DOCTOR OF PHILOSOPHY

Characterisation of the putative Type VII protein secretion system of Streptomyces spp

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Characterisation of the putative Type VII protein secretion system of *Streptomyces* spp.

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College of Life Sciences

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ABSTRACT

Streptomyces are Gram-positive bacteria belonging to the *Actinobacteria*. They are predominantly soil-dwelling saprophytic organisms. *S. coelicolor* is often used as a model organism for the study of the mechanisms underlying the processes of development and antibiotic production. *S. scabies* is one of only a few members of the genus that is pathogenic, being the causal agent of common scab of potato.

Bioinformatic analysis of the genomes of both *S. coelicolor* and *S. scabies* revealed the presence of genes encoding components of putative Type VII protein secretion systems (T7SSs). To investigate the role of these putative T7SSs in *S. coelicolor* and *S. scabies*, marked deletions were constructed in genes encoding core components of the machinery.

Phenotypic analysis revealed that deletion of genes encoding the T7SS FtsK/SpoIIIE ATPases (FSD) resulted in delayed actinorhodin production in *S. coelicolor* and slightly accelerated aerial development in *S. scabies*. In *S. scabies*, deletion of the genes encoding the WXG100 proteins, canonical substrates of T7SSs, led to a marked reduction in aerial hyphae formation and sporulation, in a media-dependent manner when compared to the wild-type strain. Closer inspection by scanning electron microscopy revealed that the spore chains of these mutant strains were under-developed and spores were irregularly sized. Strains of *S. scabies* lacking the WXG proteins (but not the FSD protein) were also resistant to lytic infection by the bacteriophage ΦC31. Taken together, these observations indicate that the major role of the WXG100 proteins in *Streptomyces* is intracellular.

Plant infection assays indicate that the T7SS of *S. scabies* does not significantly contribute to virulence in several laboratory models of plant infection.

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LIST OF ABBREVIATIONS

aa	amino acids
Amp	ampicillin
Apra	apramycin
APS	ammonium persulfate
ATP	adenosine triphosphate
Au/Pd	gold/palladium
BLAST	Basic local alignment search tool
bp	base pair(s)
BSA	bovine serum albumine
CCC	covalently closed circular
CFP-10	culture filtrate protein 10 kDa
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
cm	centimeter
CM	cytoplasmic membrane
Cml	chloramphenicol
C-terminal	carboxy terminal
C-terminus	carboxy terminus
Da	Dalton
ddH_2O	distilled/deionized water
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
DTT	dithiotreitol
EDTA	ethylenediamine tetraacetate
ESAT-6	early secreted antigenic target 6 kDa
FRT	flip recombinase target
FSD	FtsK/SpoIIIE ATPase
x *g*	relative centrifugal force in multiples of standard gravity
g	gram
GFP	green fluorescent protein
H_2O	water
HCl	hydrochloric acid
HRP	horse radish peroxidase
Hyg	Hygromycin
h	hour
IgG	Immunoglobulin G
IM	inner membrane
IPG	immobilised pH gradient
Kan	Kanamycin
kb	kilobase pairs (1000 bp)
kV	kilovolt
l	litre
LPS	lipopolysaccharide
µ	micro
M	molar
m	milli
min	minute
mRNA	messenger ribonucleic acid

MS	mass spectrometry
n	nano
nm	nanometre
nt	nucleotide(s)
N-terminal	amino terminal
N-terminus	amino terminus
OD	optical density
OM	outer membrane
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PSI	position-specific iterated
RD1	region of difference 1
RNA	ribonucleic acid
RNase	ribonuclease
RNC	ribosome nascent chain
rpm	rotations per minute
SDS	sodium dodecyl sulphate
Sec	secretory
SEM	scanning electron microscopy
SRP	signal recognition particle
Tat	twin arginine transloca se
TCA	trichloroacetic acid
TEMED	*N*,*N*,*N*′,*N*′-tetramethylethylenediamine
T_m	melting temperature
Tris	tris(hydroxymethyl) aminomethane
tRNA	Transfer RNA
U	unit
UV	ultra violet
V	volt
v/v	volume per volume
W	watts
w/v	weight per volume
YFP	yellow fluorescent protein
Δp	protonmotive force
°C	degrees Celsius

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DECLARATION

I declare that I am the author of this thesis and that, unless otherwise stated, all references cited have been consulted; that the work of which this thesis is a record of has been performed by me, and that it has not been previously accepted for a higher degree: where the thesis is based upon joint research, the nature and extent of my individual contribution is defined.

Joanna Kirsty Fyans

1. INTRODUCTION

1.1 The genus *Streptomyces* and the model organism *Streptomyces coelicolor* A(3)2

Over 900 species of *Streptomyces* have been described (http://www.ncbi.nlm.nih.gov/Taxonomy) and as such this is the largest genera of high G+C gram positive bacteria belonging to the Actinobacteria. The vast majority of streptomycetes are free-living saprophytes that are ubiquitous in soils and contribute to the "earthy" smell of the soil through production of molecules such as geosmin (Gerber & Lechevalier, 1965). Due to their saprophytic lifestyle they secrete numerous hydrolytic enzymes that enable them to break down complex organic molecules, such as lignin and chitin (Hodgson, 2000; Williamson, *et al*., 2000), and they therefore make an important contribution to carbon and nutrient cycling in the environment.

Much information has been gained about the genus through studies carried out on *Streptomyces coelicolor* A(3)2 that is often used as a model organism. As such, it was the first streptomycete to have its genome sequenced, revealing that the 8,667,507 base pairs long linear chromosome encoded 7,825 putative proteins which, at the time, exceeded that of any other sequenced bacterial genome as well as that of the lower eukaryote *Saccharomyces cerevisiae* (Bentley *et al.,* 2002). Importantly, the approximately 4.9 Mb central part of the chromosome has been shown to contain a core region of essential genes that share a high degree of synteny with other streptomycetes (Friend & Hopwood; 1971) as well as the genomes of other Actinobacteria including *Mycobacterium tuberculosis* and *Corynebacterium diptheriae* (Bentley *et al.*, 2002).

The genome sequence of several industrially important *Streptomyces* species that have since been published (Ikeda *et al.*, 2003; Ohnishi *et al.*, 2008; Song *et al.*, 2010) also revealed large linear chromosomes with coding capacity for between 7,138 and 7,898 proteins. The large coding capacity of streptomycete genomes perhaps

reflects the ability of these bacteria to successfully compete with other organisms and rapidly adapt to fluctuations in the harsh soil environment.

