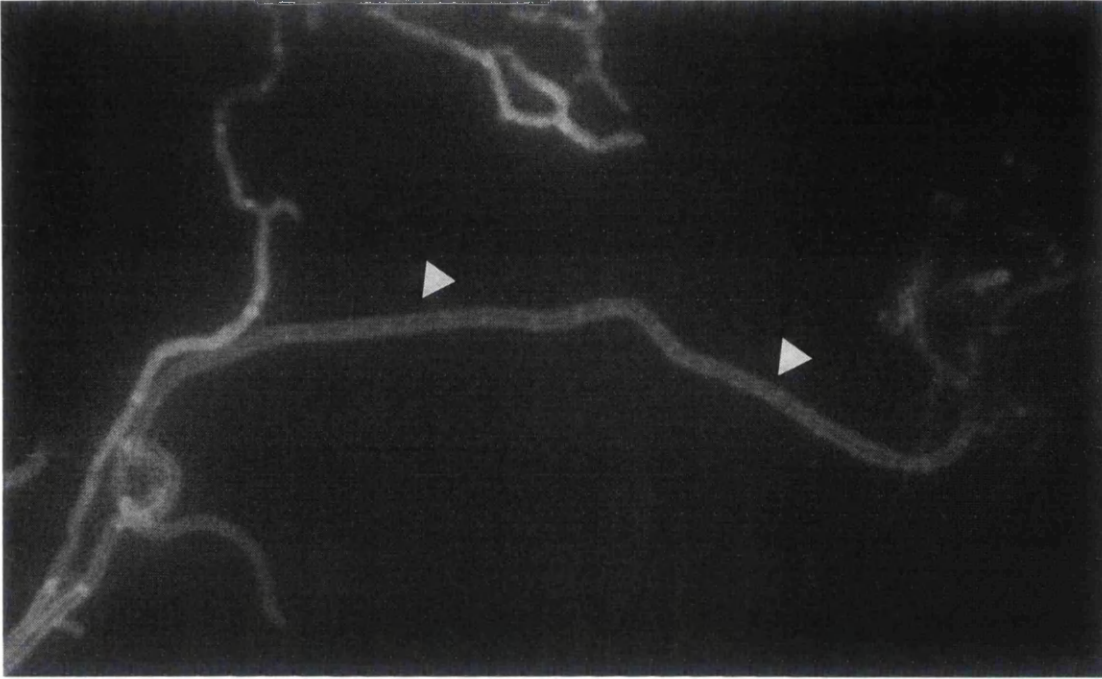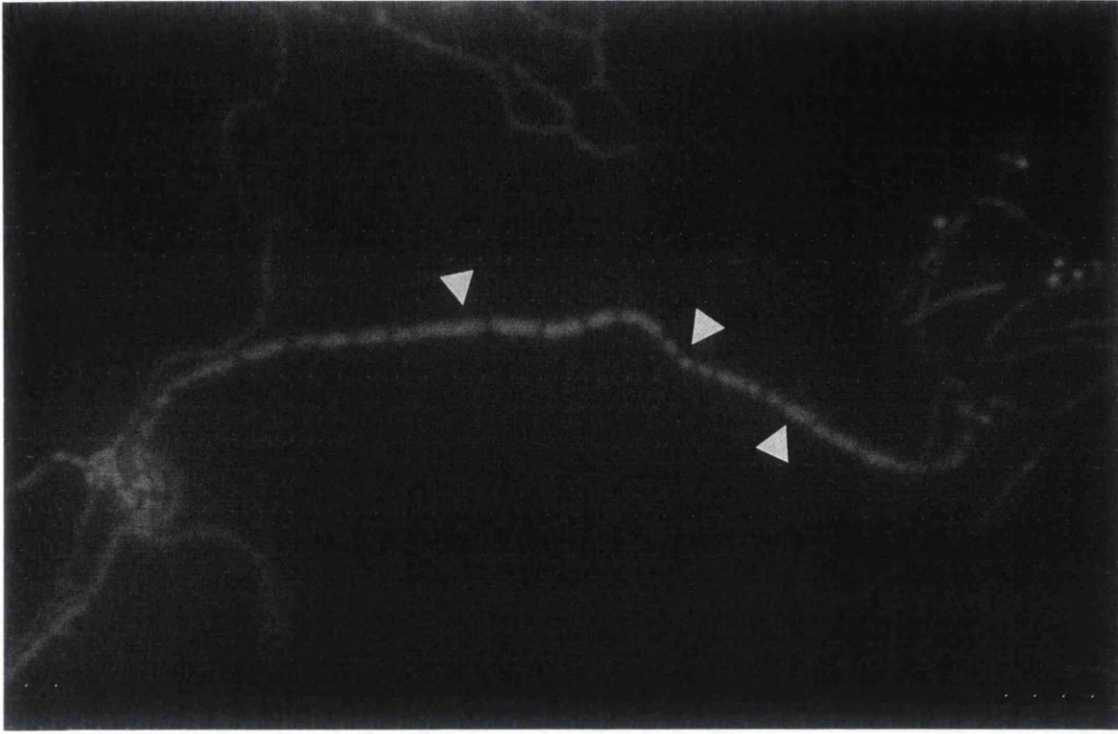
a

b

c

d

3.4.1 Statistical analysis of the spore compartment length in *esxA* and *esxB* mutants.

Microscopic analysis of the *esxA* and *esxB* mutants revealed that there was a slight variation in the pattern of septation. Three strains were analysed: The wild-type, *esxA* mutant and *esxB* mutant microscopic samples were taken at 48 h and stained with Fluo-WGA. The total number of compartments counted was: for the wild-type 415 compartments, *esxA* mutant 741 compartments and *esxB* mutant 489 compartments. The program used to measured the interseptal distance was Scion scientific available from http://www.scioncorp.com/. With the data obtained a histogram was constructed for each mutant and the wild-type strain. The data corroborated my visual observations. There seem to be irregularities in the size of the interseptal space. But the results provided me with further information about the irregularities.

In the wild-type (figure 3.9) the mode value was 1.2 μm. The total number of interseptal compartments of size 1.2 μm was 67 from a total of 415 that make up a 16 *per cent* of the total. The histogram shows a normal distribution centred around value 1.2 μm and then there is a small peak distributed between 2.1 and 2.7 μm. The normal interseptal space measured between 1.1 and 1.4 μm. The percentage of compartments that falls within the normal distribution is 97%. The small population constitutes only 2% of the whole. This indicates that 97% of the compartments fall within a normal distribution.

In the *esxA* mutant the histogram (figure 3.10) described two distinct peaks that seem to represent two populations. The mode value was 1.3 μm and for this particular interseptal size there were 84 events. Then there was another peak of 0.3 μm with 34 events. From a

total of 741 the peak of 1.3 μm constitutes an 11 *per cent* of the total and the peak of 0.3

μm represents a 4.6 *per cent* of the total. This result meant that in *esxA* the majority of the

compartments were slightly bigger than in the wild-type and also it meant that there were

a large number of smaller sized compartments below the average standard measurement.

The percentage of compartments distributed between (0.1-0.5 μm) was 7 *per cent*. The

percentage of compartments distributed between 0.6 and 2.5 μm was 92 per cent of the

total.

In *esxB* mutant the histogram (figure 3.10) also showed two distinct peaks that seem to

represent two populations of a different nature from *esxA*. The mode value was of 1.4 μm

with 42 events. Then there was another peak of 2.4-2.5 μm with 12 events. From a total

of 489 the peak of 1.4 μm constitutes an 8.6 *per cent* of the total and the peak of 2.4-2.5

μm represents a 2.4 *per cent* of the total. This result meant that in *esxB* the majority of the

compartments were slightly bigger than in the wild-type and also it meant that there were

a large number of bigger sized compartments above the average standard measurement. .

The percentage of compartments distributed between (2.2-2.9) was 9 *per cent*. The

percentage of compartments distributed between (0.6-2.5) constitutes 90 *per cent* of the

total.

I decided to use a t-Test for independent samples because the measures had a relatively

normal distribution, and although I have more than two conditions, I intended to compare

the conditions in pairs (the data obtained from each *esxA* and *esxB* mutants with the wild-

type individually). The data was tested for the differences between the means. The idea

was to prove that the differences between the *esxAB* mutants and the wild- type did not arise by chance.

The differences between *esxA* and the wild- type were significant. From table E, $t = 1.960$ at the 0.05 level of significance ($p= 5$ per cent) with ∞ degrees of freedom. The observed value of t was 2.79 that is numerically greater than 1.960. Therefore we can assume that the absence of the gene has an effect on the length of the septa.

The differences between *esxB* and the wild- type were also significant. From table E, $t = 1.960$ at the 0.05 level of significance ($p= 5$ per cent) with ∞ degrees of freedom. The observed value of t was 8.86 that is numerically greater than 1.960. Therefore we can assume that the absence of the gene has an effect on the length of the septa. The raw data for these calculations and the table for the t-Test value can be found in the Appendix.

Figure 3.8 Histogram representing the frequency of different sized septa in the wild-type.

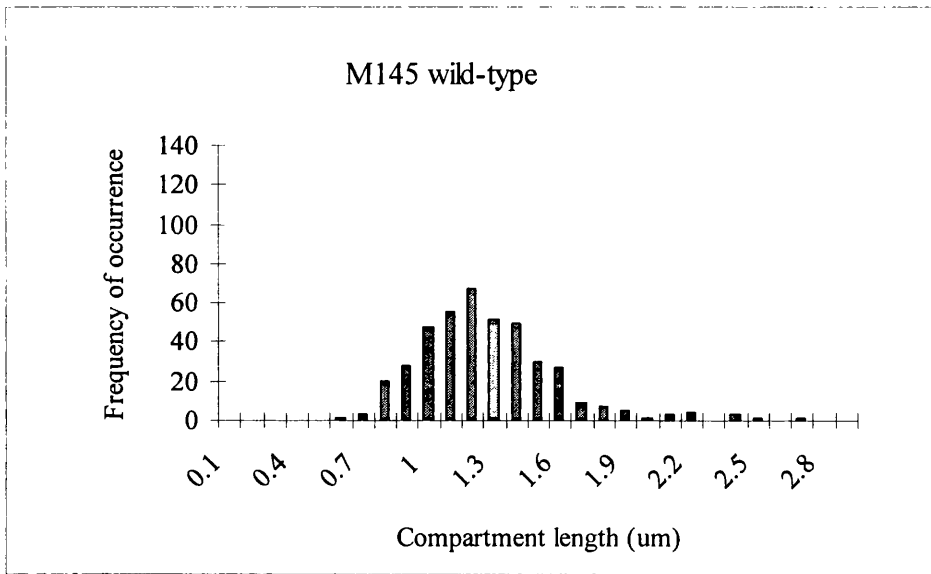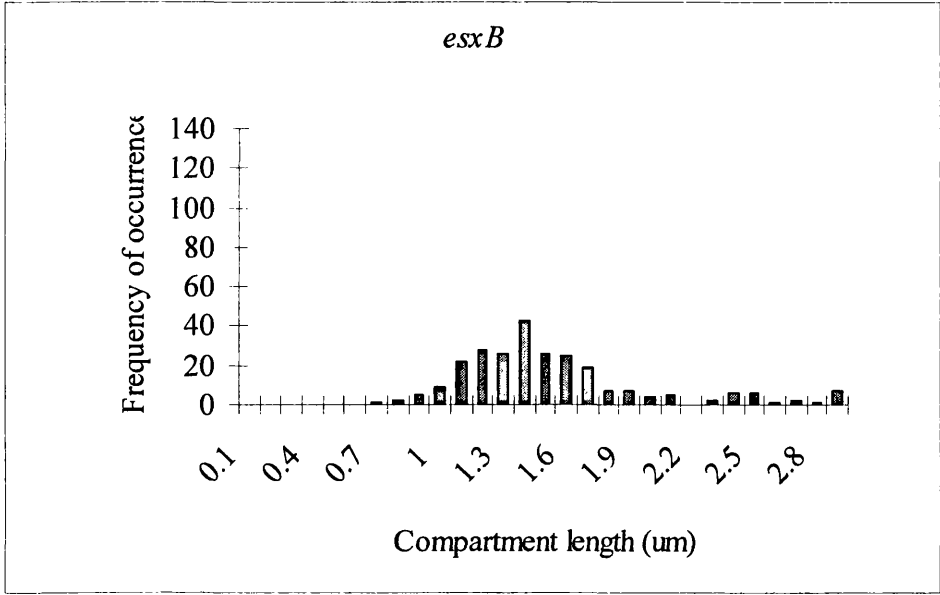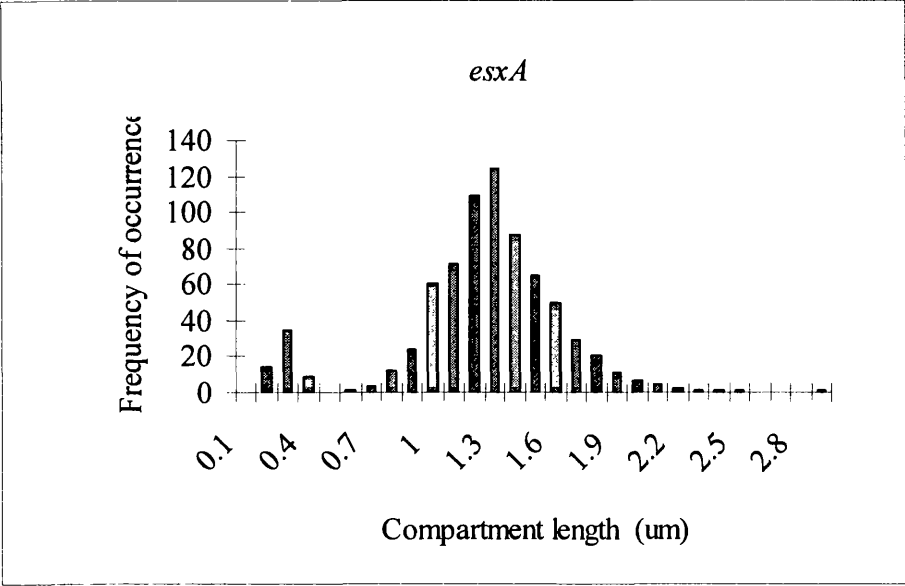**Figure 3.9 Histogram representing the frequency of different sized septa in the *esxB*.**

esxB

Frequency of occurrence

Compartment length (um)

Figure 3.10 Histogram representing the frequency of different sized septa in the

esxA **mutant.**

3.4.2 Atomic force microscopy (AFM) analysis of *esxAB* mutants spore morphology.

Atomic force microscopy (AFM) is a new imaging method discovered in 1986. Like other scanning probe microscopes, AFM utilises a sharp probe moving over the surface of a sample in a raster scan. In the case of the AFM, the probe is a tip on the end of a cantilever which bends in response to the force between the tip and the sample. I used this new imaging technique to visualize the surface of the wild- type and *esxAB* mutants and examine the differences among them (figure 3.12; 3.13 and 3.14). The results from the AFM showed striking differences between the parental strain and the mutants. The wild- type mature spores appeared smooth and full- bodied, while on the other hand the *esxAB* mutant micrographs showed sporulating hyphae that seem crumpled as if the spores were dehydrating. *esxAB* spores seem slightly thinner and the structure of the molecules that coat the hyphae seem altered somehow accumulating numerous amounts of molecules at septa interface. The differences appear to be due to less separation of the spores following cell division in the mutants. In the wild- type the cell wall is extensively remodelled at each division site and the individual spores then round-up. This does not appear to happen at 48 h in the mutants.

Figure 3.11 AFM micrograph of the wild-type after 48 h of growth on MS media without selection.

The surface of the spores in the parental strain M145 appears spherical all along the surface. There is one concavity in each spore only on one side of the spore.

0.0   2: Phase   4.1 µm

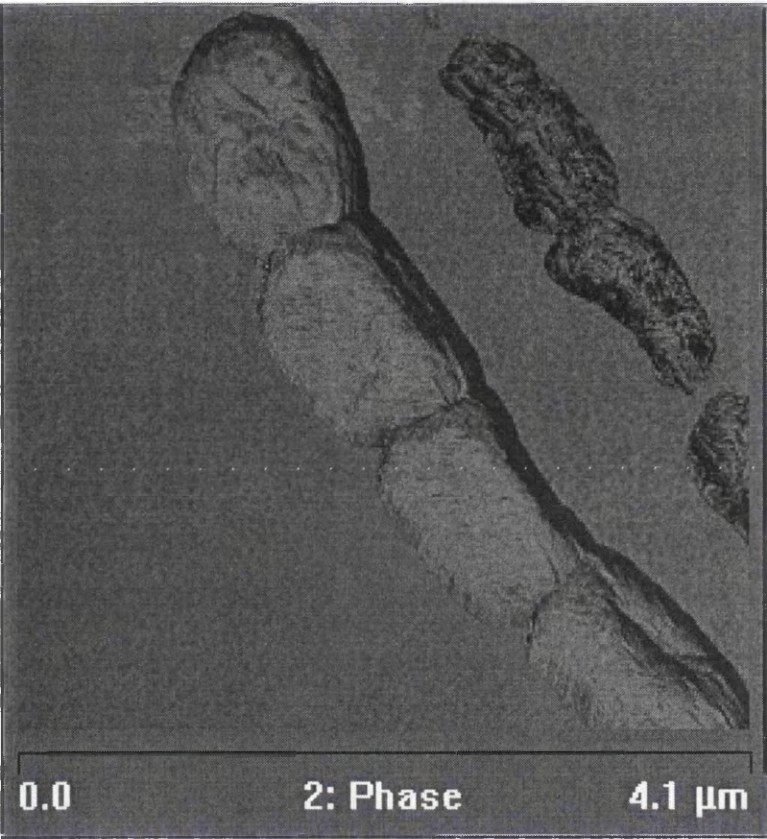**Figure 3.12 AFM micrograph of the *esxA* mutant after 48 h of growth on MS media without selection.**

The *esxA* mutant spores appear wrinkled and crumpled.
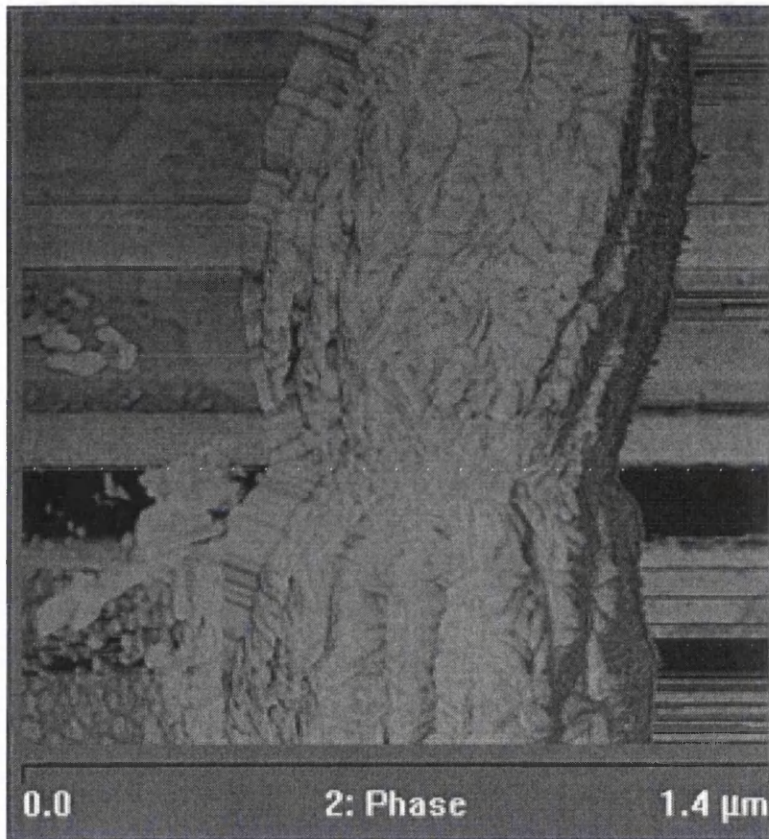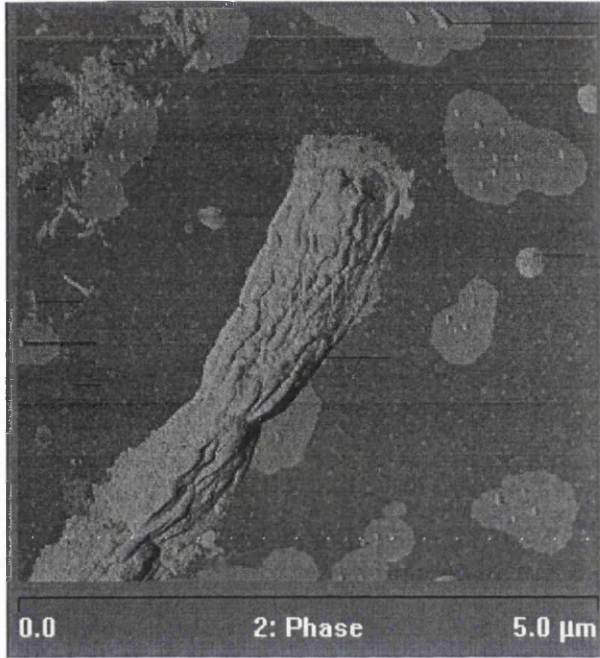
Figure 3.13 AFM micrograph of the *esxB* mutant after 48 h of growth on MS media without selection.

The *esxB* mutant spores resemble the phenotype of the *esxA* mutant being totally creased on the entire surface of the mature sporulating hyphae.

3.5 Morphological analysis of the complemented *esxA* and *esxB* mutant on solid media.

The final experiment to conclude the gene knock-outs were responsible for the phenotype was to complement the mutation. The mutant cosmid library facilitates the cloning of genes for complementation. By selecting a Tn*5062* insertion in a region flanking the gene of interest and choosing suitable restriction sites, the gene can be excised and cloned together with *aac3(IV)*, permitting positive selection (apramycin-resistance). The gene of interest was sub cloned into an integrative plasmid (section 3.3) for use in the complementation experiment.

3.5.1. Construction of an integrative plasmid containing *Streptomyces coelicolor esxA* and *esxB* genes.

Plasmid pSAHIS1 was made using pSET152 *Bam*HI- *Eco*RV as the plasmid backbone, ligated to pSAXhoI. Plasmid pSAXhoI is a derivative of pSA2 with an in-frame *Xho*I site at the end of esxA coding sequence (introduced by site – directed mutagenesis see Chapter 2). pSAXhoI digested *Bam*HI-*Xho*I was ligated to both *Xho*I-*Sma*I fragment from pET26S(+) containing a Histidine tagged and the pSET152 as the plasmid backbone (Figure 3.14). All ligated in this order, purified plasmids were subject to restriction analysis and from those containing the insert in the desired orientation one was selected for sequencing and further steps. The plasmid pSAHIS1 (Chapter 2), was modified to be able to complement the mutants *esxA* and *esxB* that were apramycin resistant. The apramycin cassette was replaced by the hygromycin cassette to create pSAHIS2 (Chapter 2). Both plasmids contain *esxA* carboxy terminal Histidine tagged and *esxB* coding

sequences under the control of their native promoter. Figure 3.15 describes the cloning procedure.

Figure 3.14 Construction of an integrative plasmid pSAHIS1 containing *esxA* carboxy terminal histidine tagged and *esxB* genes.

Plasmid pSAHIS1 was made using pSET152 *Bam*HI- *Eco*RV as the plasmid backbone, ligated to pSAXhoI (derivative of pSA2 with an in-frame *Xho*I site at the end of esxA coding sequence) digested *Bam*HI-*Xho*I and *Xho*I-*Sma*I fragment from pET26S(+) containing the Histidine tag. All fragments were ligated in this order.
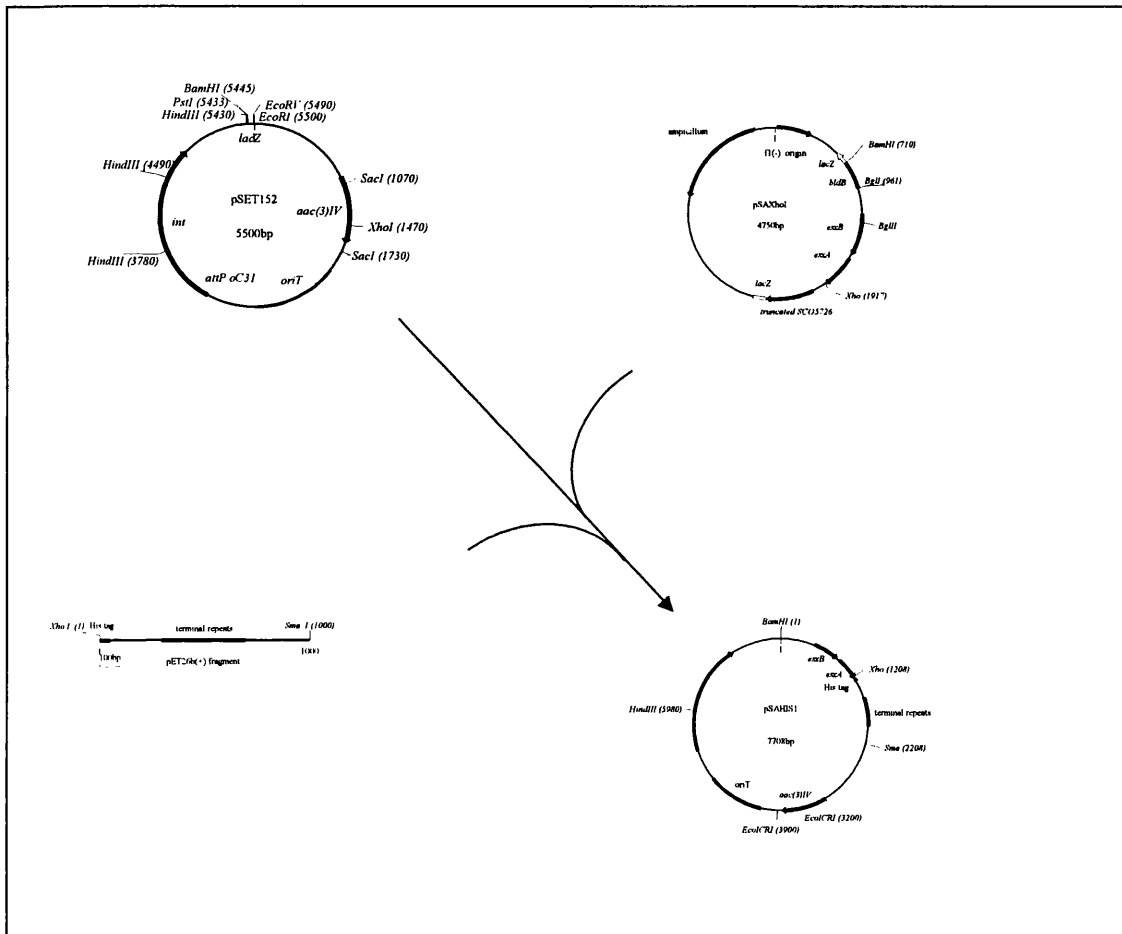
Figure 3.15 Construction of a pSAHIS2 hygromycin resistant integrative plasmid containing *esxA* carboxy terminal histidine tagged.

Plasmid pSAHIS1 (Chapter 2), was modified to be able to complement the mutants *esxA* and *esxB* that were apramycin resistant. The apramycin cassette was replaced by the hygromycin cassette to create pSAHIS2.
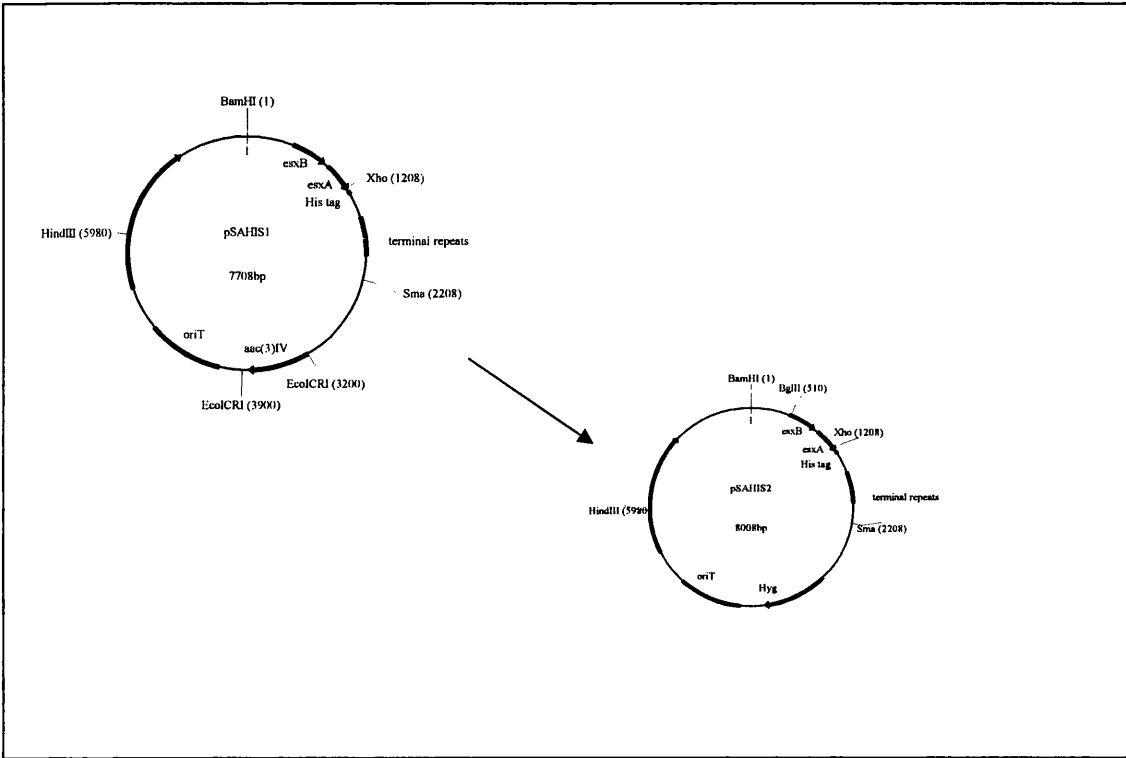
After incubation for 5 days to allow aerial development, esxA/pSAHIS2 exhibits a wild type phenotype, developing a grey aerial mycelium, confirming that *S. coelicolor esxA* phenotype is solely due to the disrupted *esxA* and *esxB* genes. This complementation restores the native dark grey pigmentation of the wild-type. The strains that contain the pSH152 integrative plasmid always look slightly lighter than the complemented strain. To account for this difference M145/ pSH152 was used as a control.

Figure 3.16 Photograph of the complemented *esxA* mutants after 48 h of growth on MS media without selection.

esxA mutant clones, top patch are M145 then anti-clockwise *esxA* mutant, then five pSAHIS2/*esxB* clones.
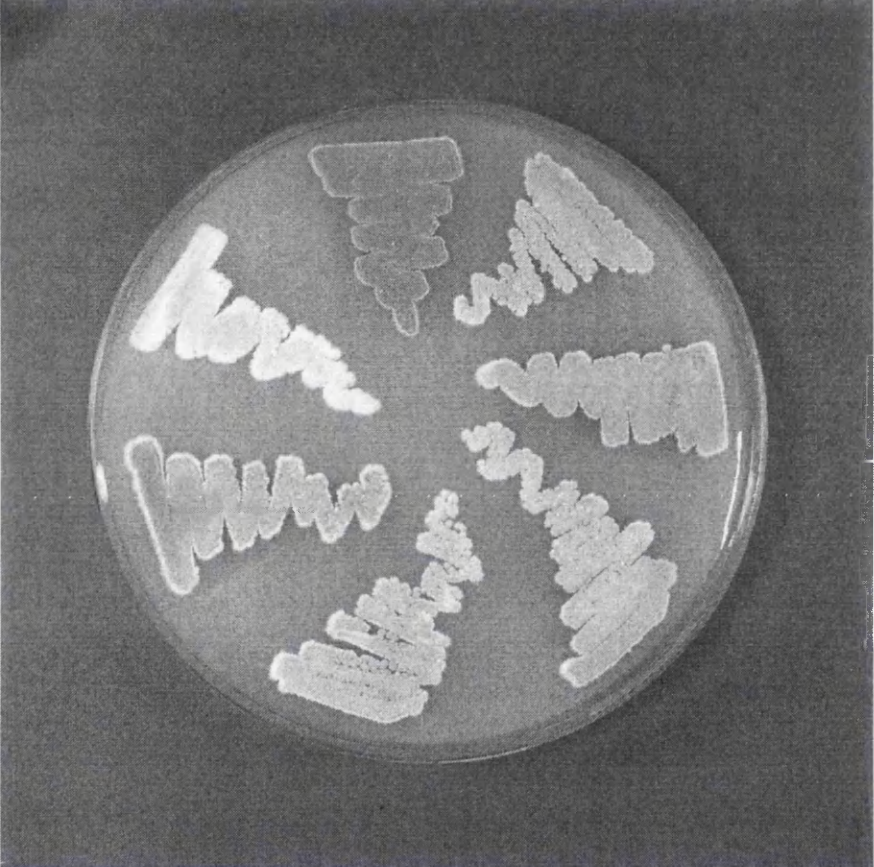
Figure 3.17 Photograph of the complemented *esxB* mutant after 48 h of growth on MS media without selection.

esxB mutant clones, top patch is M145 then anti-clockwise *esxB* mutant, then five pSAHIS2/*esxB* clones.
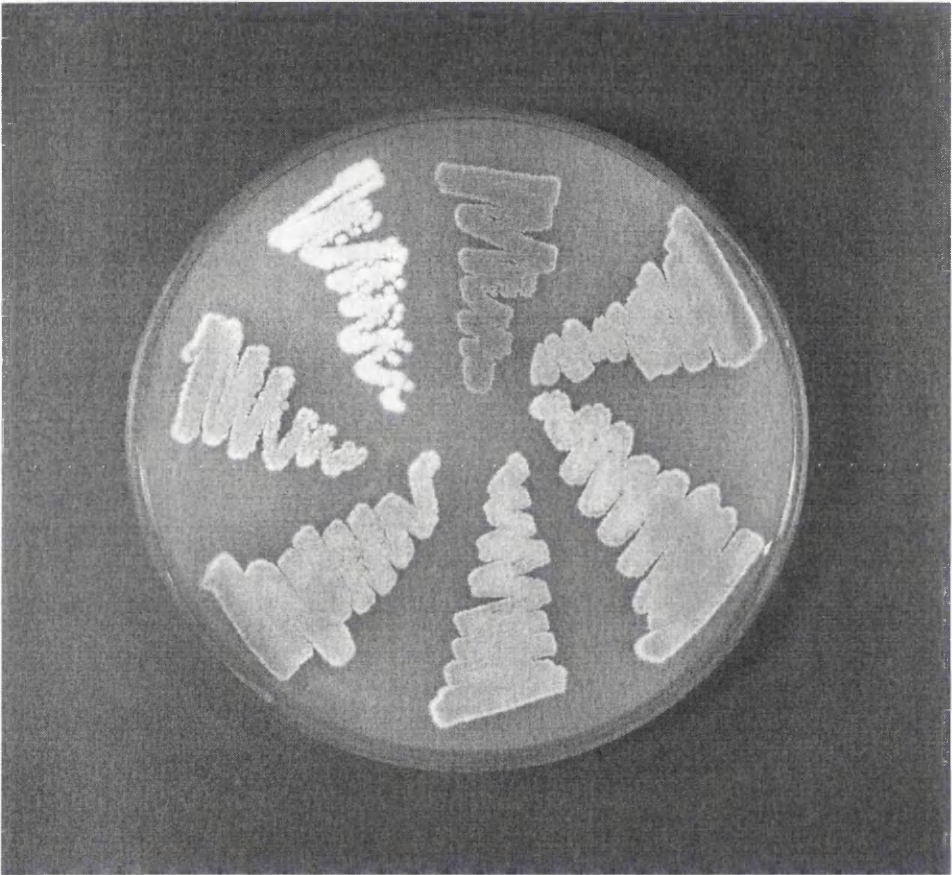
3.6 Microscopic analysis of the complemented *esxAB* mutants

The analysis of the complemented mutant was carried out exactly as for the mutant strains. Most of the analysis was performed at 48 h of growth. The microscopic observations correlate with the macroscopical results and yielded the expected results. The complemented *esxA* mutant showed an increased regularity in the shape and sizes of the spore chains. Also there seem to be an increase in the number of spore chains, this being apparent because of the increased amount of spore chains when carrying out the microscopy analysis. The analysis was completed by measuring the septa and the data analysis and histograms can be found in the Appendix in the complementation folder.