1.1.1 The developmental lifecycle of *Streptomyces*

One of the most notable features of the streptomycetes, which historically led to their misclassification as fungi, is that they undergo a developmental lifecycle that involves multicellular growth (Figure 1.1). Owing to this, the effects of gene mutations that would otherwise prove fatal in unicellular organisms can be observed in the streptomycetes. As such they are often utilised in research laboratories as model organisms for the study of cell division in closely-related Actinobacteria, including the important human pathogens *Mycobacterium tuberculosis and Corynebacterium diptheriae* (Bentley *et al.*, 2002).

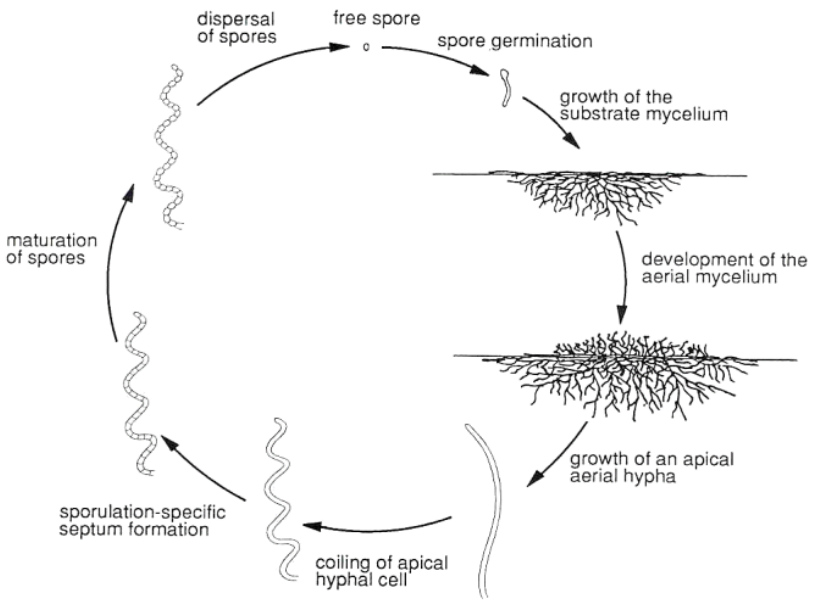

Figure 1.1: Schematic overview showing the different stages in the developmental lifecycle of the soil saprophyte *Streptomyces coelicolor*. Courtesy of Kieser *et al.* (2000).

Spore germination marks the beginning of the developmental lifecycle and occurs when the spore swells and respiration rate increases along with ATP content (Hirsch & Ensign, 1978). Transcription and translation begin in the first few minutes after germination followed by replication of chromosomal DNA approximately one hour later (Mikulík *et al.*, 1977, 2002; Bobek *et al.*, 2004). Following from this the spherical spore is converted to cylindrical hyphae through the emergence of one or two germ tubes. Growth of the vegetative mycelium then proceeds by hyphal tip extension and branching, determined by the presence of the protein DivIVA at the hyphal tip (Flärdh, 2003), reaching a rate of approximately 20 μm per hour (Jyothikumar *et al.*, 2008). Vegetative growth continues until the surface of the growth substrate is covered with a mat of mycelia. As mycelia possess infrequent cross-walls they consist of large compartments containing multiple copies of the chromosome in different replicating states (Kretschmer, 1989; Ruban-Osmialowska *et al.*, 2006).

At a certain point in the lifecycle, thought to be triggered by depletion of exogenous nutrients, development of aerial hyphae is initiated, fuelled by lysis of the vegetative mycelia. Aerial hyphae grow on the surface of the bacterial colony, giving it a fuzzy appearance. The ability of the emerging aerial hyphae to break through the aqueous environment into the air requires that *S. coelicolor* produces SapB, a peptide surfactant, when grown on rich media (Willey *et al*., 1991; Capstick *et al.*, 2007). The surface of aerial hyphae is coated in a sheath composed largely of amphipathic proteins belonging to the chaplin and, in the case of *S. coelicolor*, rodlin families (Elliot *et al.*, 2003; Claessen *et al.*, 2003) and as such the aerial hyphae possess a hydrophobic surface.

Mutant strains of *S. coelicolor* unable to form white aerial hyphae are known as *bld* mutants (due to their "bald" appearance). Early studies focussed on a number of these *bld* genes (*bldA-D*: Merrick, 1976; *bldG&H*: Champness, 1988). With the

exception of *bldA* that codes for a rare tRNA (Lawlor *et al.*, 1987) required for translation of *bldH* mRNA, these genes encode products that have been shown to be or are strongly implicated in gene regulation (Pope *et al.*, 1998; Hunt *et al.*, 2005; Elliot *et al.*, 1998, 1999, 2001; Bignell *et al.*, 2000; Takano *et al.*, 2003). Most of the *bld* mutant strains are impaired in production of SapB as shown by restoration of aerial hyphae formation by external "feeding" with purified SapB (Willey *et al.*, 1991). Growth of specific pairs of *bld* mutant strains in close proximity to each other also led to restoration of aerial hyphae formation for one of the mutants through the stimulation of SapB production (Willey *et al.*, 1993). This finding led the authors to propose a model whereby the production of SapB is controlled by an extracellular signalling cascade in which the *bld* genes fit in the following order:

$$bldA/H < bldG < bldC < bldD$$

Notably, *bldB* did not fit into this cascade as growth of the *bldB* mutant alongside other *bld* mutant strains failed to show complementation by "cross feeding" (Willey *et al.*, 1993). Since these early studies, mutations of numerous genes, including those encoding the chaplin proteins or required for their expression (Bibb *et al.*, 2000; Elliot *et al.*, 2003) as well as various enzymes and regulatory proteins, have been shown to have a *bld* phenotype (reviewed by Elliot *et al.*, 2008).