Figure 3.18 Micrograph of the complemented *esxA* mutant after 48 h of growth on MS media without selection

Figure 3.19 Photograph of the complemented *esxB* mutant after 48 h of growth on MS media without selection.

3.7 Conclusion.

These results constitute the first evidence in *Streptomyces coelicolor* of the *esxAB* being functional and possibly having a role in morphogenesis of the bacteria. Mutants of *esxA* and *esxB* display a delay *whi* phenotype and have a defect in the spore maturation phase of growth. Microscopical studies showed that the *esxA* and *esxB* mutants have in general greater sized compartments than the wild-type. The *esxA* mutant also had a population of compartments that were smaller in size. On the other hand the *esxB* mutant had a population of compartments that were larger in size about double the size of a standard wild-type compartment. The perimeter encircling the DNA usually coincides with the volume of the DNA molecule that it encloses. Both the *esxA* and *esxB* mutants were complemented with the pSAHIS2 plasmid demonstrating that their phenotypes were solely due to the disruption mutations in their respective genes.

CHAPTER IV

EXPRESSION STUDIES OF *STREPTOMYCES*

COELICOLOR esxAB.

Chapter IV. Expression studies of *Streptomyces coelicolor esxAB*.

4.1 Introduction.

In this chapter I discuss the analysis of the expression of *esxAB*. Firstly I confirmed by reverse transcriptase-PCR (RT-PCR) that the genes are co-transcribed and therefore constitute an operon. This is important to corroborate that the genes have a common purpose in the bacteria as functionally related coding frames are usually grouped together. Then I constructed a plasmid containing the promoter region of the genes. I fused this region to the *luxAB* cassette to assess the activity of the promoters. I performed these experiments in the wild-type background and in the *bldB* mutant background. I constructed the *bldB* mutant using the hygromycin resistant transposon Tn*5066*.

4.2 Co-expression of *esxAB* in *S. coelicolor*.

The ORFs encoding the proteins, *esxB* and *esxA*, are 374 and 311 bp, respectively, separated by a 37-bp DNA region (Fig. 4.1). The short intergenic space suggests that these two genes may be transcribed as one mRNA unit. This was investigated by RT-PCR with RNA isolated from cultures of *S. coelicolor*. A specific primer set was designed so that the forward primer (All2S1-primer) carried a sequence from the 5' region of *esxB* and the reverse primer (All2SA1-primer) was 715 bp downstream and had a sequence complementary to that of the 3' terminus of *esxA* (see Appendix for sequences). RNA was isolated from *S. coelicolor* after growth for 48 h in broth culture; prior to cDNA synthesis, RNA was treated with RNase-free DNase I (Roche) at 37°C for 20 min. The experiment had two steps: Reverse transcription was carried out in with the reverse primer, and the resulting products were amplified by PCR. A cDNA species was obtained from this RNA S1 obtained by amplifying using the

forward primer All2S1 and reverse primer All2AS1. The PCR reaction was carried out using the S1 cDNA species. A single amplicon of the expected size 715 bp was obtained (Fig. 4.2), and it was confirmed by nucleotide sequencing that the amplicon carried the *esxAB* gene pair. No amplicon was obtained in the absence of reverse transcription. This experiment was carried out in duplicate obtaining the same result on both experiments.

4.3 Co-expression of *esxA*, *esxB* and *SCO5726* in *S. coelicolor*.

There was a lack of homology information about the *SCO5726* gene. The coding sequence is not homologous to any sequence described in the literature. We decided to attempt to investigate whether the gene was also co-transcribed with the *esxAB* pair. The ORFs encoding the proteins, *esxA* and *SCO5726*, are 311 and 465 bp, respectively, separated by a 120-bp DNA region (Fig. 4.1). The intergenic space suggests that these two genes may or not be transcribed as one mRNA unit. This was investigated by RT-PCR with RNA isolated from cultures of *S. coelicolor*. A specific primer set was designed so that the forward primer (All3S1-primer) carried a sequence from the 5' region of *esxB* and the reverse primer (All3SA1-primer) was 1300 bp downstream and had a sequence complementary to that of the 3' terminus of *SCO5726* (Chapter 2). RNA was isolated from *S. coelicolor* after growth for 48 h in broth culture; prior to cDNA synthesis, RNA was treated with RNase-free DNase I (Roche) at 37°C for 20 min. The experiment had two steps: Reverse transcription was carried out in with the reverse primer, and the resulting products were amplified by PCR. A cDNA specie was obtained from this RNA, S2 obtained by amplifying using the forward primer All3S1 and reverse primer All3AS1. The PCR reaction was carried out using the S2 cDNA species. No amplicon was obtained for S2 cDNA, the positive

controls using DNA from plasmid pSA2633 and from M145 strain genomic DNA diluted 1:10 both produced a band of 1300 bp. No amplicon was obtained in the absence of reverse transcription. This experiment was carried out in duplicate obtaining the same result on both experiments. The results suggested that the genes were not co-transcribed as depicted in figure 4.3

Figure 4.1. Diagram representing the positions of the primers to be able to evaluate co-transcription of *esxAB* and *SCO5726*.

a, position of primers All2S1, All3S1; b, position of primer All2AS1; c, position of primer All3AS1. Note that 715 bp is the distance between the forward and reverse primers.
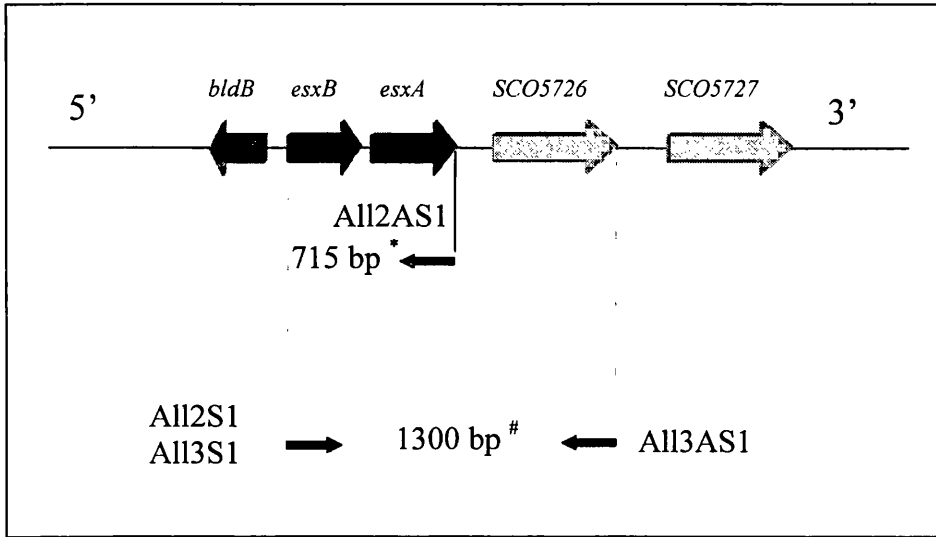
Figure 4.2. RT-PCR experiment to demonstrate co-expression of *esxA* and *esxB*.

The 1-kb ladder indicates the sizes at both sides of the gel. Lanes 2 and 3, results of amplifying PCR with water and RNA as a template. Lanes 4 and 5 positive control wild-type DNA and S1 cDNA, respectively.
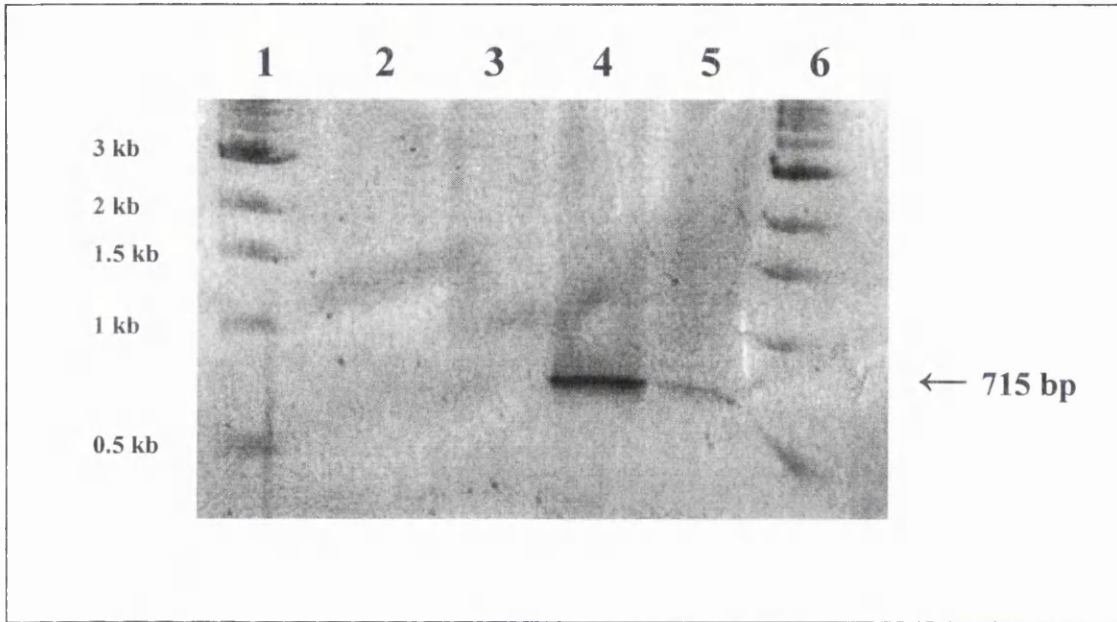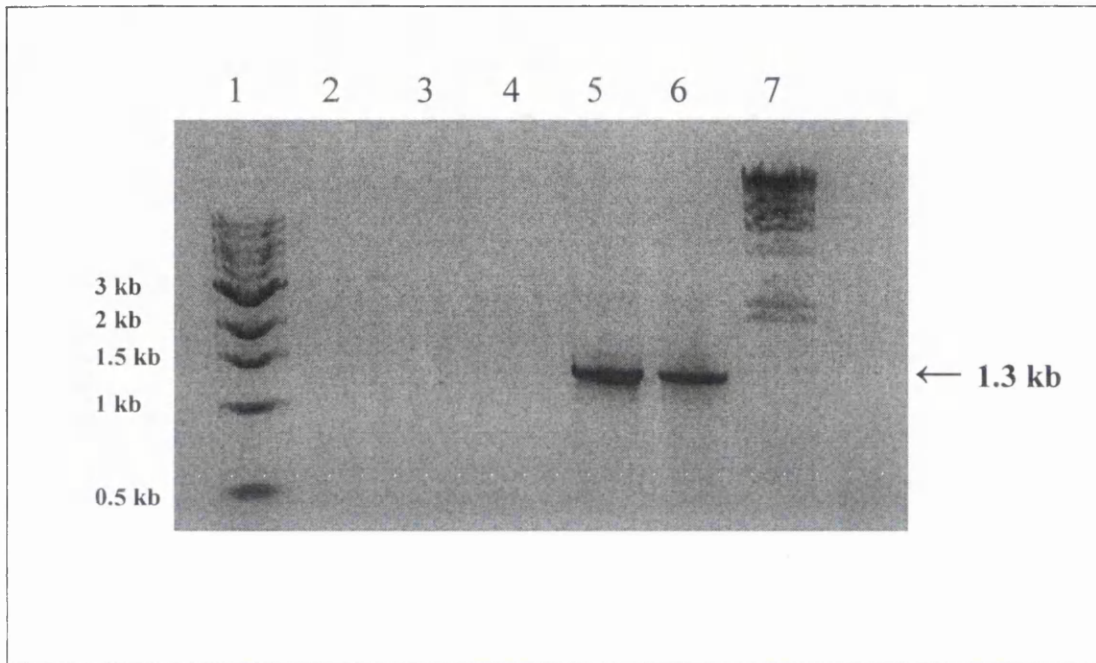
Figure 4.3. RT-PCR experiment to demonstrate co-expression of *esxA, esxB* and

SCO5726.

Lane 1, 1 kb NEB ladder. Lanes 2, 3 and 4, result of amplifying PCR with water, RNA and S2 cDNA as a template, respectively. Lanes 5 and 6 positive controls: wild-type and plasmid pSA2633 DNA, respectively. Lane 7 lambda *Hind*III ladder.

4.4 Expression studies using a transcriptional fusion to the luciferase cassette.

4.4.1 Expression studies in the wild type background.

Luciferase was preferred as the reporter gene because of its sensitivity and the possibility to perform the experiments in microtitre plates, allowing the use of various growth media and also the possibility to simultaneously measure growth and activity to normalise results.

Plasmid pSAlux1 was constructed using pRlux86 (apramycin resistant). pRlux86 was used as a negative control for the totality of the luciferase experiments. The plasmid backbone pRlux86 was restricted with *Bam*HI. A *Bam*HI/*Bgl*II 559 bp fragment from plasmid pSA2 was purified from an agarose gel and ethanol precipitated. This fragment contains the totality of the intergenic region between *bldB* and *esxB*, and 23 codons of *esxB* coding sequence. This way I could ensure that the promoter of *esxAB* would be confined in that 559 bp fragment. The *Bam*HI/*Bgl*II 559 bp fragment from plasmid pSA2 was ligated to pRlux86 restricted with *Bam*HI. The ligation reaction was electroporated into *E. coli* JM109 electrocompetent cells and plated on LB apramycin plates. Colonies carrying recombinant plasmids were grown in liquid LB apramycin and the cultures used for plasmid extraction. After restriction enzyme analysis, plasmids containing inserts were sequenced to confirm they contained the correct insert in the right orientation. The native promoter of the genes was placed in-frame with luciferase genes *luxABE*. The cloning procedure is described in figure 4.4.

Plasmids pSAlux1 and pRlux86 were introduced by intergeneric conjugation into *S. coelicolor* M145. For each of the strains, three colonies from each conjugation were selected for further studies. The preliminary experiments were carried out in solid

media and those were followed by more in-depth studies in liquid conditions using a variety of growth media. The procedure for the *lux* experiments is described in section 2.15.2.

Figure 4.4 Construction of an integrative plasmid containing the putative *esxAB*

promoter fused to the *luxAB* cassette.

Plasmid pSAlux1 was constructed using pRlux86 (apramycin resistant). A *Bam*HI/*Bgl*II 559 bp fragment from plasmid pSA2, that contained the totality of the intergenic region between *bldB* and *esxB*, and 23 codons of *esxB* coding sequence was cloned to ensure that the promoter of *esxAB* would be confined in that 559 bp fragment. The *Bam*HI/*Bgl*II 559 bp fragment from plasmid pSA2 was ligated to pRlux86 restricted with *Bam*HI.
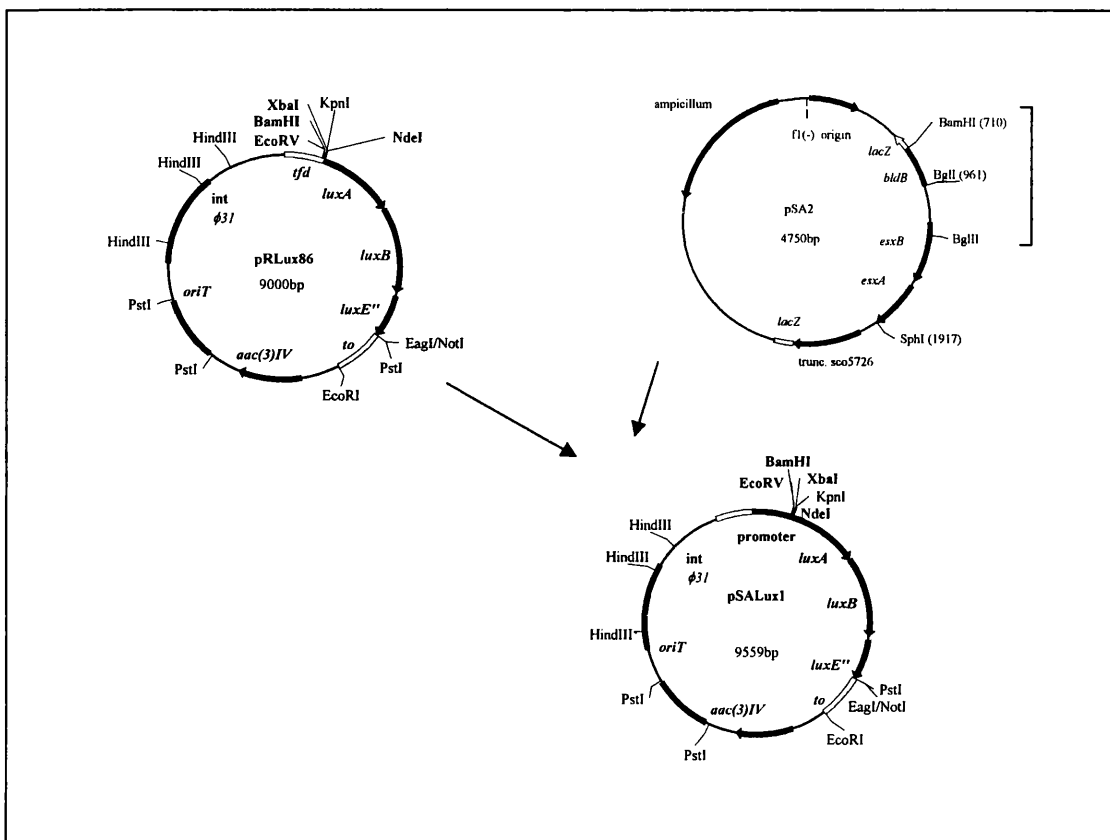
4.4.1.1 Analysis of the *esxAB* promoter in solid media

The analysis of the activity of the promoter in the wild-type was performed by growing the strain on MS media. Spore suspensions from *Streptomyces coelicolor* M145 containing pSAlux1 or pRlux86 were grown on MS solid media in 96 well black assay microplates, as illustrated in (Chapter 2). The medium was selected because of the results of the phenotypic analysis of the mutants (Chapter 3).

Luminescence was measured at 18, 24 and 48 h time points. For every biological replica, data collected were averaged and standard deviations determined. The promoter expression was plotted to be able to examine how the expression behaved as the bacterium developed.

This solid media experiment showed that in MS media the promoter reached its maximum expression at 24 hrs (Figure 4.5). Then the expression started to decreased, this preliminary experiment provided me with some clues about the pattern of expression of the promoter coinciding the maximum expression with the onset of aerial development.

4.4.1.2 Analysis of the *esxAB* promoter in liquid media

The next step was to assess the expression in more detail. I decided to utilize liquid cultures because they allow the growth rate to be accurately calculated and therefore the amount of luminescence could be corrected according to the growth of the culture. Using liquid cultures I analysed the expression of the *esxAB* promoter during vegetative growth as *S. coelicolor* does not sporulate in liquid culture. Strains of *Streptomyces coelicolor* M145 containing pSAlux1 or pRlux86 were grown on MS,

NA and TSB liquid media in sterile flasks containing stainless steel springs. At the chosen time point 1 ml of culture of each strain was withdrawn out of the flask and pipetted in triplicate into a 96 well black assay translucent bottom microplates in an orderly fashion. OD_{450} was then measured followed by the estimation of luminescence after the addition of substrate N-Decanal, as described (Chapter 2).

In the first analysis I used different growth media to evaluate the development in different environments. The results from these experiments suggested that the promoter is most active during growth in tryptone soy broth media (TSB) (Figure 4.8). When the expression was studied in conjunction with the growth rate, additional insight into the behaviour of the *esxAB* promoter was gained. In NB the promoter activity was high from the 16 h to the 24 h time point. The expression became very low at the 30 h time point and after slowly decreased until the 72 h time point (Figure 4.6). In R5 the promoter activity was high at the 16 h time point and plummeted to zero in the 24 h time point and continue at this basal level of expression until 72 h time point (Figure 4.7). The TSB plot (ratio of promoter activity versus OD) was identical to the R5 curve but the activity of the promoter reached the 100 value (Figure 4.8).

The following experiments were performed growing the strains on TSB media increasing the number of time points. I also carried out the experiments using cultures grown from a pre-culture and directly from the spore suspensions in parallel to determine whether the promoter was also active during germination. To consider the growth rate I also measured the optical density at 450 nm and calculated the ratio between the luminescence and growth rate.

The first noticeable difference between the two ways of inoculation was that the growth not surprisingly was slower when the cultures were inoculated straight from the spore suspensions. When plotting the OD alongside luminescence, the plots showed significant differences: when the cultures are grown from a pre- culture (Figure 4.9), the activity is very high from the 5 h to the 8 h time point. After the 8 h time point promoter activity decreases to very low levels. Finally the activity becomes nil 42 h. On the other hand if the cultures are grown from spore suspensions the activity of the promoter is nil until the 13 h time point (Figure 4.10). The activity is upregulated progressively until the 28 h time point and decreases but continue to be high through the 30 h time point and finally becomes nil at the 42 h time. Presumably this is due to the time lag in germination before exponential growth is achieved. To summarise, the *esxAB* promoter was active at the onset of aerial development and then the activity decreased over time indicating that it may be involved in a role on aerial development.

Figure 4.5. Analysis of the activity of the *esxAB* promoter during growth on MS solid agar using a transcriptional fusion to the *luxAB* cassette.

The red arrow indicates the onset of aerial growth.

Expression of the esxAB promoter in solid MS.
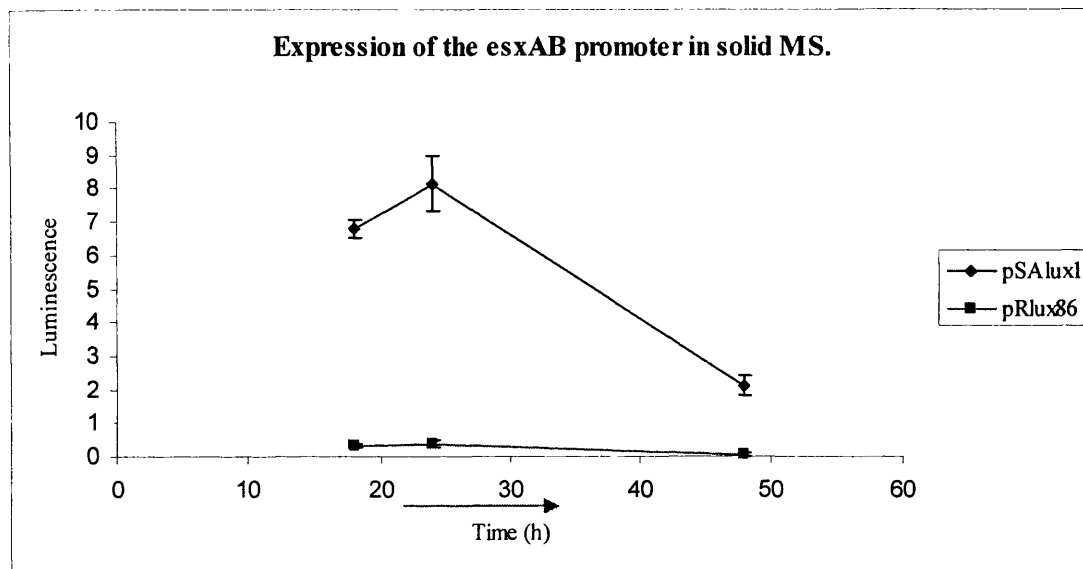
Figure 4.6 Ratio of the promoter activity versus optical density in nutrient broth.

Ratio of the luminescence versus O.D. 450 nm in NB.
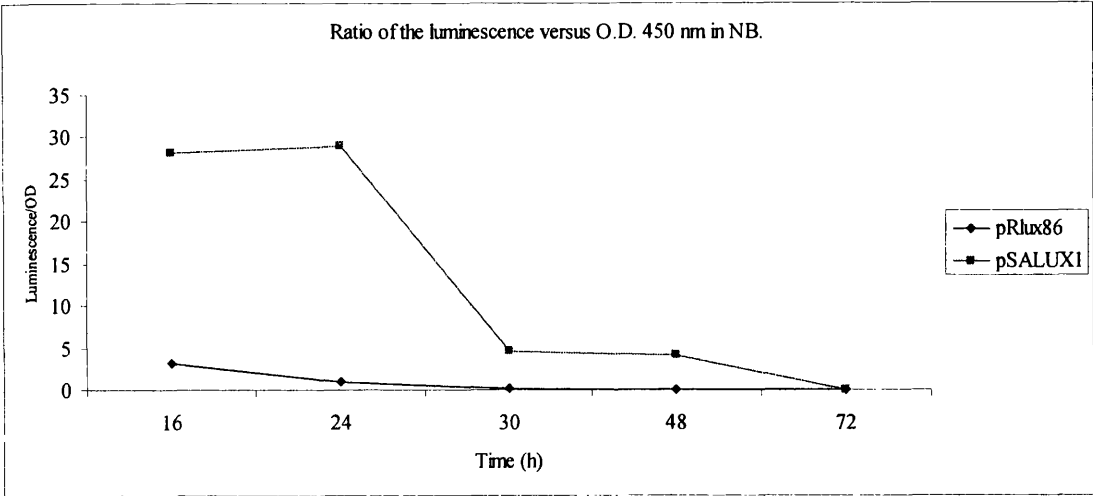
Figure 4.7 Ratio of the promoter activity versus optical density in R5 media.

Ratio of the luminescence versus O.D. 450 nm in R5 media
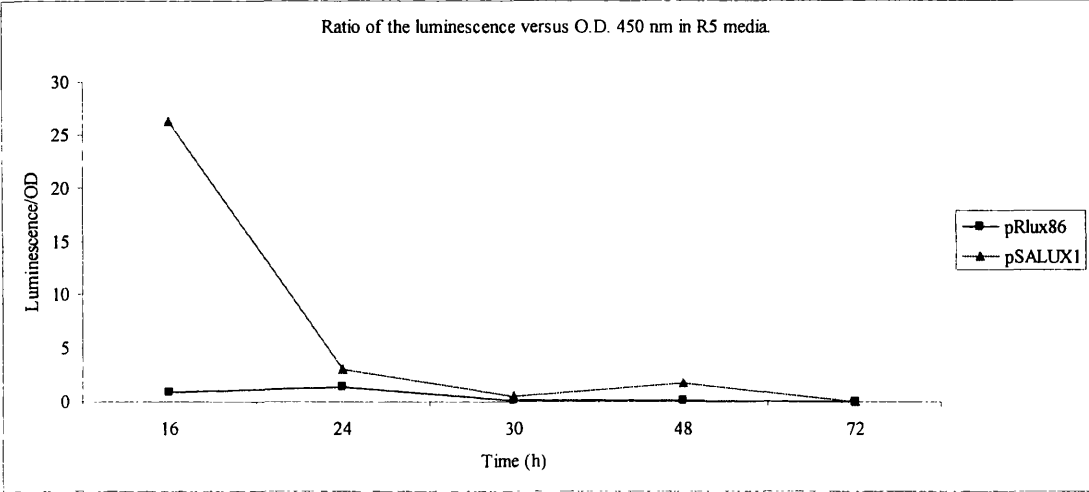
Figure 4.8 Ratio of the promoter activity versus optical density in tryptone soy broth.

Ratio of the luminescence versus OD 450 nm in TSB media.
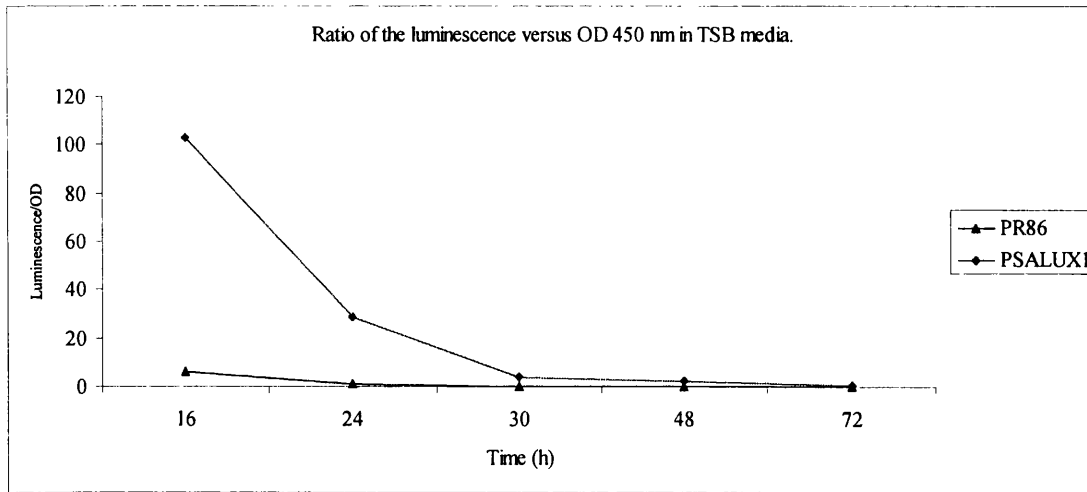
Figure 4.9 Ratio of the promoter activity versus optical density when the culture was inoculated from a pre-culture.
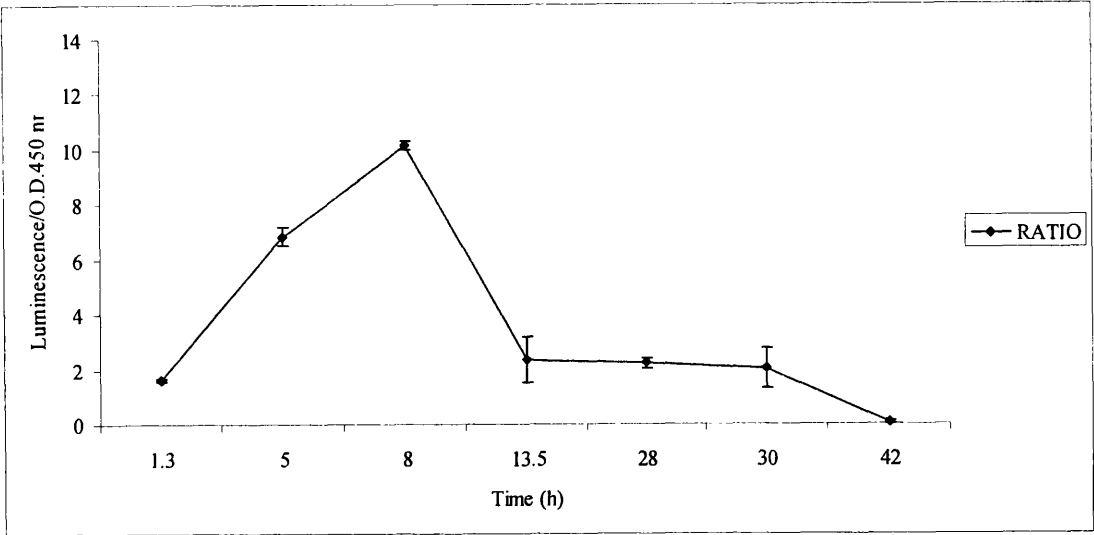
Figure 4.10 Ratio of the promoter activity versus optical density when the culture was inoculated directly from spores.
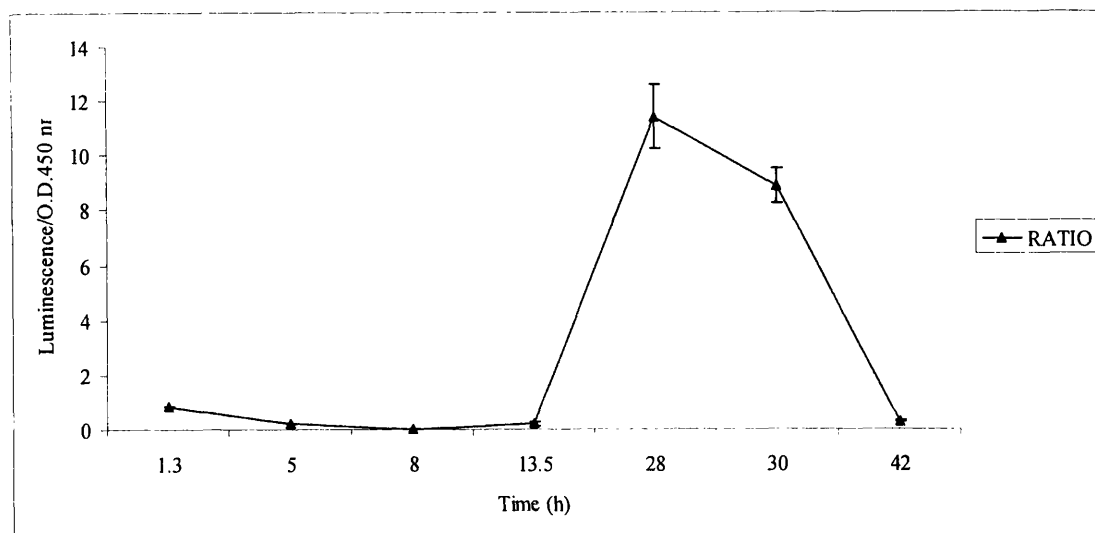
4.4.2 Expression studies in the *bldB* background.

In the wild-type background expression of the promoter starts early and activity increases progressively until the 26 h time point when it reaches the maximum activity. Activity of the promoter is down regulated from then on reaching very low activity at the 28 h time point. After that time point the activity continues to be low until it completely dies off at 42 h time point (Figure 4.11). On the other hand, on the *bldB* background the activity of the *esxAB* promoter appears to be upregulated. Activity of the promoter starts at the 23 h of growth (Figure 4.11). The promoter is only active between time points 23 h to 26 h but the activity is quite high. The modifications on the expression pattern of the promoter strongly suggest that BldB seems to have an effect on modulating the activity of the *esxAB* promoter.