Growth of aerial mycelia also proceeds by hyphal tip extension, until nutrients provided by lysis of the vegetative mycelium become limited. At this stage the aerial mycelia are multinucleate, being large enough to accommodate up to 50 copies of the chromosome (Ruban-Osmialowska *et al.*, 2006). These chromosomes are then positioned at regular intervals along the length of the aerial hyphae through the action of ParA and ParB (Jakimowicz *et al.*, 2005, 2007). Sites destined for septation are also marked at regular intervals along the length of the aerial hyphae by SsgA, and other orthologous proteins (Noens *et al.*, 2007). Synchronously, FtsZ forms a corkscrew-like

structure around the hyphal periphery leading to the formation of evenly spaced rings at the sites of septation (Schwedock *et al.*, 1997). Septation occurs through constriction of FtsZ and closure of the septa by FtsQ (Mistry *et al.*, 2008). During the process of septation, nucleoid condensation occurs through the action of several proteins such as the histone-like protein HupS (Salerno *et al.*, 2009), Smc (Kois *et al.*, 2009; Dedrick *et al.*, 2009) and Dps (for DNA protection during starvation) (Facey *et al.*, 2009). The action of FtsK also ensures that each spore contains a complete copy of the chromosomal DNA (Wang *et al.*, 2007; Ausmees *et al.*, 2007; Dedrick *et al.*, 2009).

Uninucleate spores form through a process of cell wall degradation and rounding of the spores (Flärdh *et al.* 1999). As maturation proceeds the spore wall thickens as peptidoglycan is deposited into the spore wall, which provides some resistance to adverse conditions (Noens *et al.*, 2005). In the final stages of maturation a grey spore pigmentation produced by the action of polyketide synthases is deposited in the spore wall (Davis & Chater, 1990).

Mutations that give rise to strains that are unable to form the grey pigmented spores and maintain the white appearance of the aerial hyphae are known as *whi* (white) mutants (Chater, 1972; Hopwood *et al.*, 1970). Mutations of genes encoding proteins required for differentiation of aerial hyphae into spores, such as FtsZ and SsgA, whose roles are outlined above, give rise to such a phenotype. However several of the original *whi* genes that were identified during early screens for sporulation defective strains (Hopwood *et al.*, 1970; Chater, 1972; Ryding *et al.*, 1999) have regulatory functions, analogous to the numerous *bld* genes that also have regulatory roles (Chater & Chandra, 2006; Flärdh & Buttner, 2009).

Both *whiA* and *whiB* mutants show identical pleiotropic defects and functional *whiA* and *whiB* genes are required for cessation of growth of aerial hyphae and subsequent differentiation into spores (Chater, 1972; Flärdh *et al.*, 1999; Kormanec *et*

al., 1998; Xie *et al.*, 2007). This may be explained by the fact that they are both regulatory proteins that show transcriptional interplay (Jakimowicz *et al.*, 2006) and are predicted to interact (Chater & Chandra, 2006). These proteins are required for expression of *ftsZ* (Flärdh *et al.*, 2000) and the *parAB* operon (Jakimowicz *et al.*, 2006) from promoters that are up-regulated during aerial growth or sporulation. As these gene products are required for formation of sporulation septae along the aerial hyphae, *whiA* and *whiB* mutants are unable to sporulate.

The *whiG*, *H*, *I* and *J* genes have also been shown to prevent upregulation of *ftsZ* during aerial growth and strains harbouring any of these mutations are subsequently unable to form sporulation septae (Flärdh *et al.*, 2000). WhiG is a sigma factor that controls expression of *whiH* and *whiI* that themselves code for regulatory proteins that seem to be self- and cross-regulating (Ryding *et al.*, 1998; Aínsa *et al.*, 1999). As such these three genes form a regulatory cascade. The action of WhiH is thought to be dependent on the levels of a metabolite whose downstream targets are thought to include FtsZ (Ryding *et al.*, 1998; Flärdh *et al*, 1999, 2000). WhiI has been shown to affect the expression of multiple genes and respond to an unknown ligand (Aínsa *et al.*, 1999; Tian *et al.*, 2007). One effect of mutation of *whiI* is that nucleoid condensation cannot occur therefore preventing sporulation (Aínsa *et al.*, 1999).

Other genes that appear to be important for sporulation include *whiD*, which encodes a transcription factor belonging to the same family as WhiB and appears to be important for septation (Molle *et al.*, 2000), the *whiE* locus, that encodes components of a polyketide synthase pathway responsible for production of the grey spore pigment (Davis & Chater, 1990; Kelemen *et al.*, 1998) and *whiJ* that has been partially characterised (Gehring *et al.*, 2000; Aínsa *et al.*, 2010). It is hypothesised that WhiJ is a repressor of sporulation, however, the signal it responds to and the genes it represses are currently unknown (Aínsa *et al.*, 2010).

Although some of the key structural and regulatory components required for germination, vegetative growth, aerial development and sporulation of *S. coelicolor* have been laid out here, it should be noted that this account is far from complete and the division of the lifecycle into separate stages allows for a description of each process but generally fails to highlight the overlap between each, for example BldN negatively regulates the transcription of *whiG* (Elliot *et al.*, 2001). It should also be noted that many of the molecular details underpinning the development process in *Streptomyces* are yet to be elucidated, as highlighted by the fact that many of the regulatory proteins, such as WhiI and WhiJ are thought to have unidentified interacting partners (Aínsa *et al.*, 1999, 2010). Correct temporal and spatial development of the streptomycetes is a complex process requiring the integration of both physical and chemical signals from the environment as well as self-made molecules that may be localised either intracellularly or extracellularly to influence development.

1.1.2 Antibiotic and secondary metabolite production by *Streptomyces*

Another main reason that *Streptomyces* have been intensely studied in the laboratory is due to the fact that collectively members of the genus produce nearly 70 % of all naturally-occurring antibiotics as well as other biologically active molecules such as the medically important anti-tumour agent bleomycin (Vermel, 1972). They are also widely utilised by the biotechnology industry for heterologous protein production because, in addition to being prolific protein secretors, they are able to carry out post-translational modifications, such as glycosylation, greatly enhancing the biological activity of the proteins produced (Hesketh & Chandra, 2002).

S. coelicolor is often utilised for studies into the production of antibiotics as it produces five antibiotics; methylenomycin (Mmy) (Hayes *et al.*, 1997), calcium-dependent antibiotic (CDA) (Kim *et al.*, 2004), antibiotic coelicolor polyketide (abCPK)

(Gottelt *et al.*, 2010) and two pigmented antibiotics, actinorhodin (Act) (Magnolo *et al.*, 1991) and undecylprodigiosin (Red) (White & Bibb, 1997), that are blue and red, respectively (Figure 1.2). The latter two antibiotics therefore provide an easy visual screening system for their production.

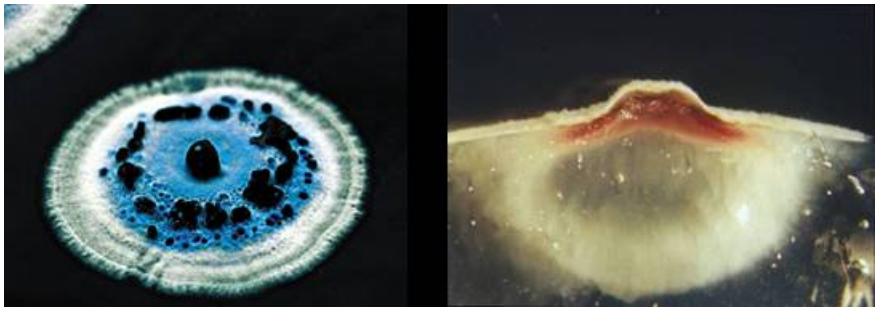

Figure 1.2: *Streptomyces coelicolor* colonies showing production of the blue pigmented antibiotic actinorhodin (left) and the red pigmented undecylprodigiosin (right). Courtesy of The John Innes Centre.

The potential for the discovery of new secondary metabolites from the *Streptomyces* genus is thought to be enormous (Watve *et al*., 2001) and indeed the *S. coelicolor* genome sequence revealed the presence of many more gene clusters encoding biosynthetic proteins required for production of secondary metabolites (Bentley *et al.*, 2002). The molecules produced by the action of two such 'cryptic' secondary metabolite clusters, coelichelin and the isogermicidins, have since been identified and characterised (Lautru *et al.*, 2005, 2007; Song *et al.*, 2006). Genes encoding the machinery to synthesise antibiotics and other secondary metabolites are often located on the arms of the chromosome, regions that have been shown to undergo extensive deletion and are dispensable for growth under laboratory conditions (Volff & Altenbuchner, 1998). However, the production of anti-microbial and anti-fungal compounds by the streptomycetes obviously confers a competitive advantage over other organisms that they encounter in their natural environment.

In *S. coelicolor*, production of antibiotics and other secondary metabolites takes place during the transition phase from vegetative to aerial growth (Chater, 1989; Gramajo *et al.*, 1993; Takano *et al.*, 1992; Kelemen & Buttner, 1998). It has been shown that mutation of many of the *bld* genes, required for production of aerial hyphae (see Section 1.1.1), also leads to defects in antibiotic production (White & Bibb, 1997; Pope *et al.*, 1998; Elliot *et al.*, 1998; Hunt *et al.*, 2005; Bignell *et al.*, 2000). Antibiotic production is also subject to control by quorum sensing, with the involvement of the γ-butyrolactone quorum sensing molecules whose detection leads to de-repression of antibiotic production (reviewed by Takano, 2006). Deletion of *relA* that encodes ppGpp synthetase required for the stringent response (Strauch *et al.*, 1991) leads to increased expression of the pathway specific regulators for Act and Red and subsequent production of these antibiotics (Chakraburtty *et al.*, 1996). Nutritional status has also been shown to regulate antibiotic production through the regulator DasR (Rigali *et al.*, 2008). Given the degree of complexity, a complete picture of the factors influencing antibiotic production cannot be formulated, however a good outline is provided by Bibb (2005).

1.2 Common scab caused by *Streptomyces scabies* and related species

The interest in antibiotic production has often overshadowed the relationship that a few members of the *Streptomyces* genus have with eukaryotic organisms that they encounter in the environment. Some of these are symbiotic and therefore are advantageous to the organism with which the relationship has been formed, for example antibiotic production by *Candidatus Streptomyces philanthi* protects the cocoon of the bee wolf wasp from fungal infestation (Kaltenpoth *et al*., 2005). Others are not so advantageous and cause disease in the eukaryotes with which they associate. By far the

most common disease caused by a streptomycete, not surprisingly given their abundance in soil, is common scab of potatoes and other root crops, caused by the plant pathogen *Streptomyces scabies* (Lambert & Loria, 1989a).

Infection of potato by *S. scabies* leads to scabby lesions of the surface of the tuber that can vary in severity, as seen in Figure 1.3. The least severe symptoms are superficial 'corky' scabbing to the surface of the potato tuber (Figure 1.3 left), producing an aesthetically less appealing potato with a reduced market value. More severe symptoms include deep-pitted and raised-corky lesions (Figure 1.3 middle & right) resulting in a potato that requires greater processing. Although the tuber yield is not greatly reduced by infection, the increased processing required and subsequent wastage greatly reduces the marketability of the crop.

Cases of common scab were traditionally thought to be endemic only in the United States but have now been reported in potato growing regions worldwide (Loria *et al.*, 1997). This is, at least in part, thought to be attributable to climate change, as warm dry growing conditions particularly favour development of the disease (Hooker, 1981; Loria *et al.*, 2006). Due to the damage caused, wide host range and global distribution it is apparent that disease caused by *S. scabies* and related species represents a significant economic problem for potato growers (e.g. Hill & Lazarovits, 2005).

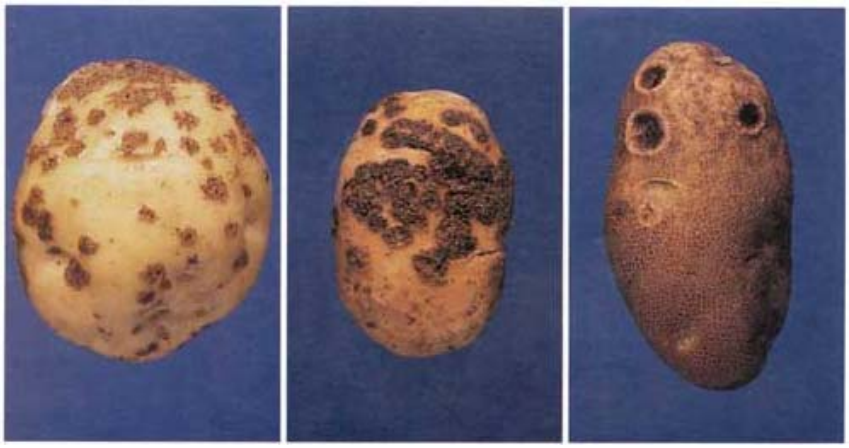

Figure 1.3: Symptoms of common scab on the surface of potato tubers. The difference in severity and type of lesion can be seen as superficial (left), raised corky (middle) or deep-pitted (right). Courtesy of Rosemary Loria.