Figure 4.11. Ratio of the promoter activity versus growth rate of the *esxAB* promoter in the *bldB* background (Tryptone Soy Broth).
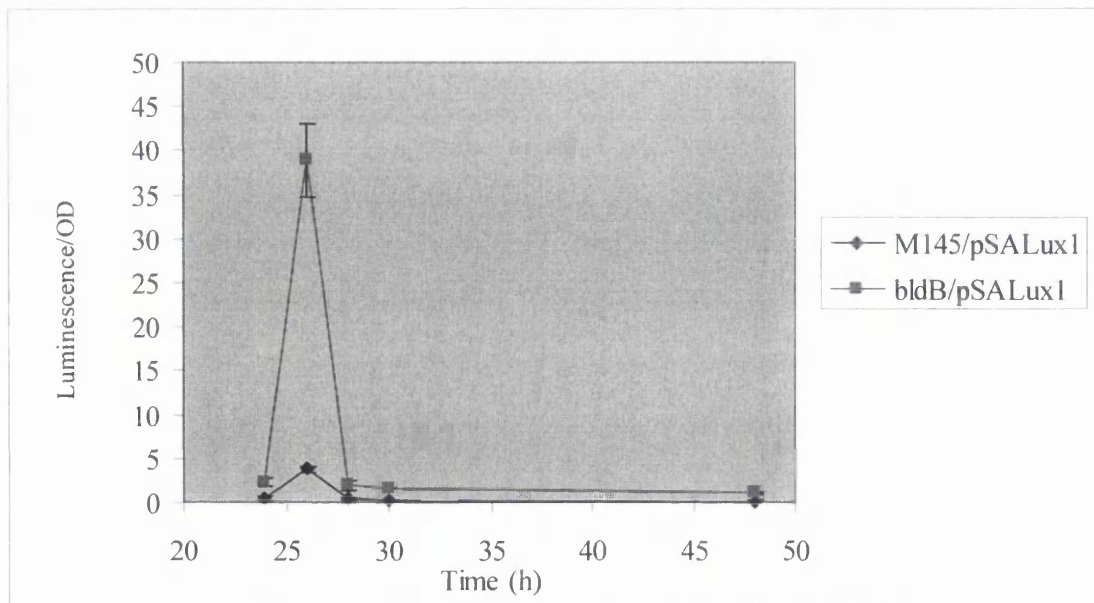
4.5 Conclusion.

The RT-PCR experiments confirm that the *esxAB* genes constitute an operon. Therefore complementation should be attainable using a two gene construct. In terms of the mutant phenotype it also corroborates the fact that disruption of *esxB* will have a polar effect on *esxA* transcription.

The promoter of the *esxAB* genes is conditionally expressed. Expression studies in solid media showed that the promoter starts to be upregulated during the onset of aerial growth. On the other hand when *S. coelicolor* is grown in liquid cultures the activity of the promoter is most active during the late exponential phase. The activity is nil until after 13 h of growth when the promoter activity is upregulated getting to the peak of expression at approximately 14 h. From then on the promoter is downregulated progressively diminishing, becoming nil after 42 h of growth. It is clear that *bldB* has a role in modulating the activity of the *esxAB* promoter. In the *bldB* background the promoter is upregulated 9 fold which indicates that BldB either directly or indirectly represses expression of this divergently transcribed operon.

CHAPTER V

PROTEIN STUDIES OF *STREPTOMYCES COELICOLOR* EsxA AND EsxB.

Chapter 5: Protein studies of *Streptomyces coelicolor esxA* and *esxB*.

5.1 Introduction.

This chapter focuses on elucidating the properties of the EsxAB proteins. Firstly since the *S. coelicolor* cluster is an ancestral cluster I analysed if the proteins were functional. I analysed the interaction of the proteins using a Bacteriomatch two-hybrid *E. coli* approach to determine if the EsxA and EsxB proteins mimic their MTB orthologues behaviour and hence form an heterodimer. I went on to explore in detail the sub-cellular localisation of the EsxAB proteins, with the idea that this could provide me with some clues to their function and also would enable me to test which member/s of the *esxI* cluster were essential for the secretion of the proteins, if secreted. The experiments where in the main performed in liquid media.

5.2 Two-hybrid interaction studies.

The BacterioMatch II two-hybrid system detects protein-protein interactions based on transcriptional activation. The EsxB protein (the bait) was fused to the full-length bacteriophage λ repressor protein (λcI, 237 amino acids), containing the amino-terminal DNA-binding domain and the carboxyl terminal dimerization domain. EsxA the target protein was fused to the N-terminal domain of the α-subunit of RNA polymerase (248 amino acids). The bait is tethered to the λ operator sequence upstream of the reporter promoter through the DNA-binding domain of λcI. When the bait and target interact, they recruit and stabilize the binding of RNA polymerase at the promoter and activate the transcription of the carbenicillin resistance gene. Downstream of the carbenicillin marker,

there is a second reporter, *lacZ* that confers that ability to metabolise β- galactosidase for extra validation of the bait and target interaction (Figure 5.1).

To be able to apply this methodology it was necessary to construct two plasmids, the bait and the target plasmids. The bait plasmid pBT would contain the λ cI repressor subunit fused in frame with the coding sequence of the *esxB* gene (Figure 5.2). It is important that the DNA encoding the bait protein be inserted so that the bait protein is expressed in the same reading frame as the λcI protein. The target plasmid pTRG should contain the α-subunit of the RNA polymerase in frame with the coding sequence of *esxA*. DNA encoding the target protein must be inserted in frame with the α-RNA polymerase protein.

Plasmid pSA2 (Chapter 2) was subjected to site- directed mutagenesis to create an *Xma*I site at the start codon of EsxB. The modified plasmid was called pSA2mt1 then a second mutation was introduced also by site- directed mutagenesis to create a *Bam*HI site at the beginning of the coding sequence of *esxA*; the resulting plasmid was called pSA2mt2. pSA2mt2 was restricted with *Xma*I and *Bam*HI to excise the desired fragment containing the *esxB* coding sequence. Then *esxB* was directionally cloned into pBT that had previously being digested with *Xma*I and *Bam*HI. Recombinant molecules resulting from the ligation reaction after being electroporated into the *E. coli* XL1-Blue MRF strain (supplied by the BacterioMatch Two-hybrid vector kit to propagate plasmids) were analysed by restriction analysis. Recombinant plasmids were then sequenced to confirm the insertion contained the complete coding sequence of EsxB (Figure 5.3).

For the construction of pTRGEST, pSA2mt2 was restricted with *Bam*HI and *Xho*I and the desired fragment containing the EsxA coding sequence was directionally cloned into pTRG that had previously being digested with *Bam*HI and *Xho*I. Recombinant molecules resulting from the ligation reaction after being electroporated into the *E. coli* XL1-Blue MRF strain were analysed by restriction analysis. Recombinant plasmids were then sequenced to confirm the insertion had the complete coding sequence of EsxA.

Prior to commencing two-hybrid interaction assays using the recombinant bait and target plasmids pBTCFP and pTRGEST, respectively I determined whether specific recombinants were suitable for detecting protein-protein interactions. Cotransformation of the BacterioMatch II reporter strain using recombinant pBTCFP plasmid and the empty pTRG vector should not produce a significant number of colonies on Selective Screening Medium. Likewise, cotransformation of the reporter strain using pTRGEST plasmid and the empty pBT vector should not produce a significant number of colonies on this medium. No significant growth was observed in either of the cotransformation experiments; therefore I proceeded to the two-hybrid interaction assays.

The recombinant plasmids pBTCFP and pTRGEST were co-transformed into the BacterioMatch II two-hybrid system reporter cells (Chapter 2). The positive control in this experiment was pBT and pTRG plasmids containing the coding sequence of proteins known to interact. The negative controls were: recombinant pBTCFP plasmid/pTRG vector; and pTRGEST plasmid/ pBT vector. Once cotransformations were plated on selective media the results strongly indicated that the EsxA and EsxB proteins interact.

The experiment was repeated three times always yielding identical results. To further validate the interaction the expression of the second reporter gene *lacZ* was examined. Colonies from fresh screening plates were transferred to selective media containing carbenicillum/tetracycline/chloramphenicol/ IPTG. The positive control, negative control and putative interacting clones, that grew on the selective media were plated. The production of β-Galactosidase was visually scored for colonies from each combination. As predicted, the positive control and putative colonies that indicated interaction of the proteins appeared blue, whereas the negative control appeared white (Figure 5.4).

Figure 5.1. Diagrammatic representation of the Bacteriomatch 2-Hybrid approach.
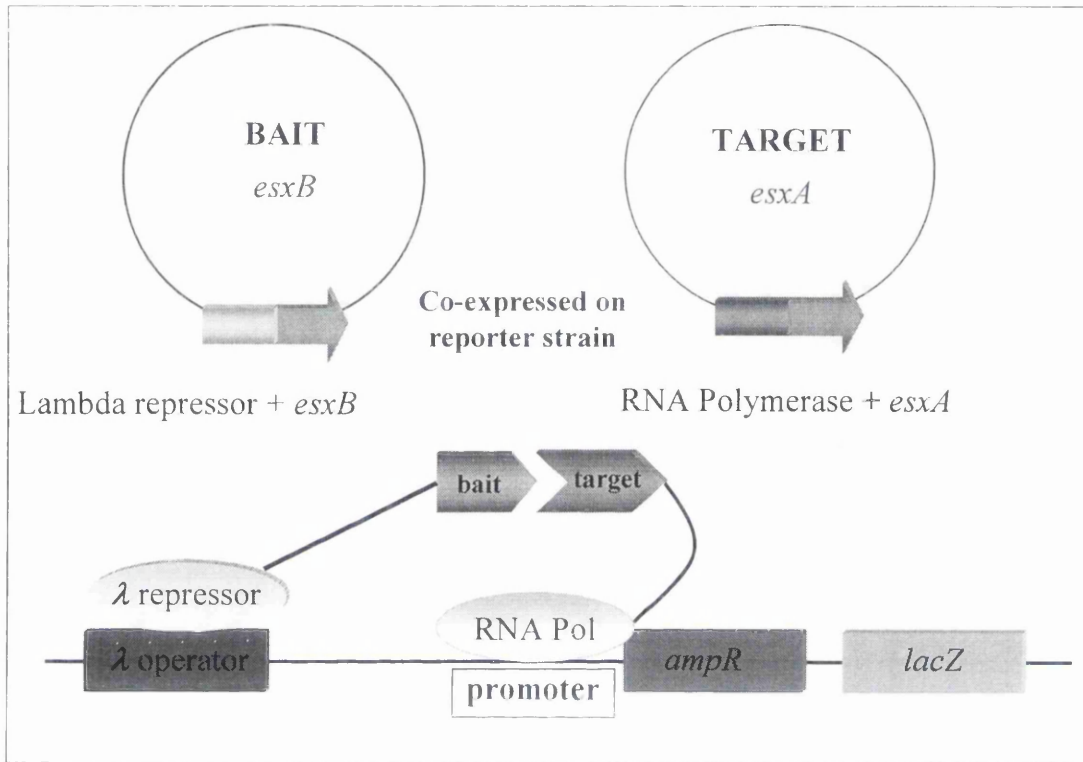
Figure 5.2 Construction of expression plasmid pBTCFP.

Plasmid pSA2 was mutagenized to create an *Xma*I site at the start codon of EsxB. The modified plasmid was called pSA2mt1 which in turn was modified to create a *Bam*HI site at the beginning of the coding sequence of *esxA*; the resulting plasmid was called pSA2mt2. pSA2mt2 was restricted with *Xma*I and *Bam*HI to excise the desired fragment containing the *esxB* coding sequence. Then *esxB* was directionally cloned into pBT that had previously being digested with *Xma*I and *Bam*HI.

AccIII (5)
Ncol (2910)
Ampicillum
Drdl (2666)
BssHII (2398)
BglII (2389)
XhoI (2368)
BamHI (2363)
SmaI (2360)
XmaI (2358)
EcoRI (2353)
NotI (2343)
MCS
pBT
3210bp
NheI (583)
XmnI (639)
SacII (835)
lambda cl
HindIII (2107)
XbaI (1425)
ClaI (1519)

fl(-) origin
BamHI (710)
lacZ
BglII (961)
ampicillum
pSA2m2
4750bp
esxB
XmaI (1194)
BglII
esxA
BamHI (1606)
lacZ
Xho (1917)

AccIII (5)
Ampicillum
Ncol (2910)
NheI (583)
XmnI (639)
BglII (2759)
XhoI (2738)
BamHI (2733)
Drdl (2666)
MCS
pBTCFP
3584bp
SacII (835)
esxB
XmaI (2358)
EcoRI (2353)
NotI (2343)
MCS
lambda cl
HindIII (2107)
ClaI (1519)
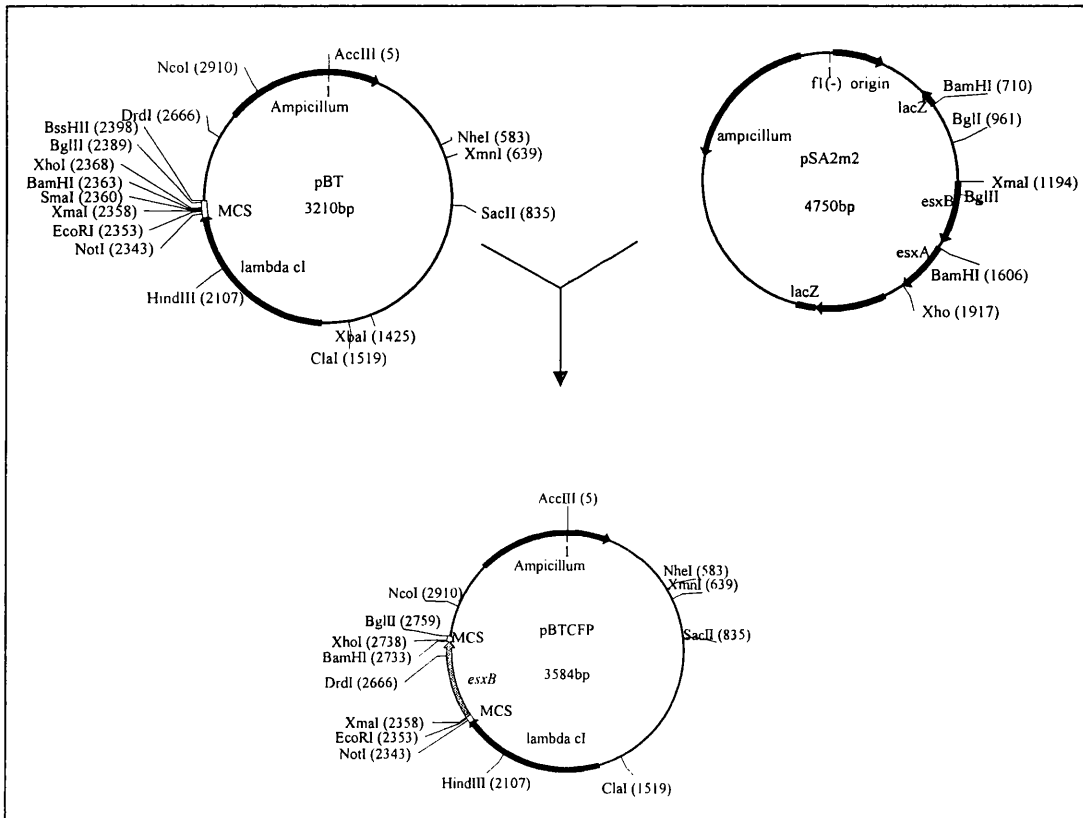
Figure 5.3 Construction of expression plasmid pTRGEST.

Plasmid pSA2 was mutagenized to create an *Xma*I site at the start codon of EsxB. The modified plasmid was called pSA2mt1 which in turn was modified to create a *Bam*HI site at the beginning of the coding sequence of *esxA*; the resulting plasmid was called pSA2mt2. For the construction of pTRGEST, pSA2mt2 was restricted with *Bam*HI and *Xho*I and the desired fragment containing the EsxA coding sequence was directionally cloned into pTRG that had previously being digested with *Bam*HI and *Xho*I
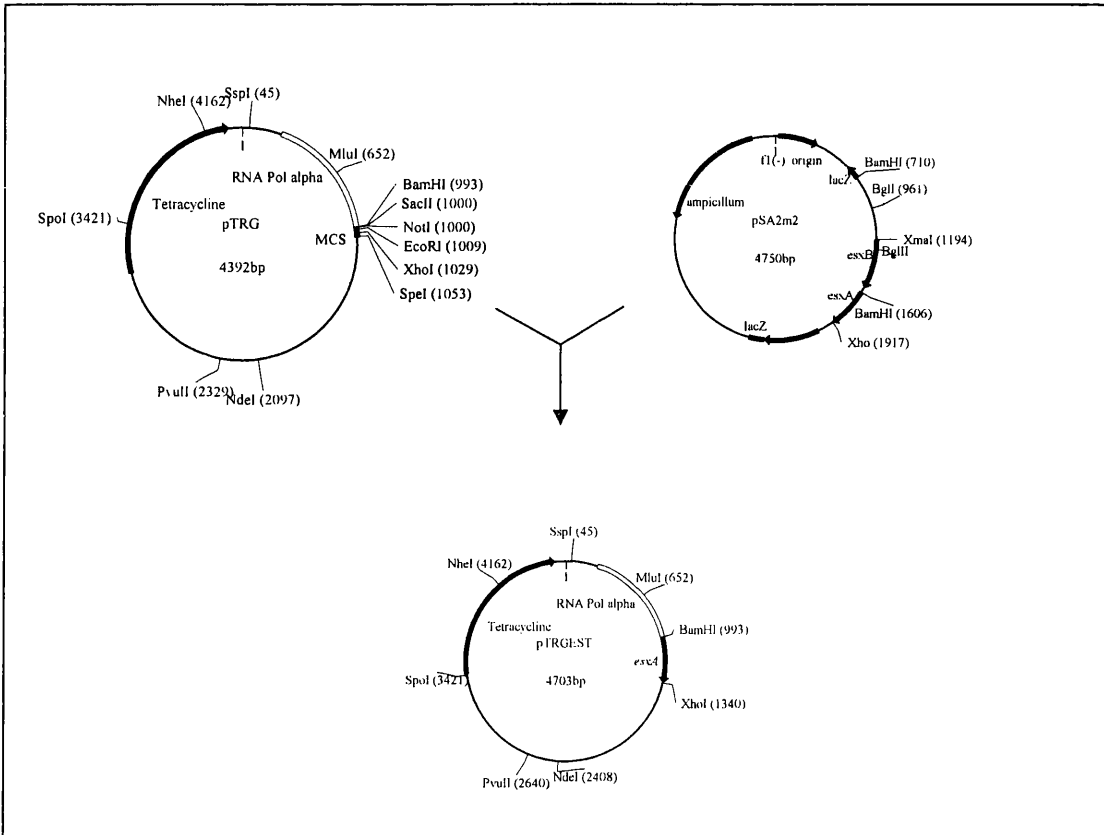
Figure 5.4. Plate showing the validation plate using the β-Galactosidase reporter.