1.2.1 Pathogenicity islands and horizontal gene transfer

The relatively recent emergence of new pathogenic species such as *S. acidiscabies* (Lambert & Loria, 1989b) and *S. turgidiscabies* (Miyajima *et al*., 1998) as well as studies on the relatedness of scab-causing species (Bramwell *et al*., 1998; Doering-Saad *et al*., 1992) suggested that the genetic factors that confer pathogenicity are shared between species. To date several species have been described that are able to induce the symptoms of common scab, some of which are listed in Table 1.1. Other less well characterised plant-pathogenic streptomycetes include *S. reticuliscabiei* that causes netted scab (Bouchek-Mechiche *et al.*, 2000; 2006) and *S. ipomoeae* that causes sweet potato "rot" or "pox" (Elliot, 1916) but these will not be discussed further here.

Species:	Distribution:	Additional info:	References:
S. scabies	Worldwide	Causative organism of "common scab". Isolated from many crops including potato, radish carrots and beet. Genome sequence available at http://www.sanger.ac.uk/Projects/S_scabies/	Lambert & Loria (1989a)
S. ascidiscabies	Possibly distributed beyond North America as also isolated from Japan and Korea	The major scab-causing pathogen in acidic soils with the ability to grow below pH 5.2. Causative organism of "acid scab"	Lambert & Loria (1989b); Tóth *et al*. (2001)
S. turgidiscabies	Isolated from locations in Japan, Korea Finland and Sweden	Causes severe erumpent legions	Miyajima *et al.* (1998); Song *et al.* (2004); Kreuze *et al.* (1999)
S. europaeiscabiei	Identified in France, also found in Korea	Causes common scab symptoms	Bouchek-Mechiche *et al.* (2000); Song *et al.* (2004)
S. stelliscabiei	Identified in France, also isolated from the eastern US and Canada	Produces star-like lesions on the surface of the tuber. Known to infect potato, carrot and radish	Bouchek-Mechiche *et al.* (2000, 2006)
S. luridiscabiei	Isolated in Korea	Produces lesions typical of common scab but does not produce thaxtomin, can grow in low pH soils	Park *et al.* (2003)
S. puniciscabiei			
S. niveiscabiei	Isolated in Korea	Produces lesions typical of common scab, can grow in low pH soils	Park *et al.* (2003)
S. caviscabies	Isolated in Québec, Canada	Produces deep-pitted lesions on the tuber	Goyer *et al.* (1996)

Table 1.1: Members of the *Streptomyces scabies* species complex that are able to elicit the symptoms of common scab. Information regarding distribution and characteristics of the individual species is provided.

Streptomyces that are able to cause common scab all share the ability to produce the phytotoxin thaxtomin (Bukhalid *et al.*, 1998; Loria *et al.*, 2006) with the exception of *S. luridiscabiei* and *S. puniciscabiesi* that do not appear to produce thaxtomins (Park *et al.*, 2003). The close proximity of the genes required for thaxtomin biosynthesis and the separate virulence factor Nec1 in the genome of scab-causing species provided a starting point for determining whether a pathogenicity island is present in the genomes of virulent species (Bukhalid *et al.*, 2002).

Pathogenicity islands (PAIs) are often found in bacterial pathogens, where genes involved in virulence are organised together. DNA sequencing of the regions surrounding *nec1* and the nitric oxide synthase gene required for synthesis of thaxtomin A led to the discovery of a large PAI with a low G+C content (Kers *et al*., 2005). This island was able to self-mobilise and site-specifically integrate into the genome of other *Streptomyces* species. When this island was transferred to two different non-pathogenic strains, the transconjugants showed varying results in the pathogenicity assays. In *S. coelicolor* transconjugants, thaxtomin was not produced, and no symptoms were observed when potato tuber slices were inoculated with the transconjugant strain. However, *S. diastatochromogenes* transconjugants did produce thaxtomin and were able to cause tissue necrosis in the same assays. It was thought that this was due to differences in the function of the PAI depending on the genetic background of the strain, although this warrants further investigation (Kers *et al*., 2005). This PAI is believed to be the reason for emergence of new scab-causing *Streptomyces* spp.

During sequencing of the PAI, other genes were discovered that encoded proteins with high homology to those known to function in virulence of other species (Kers *et al*, 2005). One such gene encoded the tomatinase enzyme that is known to play a role in the suppression of plant defences (Bouarab *et al*., 2002). Further genes, found only in the PAI from *S. turgidiscabies*, were the *fas* operon that is normally encoded on

a linear plasmid in the Gram positive bacterium *Rhodococcus fascians* and is involved in the production of leafy galls by synthesis of a cytokinin (Goethals *et al*., 2001). Given the apparent later acquisition of the *fas* genes into the PAI and the amenability of streptomycete genomes to the acquisition of genes by horizontal gene transfer (Bentley *et al*., 2002), there may be many more genes encoding for proteins that have potential to function in virulence.

1.2.2 Virulence determinants of common scab infection

Pathogenic bacteria produce a number of molecules that are delivered to the host cell where they interact at the molecular level to enhance infectivity. These are known as virulence factors or determinants. Some of the virulence determinants produced by scab-causing *Streptomyces* are described below.

1.2.2.1 Phytotoxins produced by scab-causing streptomycetes

As discussed above in Section 1.1.2, a number of secondary metabolites are produced by non-pathogenic members of the *Streptomyces* genus that confer to the organism a selective advantage in the environment by, for example, eliminating other microbes present in the soil that may compete for nutrients. However, plant pathogenic bacteria are renowned for their ability to produce secondary metabolites that function as phytotoxins, enhancing the infectivity of the organism (Strange, 2007). *S. scabies* has been shown to produce two such secondary metabolites, thaxtomins and a coronafacic acid (CFA)-like molecule that are each described below.

1.2.2.1.1 Thaxtomin

The primary virulence determinant of *S. scabies* is thaxtomin A (Loria *et al.*, 1995; King *et al.*, 2001; King and Calhoun, 2009). Thaxtomins are unique nitrated

dipeptides (King *et al*., 1989; King *et al*., 1992) with thaxtomin A being the most abundantly produced of the thaxtomins by a ratio of about 20:1 (King *et al*., 1991). The structure of thaxtomin A can be seen in Figure 1.4. The application of thaxtomin alone has been shown to be sufficient for induction of necrosis on immature potato tubers (Lawrence *et al*., 1990) and has also been demonstrated to inhibit seedling growth (Loria *et al*., 2006). The genes required for synthesis of thaxtomin A were identified (Healy *et al*., 2000; Kers *et al*., 2004) and strains harbouring mutations in these genes were shown to be avirulent on potato tubers (Healy *et al.*, 2000).

As thaxtomins are produced when strains are grown in liquid media (Loria *et al.*, 1995), the ability of scab-causing streptomycetes to produce thaxtomin is not dependent on colonisation of a living host. Media containing plant cell wall components or complex carbohydrates were shown to stimulate thaxtomin production (Loria *et al*. 1995; Wach *et al*. 2007; Joshi *et al*. 2007b), whereas the addition of glucose suppresses its production (Babcock *et al*., 1993; Loria *et al*. 1995; Wach *et al*. 2007).

The production of TxtR, a transcriptional regulator of all genes known to be involved in thaxtomin production (Healy *et al*. 2000; Kers *et al*. 2004), is dependent on the presence of cellobiose, the smallest precursor of cellulose which is probably released during expansion of plant tissue, and cellobiose is also a ligand for TxtR (Joshi *et al*. 2007b).

It was initially demonstrated that the effect of thaxtomin A is similar to that of herbicides known to be inhibitors of cellulose synthase (King *et al*., 2001) and it was later found that pre-incubation of seedlings with thaxtomin reduced the incorporation of ^{14}C-glucose into cell walls, showing that it is indeed an inhibitor of cellulose synthase (Scheible *et al.*, 2003). This explains why active expansion of plant tissue is required for the establishment of infection.

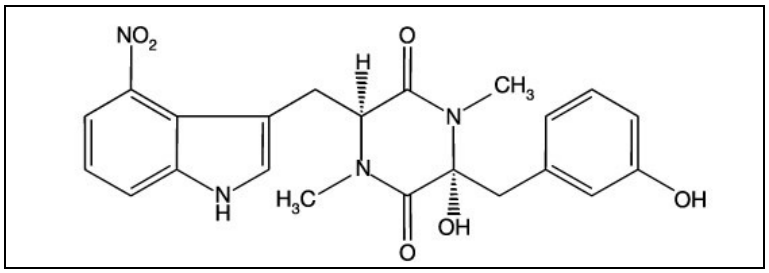

Figure 1.4: Molecular structure of Thaxtomin A

1.2.2.1.2 Coronafacic acid-like molecule

Bioinformatic analysis of the *S. scabies* 87-22 genome has revealed the presence of a secondary metabolite biosynthetic gene cluster that is predicted to synthesize a CFA-like molecule (Bignell *et al.*, 2010). CFA is a component of the coronatine (COR) phytotoxin produced by another plant pathogen, *Pseudomonas syringae,* which contributes to lesion size and bacterial load in several plant hosts (Bender *et al.*, 1987). The structure of COR can be seen in Figure 1.5. COR has been shown to act as a molecular mimic of jasmonate derivatives (Katsir *et al.,* 2008b; Melotto *et al.*, 2008), which are signalling molecules involved in plant immunity (Katsir *et al.,* 2008a). Pathogens that produce COR are able to suppress plant defence responses leading to enhanced infection (Bender *et al.*, 1999). Another component of COR is coronamic acid (CMA), a derivative of isoleucine (Bender *et al.*, 1999). Although *S. scabies* lacks the biosynthetic genes required for production of CMA, the CFA-like biosynthetic cluster encodes a homologue of the protein that is responsible for ligating CFA to CMA (Bignell *et al.*, 2010) and therefore may produce COR-like molecules whereby the CFA-like molecule is ligated to an amino acid such as valine, isoleucine, serine or threonine, which has also been suggested to occur in *P. syringae* (Bender *et al.*, 1999). It should be noted that the genome of *Pectobacterium atrosepticum* strain SCRI1043,

the causative organism of soft rot and black leg of potato, encodes a similar gene cluster encoding a CFA-like molecule (Bell *et al.*, 2004) and this organism has been shown to produce CFA-like amino acid conjugates (Ravendale & Toth, unpublished).

A number of important differences exist between the CFA biosynthetic gene clusters of *S. scabies* and *P. syringae* leading to the conclusion that the CFA-like metabolite produced by *S. scabies* is likely novel in structure in comparison to the *P. syringae* CFA molecule (Bignell *et al.*, 2010).

Growth of *S. scabies* in media containing plant derived material, known to support the production of other virulence associated secondary metabolites, leads to expression of the CFA-like cluster. The primary promoter region of the CFA-like gene cluster is also activated during colonisation of living plant tissue by *S. scabies*. When taken together these suggest that the CFA-like secondary metabolite is produced during host microbe interactions (Bignell *et al.*, 2010). Mutational analysis and over-expression studies of the CFA-like biosynthetic gene cluster indicated that although the resulting metabolite is not essential for infection, it is important for the development of disease symptoms (Bignell *et al.*, 2010).

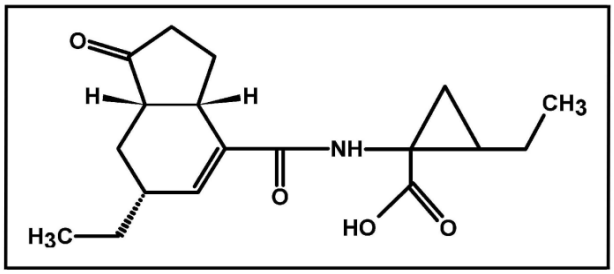

Figure 1.5: Molecular structure of COR phytotoxin

1.2.2.2 Proteins produced by scab-causing streptomycetes

1.2.2.2.1 Nec1

Nec1 is encoded in the same genetic region as the thaxtomin biosynthetic enzymes, on the PAI that is present in many scab-causing *Streptomyces* species (Bukhalid *et al.*, 2002; Kers *et al.*, 2005). Nec1 is a secreted protein with two translational start sites, both of which show N-terminal processing following secretion (Joshi *et al*., 2007a). Joshi and colleagues (2007) also showed that the extracellular fractions of cultures from *nec1* mutant strains of *S. turgidiscabies* demonstrated greatly reduced necrogenic activity on potato tissue slices when compared to the wild type strain, as well as reduced virulence in whole plant assays on *Arabidopsis*, tobacco and radish.