Positive control pBT-LGF2 & pTRG-GAL11P*(1)

pBTCFP & pTRGEST§
Clones (2- 5)

pBT& pTRG (6)

*pBT-LGF2: dimerization domain of the Gal4 transcriptional activator; pTRG-GAL11P:Gal11 protein
§pBTCFP: EsxB fused to full-length bacteriophage λ repressor
pTRGEST: EsxA fused to N-terminal domain of the α subunit of RNA polymerase.
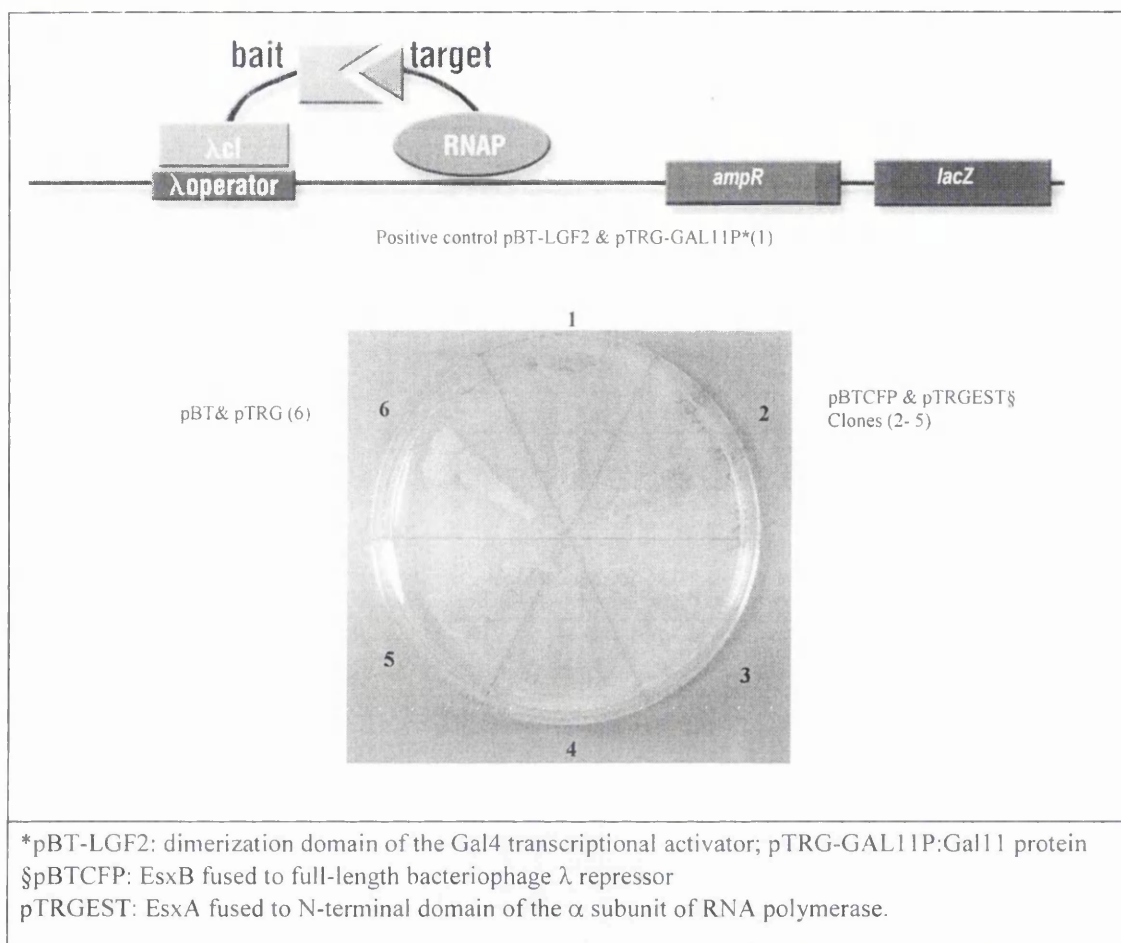
5.3. Sub-cellular localisation of EsxA using a translational fusion to a hexa-histidine tag.

Prior to commencing experiments to localise EsxA I intended to detect EsxB. A polyclonal antibody was purchased raised against the EsxB protein. Several approaches were taken to detect the protein: using solid and liquid media to grow the strains, different sampling time points etc. Finally the antibody finished and these preliminary experiments were still not successful.

In order to localise EsxA I employed an anti- His antibody readily available from Qiagen. The hexa- histidine tag alternative was cheaper to purchase and also the detection was facilitated because the method of detection consists of a single antibody fused to the horseradish peroxidase enzyme eliminating the need of a secondary antibody. A plasmid pSAHIS1 (Figure 5.6) was constructed that contained EsxA C- terminus fused to a Hexa-histidine tag. Three plasmids were used for this cloning experiment: pET26b(+) digested *Xho*I and *Xma*I, provided the Histidine tag followed by terminal repeats; pSAXhoI a derivative of pSA2 mutagenised to introduce a *Xho*I site at the end of the *esxA* coding sequence; and pSET152 provided the plasmid backbone and was digested *Bam*HI and *Eco*RV to linearise the plasmid. The three vector fragments were ligated directionally and putative recombinant colonies investigated by restriction enzyme and sequence analysis. This plasmid was transferred into *S. coelicolor* M145 by intergeneric conjugation. At last the M145/pSAHIS1 was ready to be tested and I was eager to continue the studies. I had to decide which method I was to use for the purification step. Reviewing the literature I searched for protocols to concentrate proteins contained in the culture filtrate. Most of the

protocols consisted of precipitating the protein so that it would come out of solution. One of the several protocols I found suggested to precipitate the supernatant with Trichloroacetic acid (TCA), the method was practical and yielded results. Prior to the precipitation method I tried to enrich for proteins of molecular weight below 20 kDa using MW filters but I failed to detect any protein. Exploiting the data from the analysis of gene expression (Chapter 4), I could choose optimal conditions with which to grow the cultures to detect the His- tagged protein.

5.3.1   Localisation of EsxA in *Streptomyces coelicolor* M145.

The initial experiment was performed by growing M145/pSAHIS1 strain in liquid culture using the tryptone soy broth (TSB) as the luciferase experiments suggested that the expression of the *esxAB* promoter was very active on this particular culture media. I obtained the samples at 24 h at the peak of expression (when the cultures were inoculated from spore suspensions). The volume of culture grown was also critical and I found that 50 ml of culture was the right volume. This volume, when transferred to sterile Falcon tubes produces about 45 ml of supernatant for the TCA precipitation of extracellular proteins and 5 ml of cells to use for the intracellular detection (Chapter 2). The first experiment (Figure 5.5) was performed extracting protein from the intracellular and extracellular fractions. The amount of protein although visualised using Coomassie staining was not quantified at this stage. I was able to detect the EsxA::His protein in both fractions; this was a huge step forward, as experiments using the anti-EsxB polyclonal antibody only yielded detection on one occasion. The positive control on this experiment was CrgA::His that had previously being successfully detected in-house. The

experiment was repeated yielding the same results before proceeding to further analysis. The plasmid introduced by intergeneric conjugation to carry out this work was pSAHIS1 that is apramycin resistant (Figure 5.6). This plasmid was ideal for experiments performed on the wild- type strain but it could not be used on the mutants that were apramycin resistant like the pSAHIS1 plasmid. To solve this problem plasmid pSAHIS2 was constructed by replacing the apramycin cassette of the pSAHIS1 plasmid with a hygromycin cassette (Figure 5.7). The pSAHIS2 construct was introduced into the wild-type strain and the *esxAB* mutant strains. The succeeding western experiments were carried out using the pSAHIS2 hygromycin construct. This part of the analysis concluded that the EsxA protein was secreted and could be readily detectable in the intracellular and extracellular fraction in the wild- type background. In addition this experiment provided the first evidence that EsxA::His$_6$ was fully functional as when the plasmid was conjugated into the *esxB* mutant the protein could be detected in the western blot and so the construct was used for the complementation studies (Chapter 3).

Figure 5.5. Western blot detecting EsxA::His in the wild-type M145 background.

Lane 1, positive control CrgA::His; <u>Intracellular fraction</u>: *Lane 2*, M145; *Lane 3*, M145/pSAHIS1; <u>Extracellular fraction</u>: *Lane 4*, M145; *Lane 5 & 6*, M145/pSAHIS1 array of samples obtained in different precipitation experiments; *Lane 8*, positive control CrgA::His$_6$.
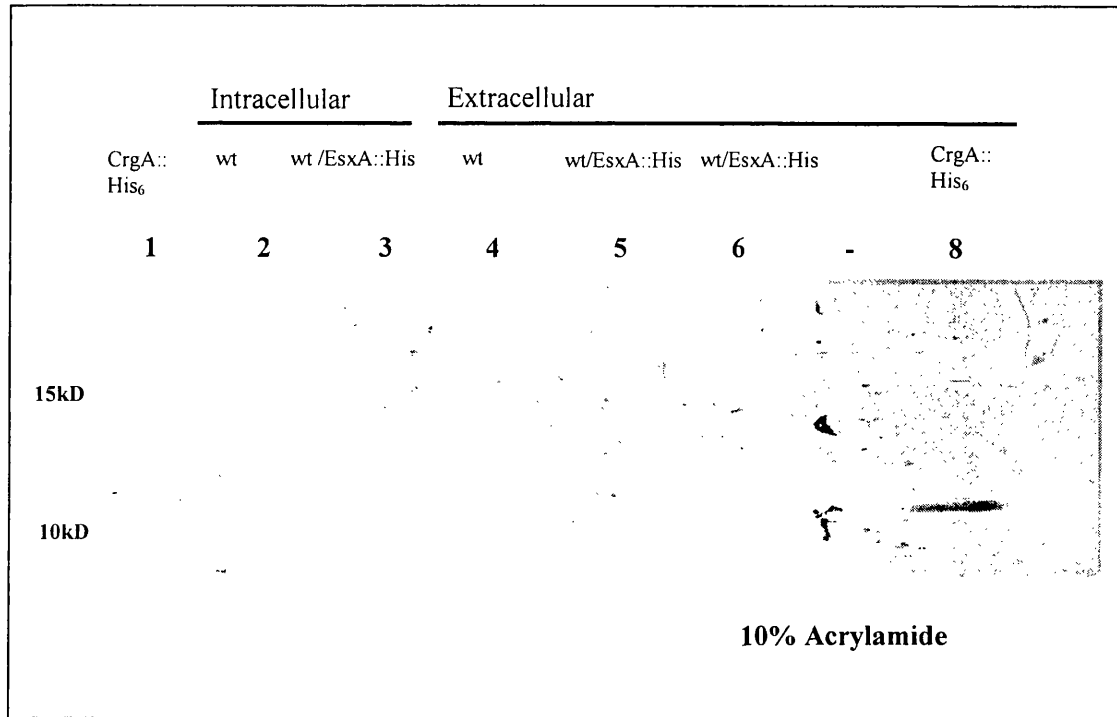
Figure 5.6 Construction of an integrative plasmid pSAHIS1 containing *esxA* carboxy terminal histidine tagged and *esxB* genes.

Plasmid pSAHIS1 was made using pSET152 *Bam*HI- *Eco*RV as the plasmid backbone, ligated to pSAXhoI (derivative of pSA2 with an in-frame *Xho*I site at the end of esxA coding sequence) digested *Bam*HI-*Xho*I and *Xho*I-*Sma*I fragment from pET26S(+) containing the Histidine tag. All fragments were ligated in this order.
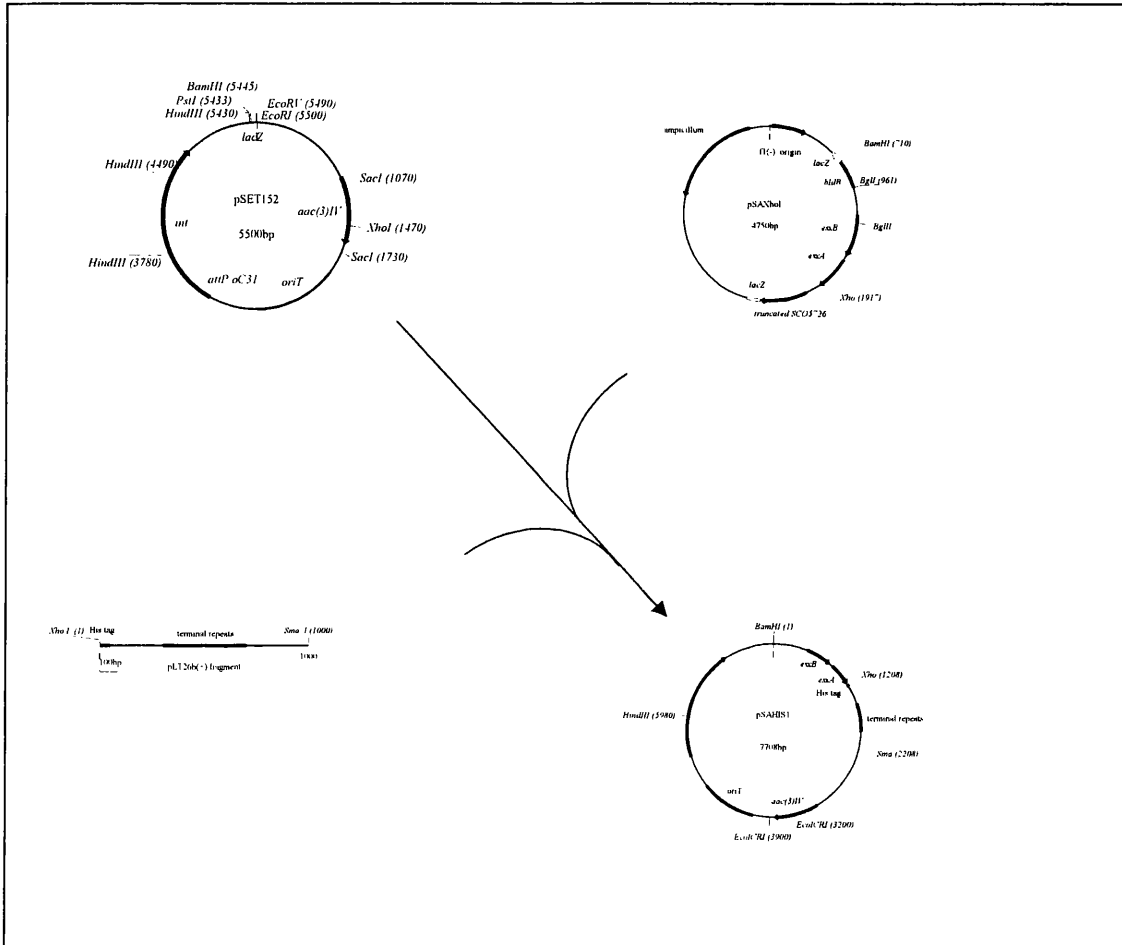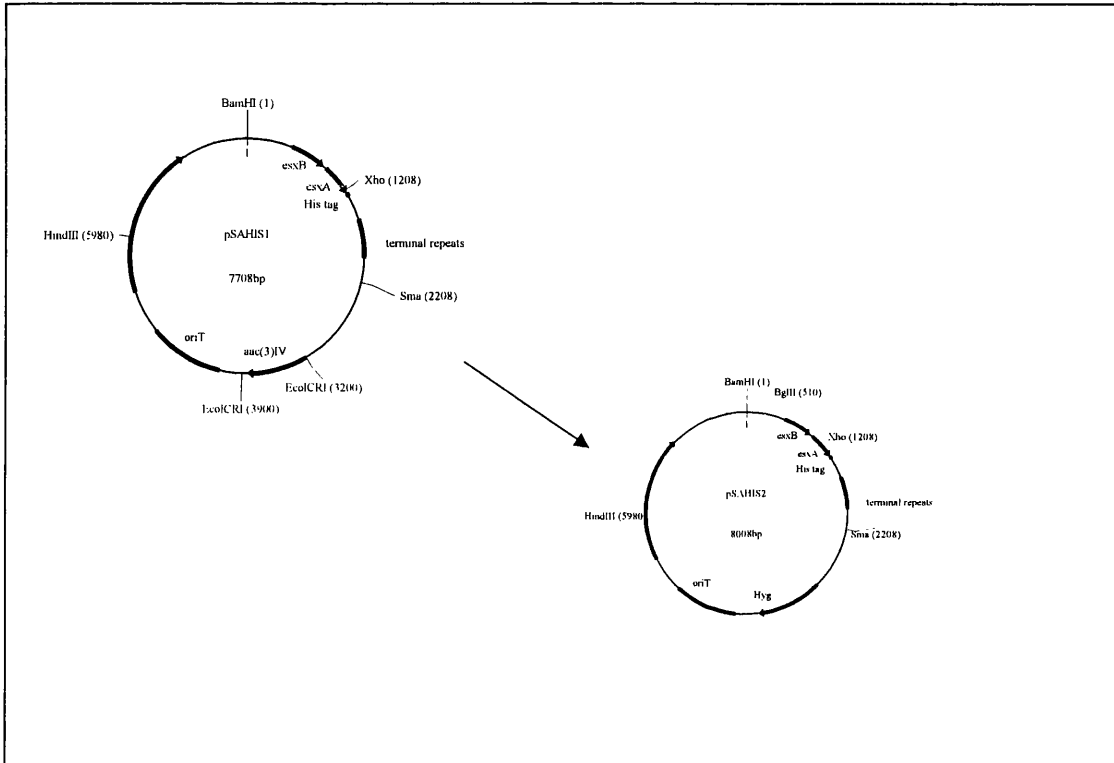
Figure 5.7 Construction of a pSAHIS2 hygromycin resistant integrative plasmid containing *esxA* carboxy terminal histidine tagged.