Analysis of the N-terminal region of Nec1 has shown that it possesses a signal sequence that may lead to secretion via either the Sec or the Tat pathway. Experimental testing of this signal sequence using the agarase assay, a genetic reporter assay allowing the identification of Tat signal peptides (Widdick *et al.*, 2008), has indicated that the Nec1 signal peptide does not target to the Tat pathway and therefore Nec1 is most likely secreted via Sec (Mann, 2009). Nec1 is an unusual protein that has no known homologues in the protein database, and it is thought to act by suppressing the plant defence response (Bukhalid & Loria, 1997; Joshi *et al.*, 2007a).

1.2.2.2.2 Tomatinase

During sequencing of the *S. turgidiscabies* PAI, a gene was discovered that encodes for a putative tomatinase (Kers *et al.*, 2005). Tomatinase enzymes are normally found in fungal plant pathogens of tomato such as *Septoria lycopersici* and *Fusarium oxysporum* f. sp. *lycopersici*. In these organism tomatinase has been implicated as being important for infection of tomato (Pareja-Jamie *et al.*, 2008) Tomatinases are

enzymes that act as glycosyl hydrolases to break down α-tomatin (Bouarab *et al*., 2002), an anti-microbial compound produced by plants (VanEtten *et al.*, 1994). Tomatinases are also known to be involved in the suppression of the induced plant defence response (Bouarab *et al*., 2002; Ito *et al.*, 2004). Although mutation of the *S. scabies* tomatinase gene *tomA* rendered the bacteria sensitive to α-tomatine (during aerial growth), indicating that the enzyme is active, it appears to be dispensible for infection (Seipke & Loria, 2008).

1.3 Bacterial protein translocation systems

In both prokaryotic and eukaryotic organisms proteins must be targeted to their site of action, be it cellular or extracellular, so that they can correctly perform their physiological functions. In bacteria (and eukaryotes) many secreted proteins perform essential functions and contribute to a wide range of physiological processes such as nutrient acquisition from the environment and respiration. In the case of pathogenic bacteria proteins are produced that function as virulence factors and therefore must be located extracellularly in order to interact with their host cell target and successfully establish infection.

Delivery of proteins from their site of synthesis to the extracellular environment is known as ‘secretion’. Proteins that are destined for secretion perform diverse functions and therefore belong to many classes. Taken together with the fact that membrane architecture differs between bacteria, a number of systems have evolved for efficient movement of proteins across these membranes, referred to as ‘export’ or ‘translocation’. A brief outline of these can be found in this section and a schematic representation of these can be seen in Figure 1.6.

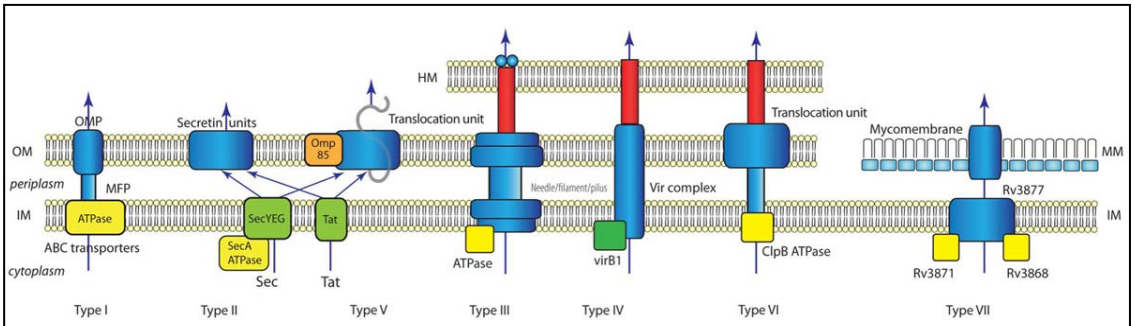

Figure 1.6: Schematic representation of protein secretion systems currently described from Gram-positive and Gram-negative bacteria. HM: Host membrane; OM: outer membrane; IM: inner membrane; MM: mycomembrane; OMP: outer membrane protein; MFP: membrane fusion protein. ATPases and chaperones are shown in yellow. Courtesy of Tseng *et al.* (2009).

1.3.1 Protein translocation systems in Gram-negative bacteria

In Gram-negative bacteria, such as the model prokaryote *E. coli*, the cell envelope comprises two membranes. The cytoplasmic membrane (CM) is composed of a phospholipid bilayer whereas the outer membrane (OM) is an asymmetric bilayer containing phospholipid and lipid A, which serves to anchor an outer layer of lipopolysaccharide (LPS) to the cell surface. The region between the CM and the OM is the periplasm and contains the cell wall that is made up of a thin layer of peptidoglycan. Proteins performing a periplasmic function must be translocated across the CM only. However, for extracellular proteins, transport across the CM, the periplasm and the OM must be achieved to reach the extracellular environment.

Gram-negative bacteria possess a number of different systems that are able to transport proteins across the cell envelope, either as a single or a two step process. The Sec and the Tat machineries, located in the CM, are found in both Gram-positive and Gram-negative bacteria whereas the Type I-VI protein translocation systems are unique to Gram-negative bacteria. In addition, two of these systems, Type III and IV, are particularly important to the virulence of pathogenic bacteria as their machineries penetrate eukaryotic cell membranes allowing delivery of virulence factors directly into the host cell.

1.3.1.1 The Type I secretion system (T1SS)

The first protein known to be secreted by a T1SS is α-haemolysin, encoded by *hlyA* from uropathogenic *E. coli* (Goebel & Hedgpeth, 1982). This protein is required for virulence and has been shown to form pores in host cell membranes, resulting in lysis (Bhakdi& Tranum-Jensen, 1988; Hughes *et al.*, 1992; König *et al.*, 1994). Two other genes, *hlyB* and *hlyD*, are also required for secretion (Goebel & Hedgpeth., 1982; Welch *et al.*, 1983; Mackman & Holland, 1984). It was later found that α-haemolysin is targeted for secretion by means of a signal sequence at its C-terminus (Nicaud *et al.*, 1986) and that this C-terminus was able to direct export of a chimeric protein (Mackman *et al.*, 1987). The proteins encoded by *hlyB* and *hlyD* are components of a secretion apparatus present in many Gram-negative bacteria.