Plasmid pSAHIS1 (Chapter 2), was modified to be able to complement the mutants *esxA* and *esxB* that were apramycin resistant. The apramycin cassette was replaced by the hygromycin cassette to create pSAHIS2.

5.3.2 Sub-cellular localisation of EsxA different mutant backgrounds.

To progress it was necessary to know whether there were any essential component/s for the secretion of EsxA. Fortunately I had made an array of mutants from the *esx1* cluster of *S. coelicolor* and I only had to introduce the pSAHIS2 plasmid by intergeneric conjugation to continue to the next phase of the analysis. The pSAHIS2 plasmid was conjugated into three mutants in which other genes of the *esx1* cluster were disrupted and into the *esxB* mutant. I chose three conserved genes from the *esx1* cluster, some that literature suggested that were essential for EsxAB secretion in *M. tuberculosis* and some that were not or that at the time it was still not clear how they contributed to the putative secretion system. The first gene was *SCO5721* encoding an amino-terminal transmembrane protein that contains an ATP/GTP binding site; the second gene was *SCO5722* encoding a subtilisin like cell wall associated serine protease; finally I selected *SCO5734* encoding a DNA segregation ATPase, FtsK-like chromosome partitioning protein (3x ATP/GTP binding sites) with an amino-terminal transmembrane domain.

I also wanted to know if EsxA protein would be secreted in the absence of EsxB its known interacting partner. To examine if EsxA secretion was dependent upon EsxB I introduced a deletion on the pSAHIS2 plasmid creating the new plasmid pSAHIS3 (Figure 5.8) that contains a deletion that disrupted the *esxB* reading frame, hopefully rendering the *esxB* transcript aberrant. This plasmid was conjugated into M145, *esxA* and *esxB* strains.

The whole experiment was executed as in the wild-type experiments. For this western experiment I intended to load the same amount of protein for each sample (Figure 5.9). This western consisted only of the proteins extracted from the extracellular fraction although the cells were kept at -70° C for later examination. The results were encouraging as they suggested that two of the three proteins I selected for the study were indeed essential for the secretion of EsxA. SCO5721 an amino-terminal transmembrane protein and SCO5734 DNA segregation ATPase were required for the secretion. When the pSAHIS3 construct was introduced into the M145 strain there was a reduction in the secretion of EsxA::His$_6$. A possible explanation for that was that the deletion in *esxB* has a polar effect reducing the expression of *esxA* and/or, as I was only detecting the EsxA::His$_6$ protein, if dimerisation with EsxB is a requirement for EsxA secretion, both the EsxA and EsxA::His$_6$ counterpart for binding had to compete with EsxB hence the decreased concentration of EsxA::His$_6$ protein extracellularly. More importantly the experiment showed that the *esxB*/pSAHIS3 strain did not secrete EsxA::His$_6$. The next step was to confirm whether the protein was still synthesized but kept intracellularly as a result from the inability of the incomplete secretion system to function correctly.

The intracellular samples were processed and visualised in the Coomassie stain (Figure 5.10) to estimate that the samples were of good quality. Samples were then analysed using the Bradford quantification method to estimate the amount of sample to add into the western experiment. Equivalent amount of protein was loaded into each well. The results showed that EsxA protein could be detected intracellularly in *SCO5722, SCO5721* and *SCO5734* mutant backgrounds (Figure 5.10). The experiment also showed that in the

wild- type background the pSAHIS3 construct affected the accumulation of EsxA as limited amounts of the protein were detected. No protein was detected in the *esxB/* pSAHIS3 possibly due to the fact that the polar effects on both constructs (the plasmid and the chromosome) completely abolish the expression of the *esxA* gene. Subsequently the experiments were repeated, to confirm the results and also to test the *esxA/* pSAHIS3. Until now I could not explain how the disruption of the *esxB* gene could affect so dramatically the accumulation of EsxA::His$_6$ intracellularly.

Figure 5.11 shows the results from an extracellular fraction Western blot experiment corroborating the above results. This experiment incorporates the *esxA*/pSAHIS3 strain. The experiment shows that in the SCO5722 mutant, EsxA::His$_6$ was secreted in normal amounts. On the other hand, both the SCO5721 amino-terminal transmembrane protein and SCO5734 DNA segregation ATPase are required for efficient secretion. In the M145/pSAHIS3 strain EsxA::His$_6$ detection was low too. The *esxB*/pSAHIS3 strain did not secrete EsxA::His$_6$, suggesting that EsxA could not be secreted in the absence of EsxB. The *esxA*/pSAHIS3 strain secreted EsxA::His$_6$ in barely detectable low quantities.

The results from the intracellular fraction western blot experiment (Figure 5.12) corroborate the results obtained in the previous analysis (Figure 5.10) incorporating the *esxA*/pSAHIS3 strain. The results showed that EsxA::His$_6$ protein could be detected intracellularly in *SCO5721, SCO5722* and *SCO5734* mutant backgrounds. The experiment also showed that in the wild- type background the pSAHIS3 construct affected the accumulation of EsxA::His$_6$ as there was limited amount of the protein being

detected. In the *esxB/* pSAHIS3 strain there was no detection of the EsxA::His$_6$ protein.

For the *esxA/* pSAHIS3 construct only minor amounts of the EsxA::His$_6$ protein could be

detected.

Figure 5.8. Construction of an integrative plasmid pSAHIS3 containing *esxA*

carboxy terminal histidine tagged with a deletion in the *esxB* gene that alters the

reading frame.

Plasmid pSAHIS2 was modified by restricting *Bgl*I and religating, this procedure generated a three basepairs deletion in *esxB* coding sequence that would affect the reading frame and possibly abolish *esxB* normal translation. The resulting plasmid was called pSAHIS3.

BamHI (1)
BglII (510)
esxB
Xho (1208)
esxA
His tag
pSAHIS2
terminal repeats
HindIII (3980)
Sma (2208)
8008bp
oriT
Hyg

BamHI (1)
ΔBglII
esxB
Xho (1208)
esxA
His tag
pSAHIS3
terminal repeats
HindIII (3980)
Sma (2208)
8005bp
oriT
Hyg

Figure 5.9. Western blot using anti-His antibody to detect EsxA::His tag in different

mutant backgrounds from the extracellular fraction.

a, Coomassie. b, Western blot: Lanes: 1, M145/pSAHIS1; 2, M145/pSAHIS1 (different samples tested); 3, *esxB*/pSAHIS2; 4, *SCO5734* (*FtsK*-like)/pSAHIS2; 5, *SCO5722* (*Serine protease-*)/pSAHIS2; 6, *SCO5721 (ATP/GTP binding site-*)/pSAHIS2; 7, M145/pSAHIS3 (Δ*Bgl*II alteration of the reading frame in *esxB*); 8, *esxB*/ pSAHIS3.
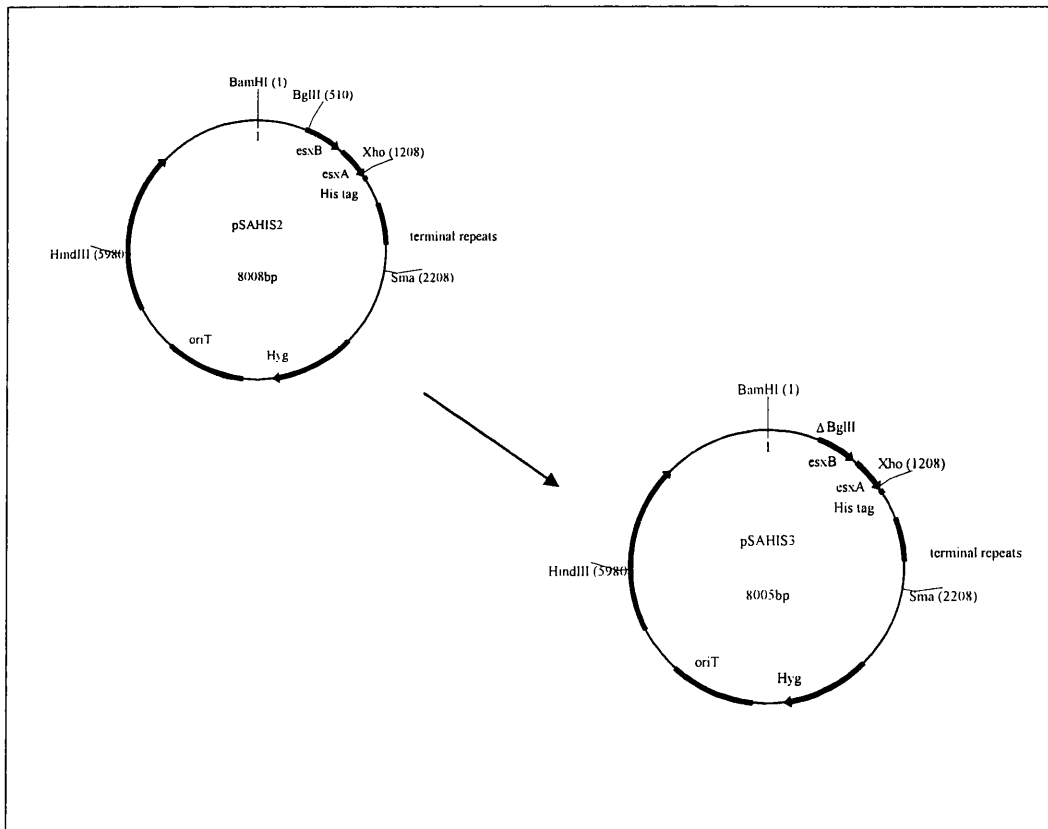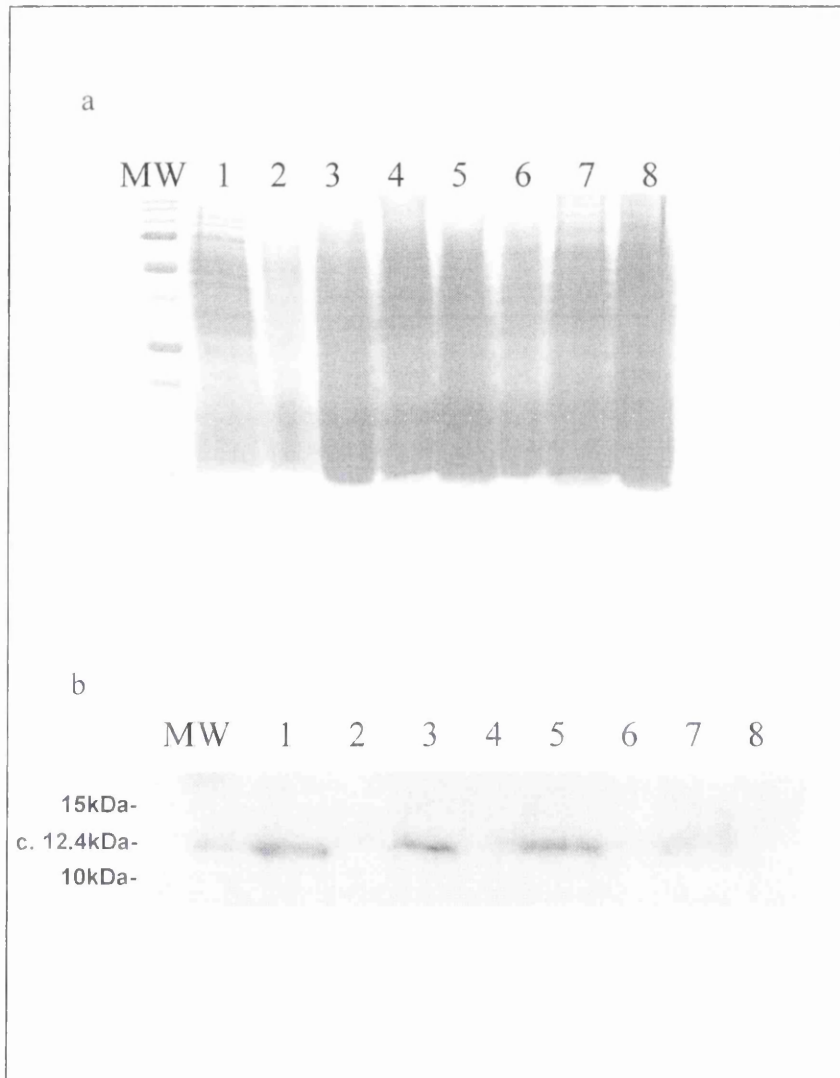
Figure 5.10. Coomassie stained intracellular samples and western blot using anti-His antibody to detect EsxA::His$_6$ in different mutant backgrounds from the intracellular fraction.

a, Coomassie: Lanes: 1, Molecular weight marker; 2, *SCO5722* (*Serine protease-*)/pSAHIS2; 3, *SCO5721* (*ATP/GTP binding site-*)/pSAHIS2; 4, *SCO5734* (*FtsK-*like)/pSAHIS2; 5, M145/pSAHIS3 6, *esxB*/ pSAHIS3. b, Western blot: Lanes: 1, *SCO5722* (*Serine protease-*)/pSAHIS2; 2, *SCO5721* (*ATP/GTP binding site-*)/pSAHIS2; 3, *SCO5734* (*FtsK-*like)/pSAHIS2; 4, M145/pSAHIS3; 5, *esxB*/ pSAHIS3.
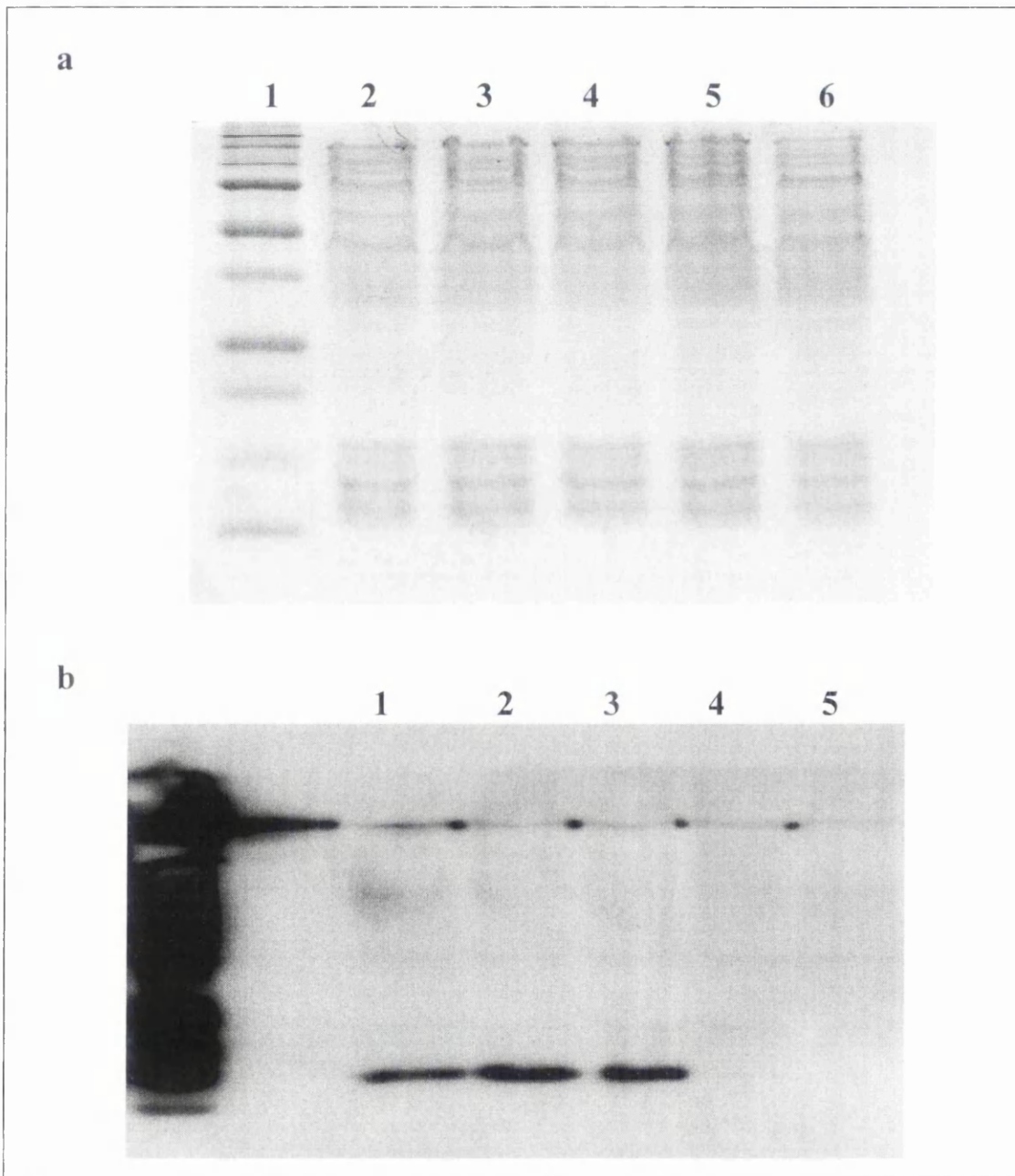
Figure 5.11. Coomassie stain and western blot detecting EsxA::His$_6$ in different mutant backgrounds from the extracellular fraction.

a. Coomassie; Lanes 1, Molecular weight marker; 2, M145/pSAHIS2; 3, *SCO5722* (*Serine protease*)/pSAHIS2; 4, *SCO5721* (*ATP/GTP binding site-*)/pSAHIS2 *esxB*/pSAHIS2; 5, *SCO5734* (*FtsK*-like)/pSAHIS2; 6, M145/pSAHIS3; 7, *esxB*/pSAHIS3; 8, *esxA*/ pSAHIS3; 9, *esxB*/pSAHIS2.

b, Western blot: Lanes 1, M145/pSAHIS2; 2, *SCO5722* (*Serine protease*)/pSAHIS2; 3, *SCO5721* (*ATP/GTP binding site-*)/pSAHIS2 *esxB*/pSAHIS2; 4 *SCO5734* (*FtsK*-like)/pSAHIS2; 5, M145/pSAHIS3; 6, *esxB*/pSAHIS3; 7, *esxA*/ pSAHIS3; 8, *esxB*/pSAHIS2.
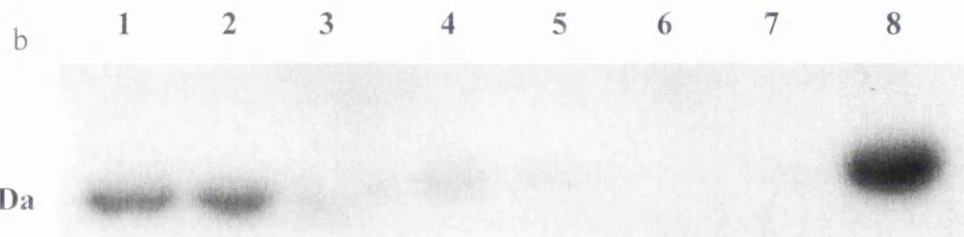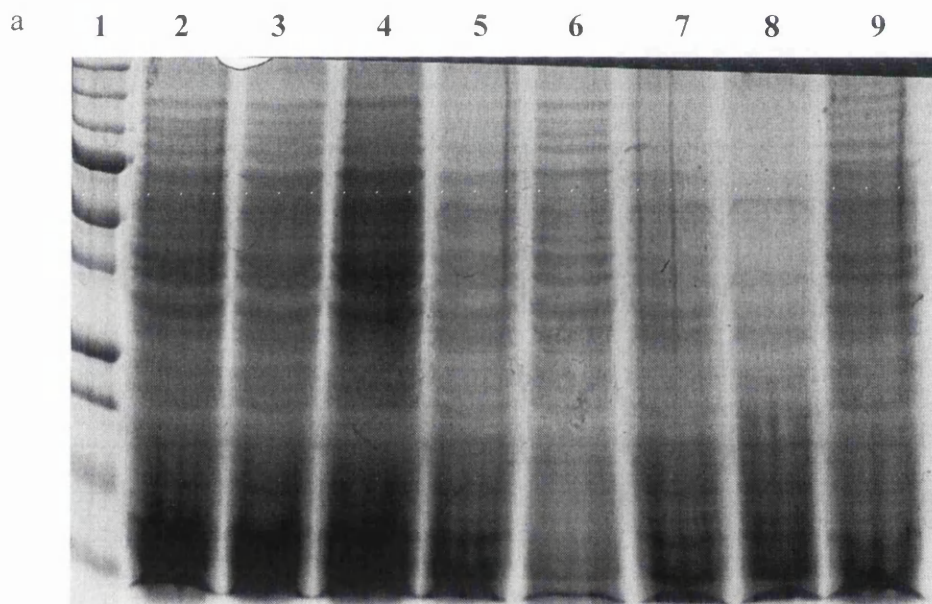
Figure 5.12. Western blot and coomassie stain detecting EsxA::His tag in different

mutant backgrounds from the intracellular fraction.

a. Coomassie; b. Western blot.

Lanes 1, Molecular weight marker, 2, M145/pSAHIS2; 3, *SCO5722 (Serine protease-*)/pSAHIS2; 4, *SCO5721 (ATP/GTP binding site-)*/pSAHIS2; 5, *SCO5734 (FtsK-*like)/pSAHIS2; 6, M145/pSAHIS3; 7, *esxB*/pSAHIS3; 8, *esxA*/ pSAHIS3; 9, *esxB*/pSAHIS2.
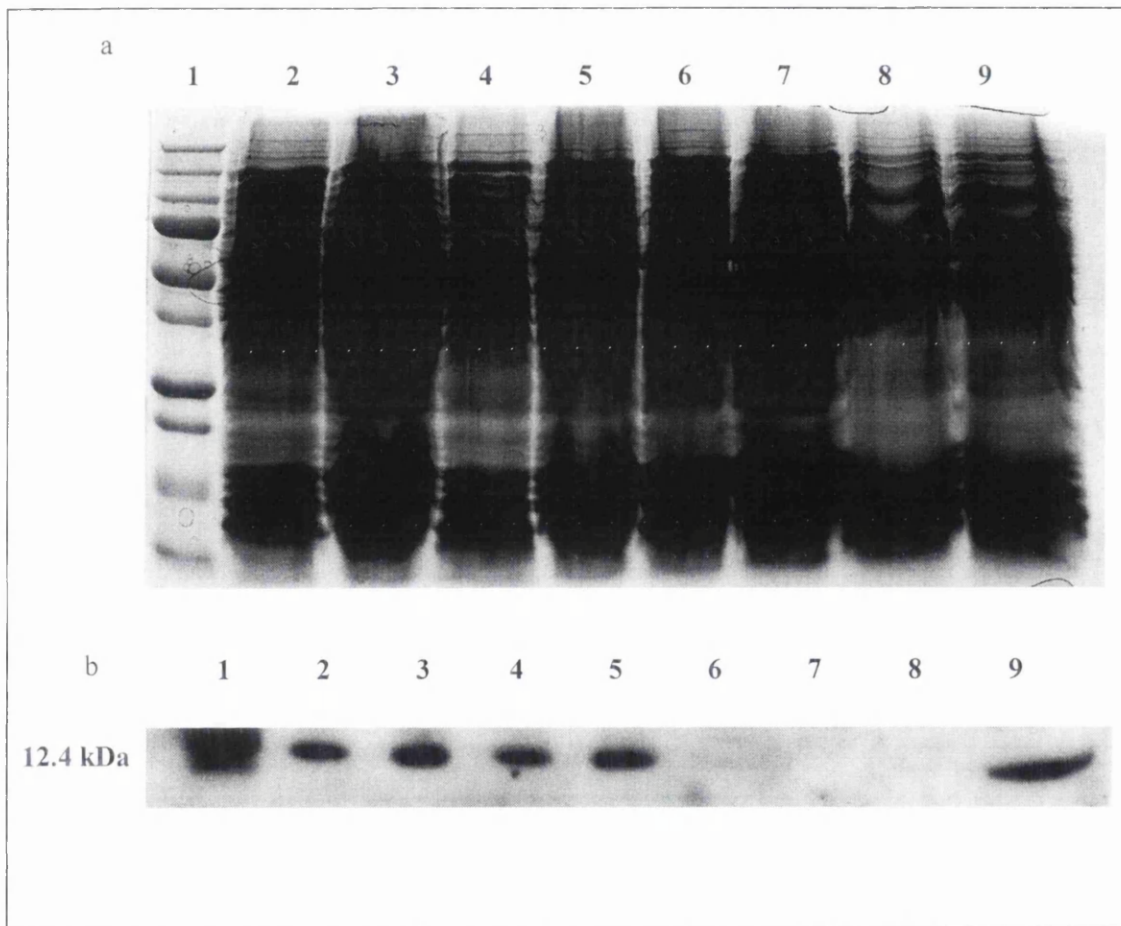
5.4 Conclusion

Two-hybrid analysis confirmed that EsxA and EsxB interact. Western hybridisation studies indicate that EsxA protein is secreted into the extracellular milieu. There is also evidence provided by this analysis suggesting that the EsxA protein is secreted by a dedicated secretory apparatus of which I have identified two key components and without them the protein fails to be secreted. It also appears that in the absence of EsxB there is a decrease of EsxA in the intracellular fraction, possibly due to protein turnover.

CHAPTER VI

DISCUSSION

Chapter 6. Discussion.

The investigations completed in this thesis present the functional characterisation of the *esx1* alternative protein secretion system in *S. coelicolor*. The system is analogous to the novel tat/sec independent secretion systems described in several mycobacteria and is only existent in Gram positive bacteria. The system was initially pointed out by Gey van Pittius et al. 2002 who carried out a comparative genetic study based in the *M. tuberculosis* cluster. The *S. coelicolor* locus closely resembles *M. tuberculosis* Region 4 locus that is believed to be the original ancestor of the cluster. Gey van Pittius et al. discovered that the cluster only featured in high-G+C Gram- positive bacteria, although they noted that multiple duplications of the cluster have taken place only in *Mycobacterium*.

6.1 *S. coelicolor esxAB*, particular function of the genes in the bacteria.

The first part of this discussion is focused in the phenotype of the mutants in *S. coelicolor*. The *esxAB* genes are affected in morphological development. Their absence leads to delayed development of aerial hyphae of the organism and defects in sporulation. There is retardation in the maturation of the spores that is very noticeable as immature spores lack the polyketide spore pigment. As a consequence of this, the mutants appear light grey even when the spores have fully developed. The phenotype of the mutants is apparent macroscopically and more importantly microscopically. In the micrographs of the mutants it is evident that there is a reduced number of spore chains as compared to the number of spore chains in the M145 wild- type. Interestingly the microscopic phenotype of the *esxA* and *esxB* mutants is that septation of the aerial hyphae occurs in an irregular

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mode. Nevertheless each mutant has a specific phenotype. The *esxA* mutant is characterized by irregular pre- spore compartments and also that a significant percentage of the compartments are significantly smaller than the norm. On the contrary the *esxB* mutant is typified by the presence of larger compartments. Both mutants also contain an unusual high number of ghost compartments devoid of DNA (Chapter 3). The mutations of the *esxAB* genes were successfully complemented using a plasmid pSAHIS2 containing the two genes. This construct was also use successfully to detect the protein *in vivo*. The phenotype of the *SCO5724* mutant had been previously described by Piret et al. 1985. Piret isolated recombinant phages (obtained from a phage library of wild-type *S. coelicolor* DNA), which restored wild-type morphological development to several *bldB* mutants. Piret's study focused on *bldB*, but the mutant of the gene divergently transcribed, *esxB*, was also obtained and reported as being delayed in sporulation. Interestingly, deletion of the *esx1* genes affects morphology of mycobacteria. The earliest study to report alterations in colony morphology of RD1 deletion mutants was described by Calmette on 1927, and since then (Pym, Brodin et al. 2002) and later (Brodin, Majlessi et al. 2004) described the phenotype in *M. tuberculosis* and *M. microti*. In addition to the phenotypic and complementation analysis, I confirmed that the genes constitute an operon. The expression studies linked the *esxAB* genes to the onset of sporulation providing more clues that associate the genes as having a developmental function. The genes were not expressed during germination or later phases of growth. The activity of the promoter was only present during the commencement of sporulation (Chapter 4).

Gene expression of the *esxAB* genes at the transcriptional level is affected by the pleiotropic regulator BldB. *bldB* mutants have a bald phenotype, are defective in antibiotic production and de-regulated in catabolite repression (Pope, Green et al. 1996). BldB either directly or indirectly represses the activity of the *esxAB* promoter (Chapter 4). The *bld* genes are know to be the key component that through an extracellular signalling cascade define the onset of aerial mycelium formation. The phenotype of the mutants being delayed in aerial development together with the evidence that BldB modulates the *esxAB* promoter activity suggests that the genes have a morphological role in the bacteria.

Recent research indicates that the CFP-10 (EsxB of *M. tuberculosis*) protein interacts with members of the Signal Recognition Pathway (SRP) and components of the sec/YEG translocon (Singh, Mai et al. 2006). CFP-10 protein has been demonstrated to interact with Rv0686, a member of the SRP-GTPase family. Other members of this family include SRP and its receptor SR. CFP-10 interacts with FtsQ (Rv2151c), a cell division protein too; it is believed that FtsQ might be responsible for the targeting of CFP-10 to the sec/YEG translocon. CFP-10 also interacts with ClpC1 (Rv3596c), a protein that is involved in secretion, gene regulation, protein refolding and degradation. (Singh, Mai et al. 2006) provided the first evidence that the specialised secretion system could be linked to evolutionary conserved SRP and sec/YEG systems.

This new evidence provides an interesting background to the work performed in this thesis, linking the *esxAB* genes with secretory components and cell division proteins. It

could be argued that the *S.coelicolor esxAB* mutant phenotypes resulted as a consequence of abolished EsxAB complex secretion. This was proven not to be the case as mutants of the *SCO5722* and *SCO5734* genes defective in secretion did not have any obvious phenotype on MS media. This indicates that EsxAB might perform an intracellular role. This is supported in view of the fact that there is detectable accumulation of EsxA::His$_6$ intracellularly in the wild- type.

Converse *et al.* 2005 argues that the Snm pathway may be regulated, like some sec-independent pathways from gram negative bacteria. This was proposed because the *M. tuberculosis* EsxAB proteins are not secreted in every growth conditions, only being secreted in certain culture media.

EsxB interacts with the intracellular cell division protein FtsQ potentially modulating its activity. In the absence of intracellular EsxB, FtsQ function may be affected. This might explain irregular cell division of aerial hyphae.

6.2. Analogy of the putative sec/tat independent secretion system with other mycobacteria.

The esx1 system from *S. coelicolor* resembles the ESAT apparatus from *Mycobacterium tuberculosis* and also the Snm secretion system of *Mycobacterium smegmatis* described by (Converse and Cox 2005). The *esx1/snm* secretion system is specific for members of the 100 residue protein family represented by ESAT-6. The locus is composed of genes encoding ATP binding proteins, putative ABC transporters, serine proteases and other membrane associated proteins. Western hybridisation studies in *S. coelicolor* indicated

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that EsxA protein is secreted into the extracellular milieu. My analysis of the locus confirmed that EsxB, SCO5722, and SCO5734 were all essential for effective EsxA secretion and/ or stability. It seems that in the absence of EsxB there is a decrease of EsxA in the intracellular fraction. All the evidence suggests that the system is fully functional and analogous to others described in the literature (Table 6.1). In *M. tuberculosis* inactivation of gene *pe35* (Rv3872) impaired expression of CFP-10 and ESAT-6, suggesting a role in regulation. Genes *Rv3868, Rv3869, Rv3870, Rv3871,* and *Rv3877* encoding an ATP-dependent chaperone and translocon were essential for secretion of ESAT-6 and CFP-10 in contrast to *ppe68, Rv3873* and *Rv3876,* whose inactivation did not alter secretion of ESAT-6 (Brodin, Majlessi et al. 2004).

Why would be the ESAT system be conserved in non- pathogenic bacteria when the system is so strongly associated with virulence in *M. tuberculosis*? It is well documented that bacteria devise dedicated secretion apparatus for the exporting of effector molecules to mediate infection. Those systems have been extensively studied in Gram negative bacteria. (Champion, Stanley et al. 2006) reviewed that *Mycobacterium tuberculosis* uses the esx1/snm system to deliver virulence factors into host macrophages during infection. The extracellular function of EsxAB in *S. coelicolor* is yet to be elucidated.

Table 6.1 Homology between the components of the Snm secretion system in *S. coelicolor* and *M. tuberculosis.*

S. coelicolor genes	Essential	M. tuberculosis genes	Essential
SCO5717c	Not tested	Rv3876 (snm3)	Yes
SCO5721	Yes	Rv3869 (snm6)	Yes
SCO5722	No	Rv3883	No
SCO5724	Yes	Cfp-10	Yes
SCO5725	n/a	Esat-6	n/a
SCO5734	Yes	Rv3870 (snm1)/Rv3871(snm2)	Yes
SCO5735	Not tested	Rv3877(snm4)	Yes

6.3 Conclusion.

The model organism *S. coelicolor* has proved to be a good working model to understand the Snm (secretion in mycobacteria) secretion apparatus. The results indicate that the modus operandim of the *M. tuberculosis, M. smegmatis* and *S. coelicolor* systems are analogous.

Future work would include characterisation of the interactions in *S. coelicolor* between the proteins from the cluster to determine which proteins interact with which. It also would be interesting to continue to explore which components of the system are dispensable for the secretion. The study could be extended by trying to export the *M. tuberculosis* orthologues. The components that are essential for the secretion of the substrates are also analogous to those of *M. tuberculosis*. I believe that possibly *S. coelicolor* could be an excellent host for the production of recombinant peptides or proteins from *M. tuberculosis* to be used as recombinant vaccines.

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