The T1SS, which is related to ATP-binding cassette (ABC) transporters, transverses the entire cell envelope. The secretion apparatus is composed of three components; the ABC-related protein located in the CM, the membrane fusion protein (MFP) that spans the periplasm and the outer membrane factor (OMF) that, as the name suggests, is located in the OM (Dinh *et al.*, 1994; Paulsen *et al.*, 1997). These three components interact with each other to form a channel through the cell envelope. Structural evidence suggests that the OMF undergoes conformational change allowing opening of the channel during substrate transport (Koronakis *et al.*, 2000; Akama *et al.*, 2004).

It has been shown that the substrate binds to the ABC protein through interactions with its C-terminus and this promotes assembly of the secretion apparatus, ATP hydrolysis and subsequent secretion of the substrate (Létoffé *et al.*, 1996; Thanabalu *et al.*, 1998). The C-terminal location of the signal sequence indicates that the entire protein is synthesised prior to secretion which raises questions regarding the conformational state of T1SS substrates. It is now known, through studies carried out

on *Serratia marcescens* HasA, that T1SS substrates may be held in a loosely folded or unfolded state by the SecB chaperone (Delepelaire & Wandersman, 1998), although this does not appear to play a role in delivery of the substrate to the transport apparatus (Sapriel *et al.*, 2003; Wolff *et al*., 2003).

Various T1SS substrates have been described with a diverse range of functions that includes lipases, proteases and haemophores, such as HasA from *S. marcescens* (Létoffé *et al.*, 1994a,b), that scavenges extracellular iron in the form of haem (Létoffé *et al.*, 1994a,b). These substrates have also been shown to contain a signal sequence of approximately 60 amino acids at their C-terminus. Most substrates contain a repeated amino acid stretch, GGXGXDXXX, just prior to the signal sequence that is believed to bind calcium ions and to assist folding in the extracellular space following secretion (Hui *et al.*, 2000). As well as being functionally diverse, these proteins have also been noted to differ greatly in size (reviewed by Delepelaire, 2004). However, a strict consensus sequence for secretion has not been defined and recent work suggests that multiple regions throughout T1SS substrates may be important for binding to the ABC protein (Masi & Wandersman, 2010).

1.3.1.2 The Type II secretion system (T2SS)

The T2SS was first discovered in *Klebsiella pneumoniae* when localisation of pullulanase, encoded by *pulA*, to the surface of the OM was shown to be dependent on coordinated expression with other genes (d'Enfert *et al.*, 1987a,b). Subsequent studies involving expression of *pulA* and neighbouring genes in *E. coli* and mutational analysis in *K. pneumoniae* led to the identification of the '*pul*' genes encoding components of a system required for pullulanase secretion (d'Enfert *et al.*, 1987a,b; d'Enfert & Pugsley, 1989; Pugsley & Reyss 1990; Reyss & Pugsley, 1990; Possot *et al.*, 1992). This became known as the T2SS and similar systems have since been identified and

characterised in numerous Gram-negative bacteria such as *Burkholderia glumae* (Kang *et al.*, 2008), *Erwinia chrysanthemi* (Kazemi-Pour *et al.*, 2004) and *Ralstonia solanacearum* (Poueymiro & Genin, 2009).

T2SSs have been shown to export substrates of different classes including proteases (Scott *et al.*, 2001; Sikora *et al.*, 2011), lipases (Aragon *et al.*, 2000), chitinases (Folster & Connell, 2002), aminopeptidases and metalloproteases (Rossier *et al.*, 2008). The T2SS is present in many plant pathogens and has been shown to secrete proteins that contribute to pathogenesis, largely through degradation of plant cell walls (reviewed by Gopaljee *et al.*, 2005).

The T2SS apparatus is composed of 12 conserved components; a cytoplasmic ATPase that is anchored to the membrane through interactions with three integral CM proteins, five proteins that comprise both major and minor pseudopilins, a peptidase that is located in the CM required for pre-assembly processing of the pseudopilins, a 'secretin' protein that oligomerises to form pores in the outer membrane and a further protein that links components of the CM and the OM (for a recent review see Cianciotto, 2009).

Although the secretion apparatus spans the cell envelope it does not form a continuous channel across the periplasm, as is the case for other secretion systems. Instead substrate proteins are secreted in a two-step process that depends on either the Sec or Tat export machinery to export proteins across the CM to the periplasm (these systems are described in more detail in Section 1.3.2). This is highlighted by the fact that over-expression of T2SS substrate proteins or absence of T2SS components not only prevents secretion of substrates to the extracellular environment but leads to their accumulation in the periplasm (Burr *et al.*, 2001; Voulhoux *et al.*, 2001).

Once in the periplasm substrates are recognised by the T2SS apparatus. Although the precise signal that directs proteins for secretion by the T2SS is unknown, given that

the proteins are folded into their tertiary structure prior to secretion it is thought that multiple regions of the protein are required to make contact with components of the T2SS apparatus (Francetic & Pugsley, 2005). Following interactions of the substrate with the secretion apparatus, the T2SS ATPase hydrolyses ATP leading to a conformational change that is thought to polymerise the pseudopilins into a pseudopilus to push the substrate out through the pore formed by the secretin in the OM (Cianciotto, 2009).

1.3.1.3 The Type III secretion system (T3SS)

The T3SS is widely known for the role it plays in the delivery of effectors, proteins that are key to the virulence of the majority of Gram-negative pathogens of plants and animals, into host cells. The wide distribution of the T3SS amongst pathogenic bacteria has resulted from horizontal gene transfer, with genes encoding effector proteins and secretion apparatus components being found on large plasmids and PAIs e.g. *Salmonella* pathogenicity island 2 (SPI2) (Shea *et al.*, 1996).

It should be noted that many components of the T3SS show significant similarity to the flagellar export system, from which the T3SS is widely accepted to be evolved (He *et al.*, 2004).

Effector proteins are generally involved in establishment of infection and have been shown to have numerous functions varying from restructuring of the host cytoskeleton, (Zhou *et al.*, 1999a,b) to activating transcription of host genes to promote infection (Sugio *et al.*, 2007). Perhaps the most famous example of an effector in terms of plant pathology is AvrA from the plant pathogen *Pseudomonas syringae pv. glycinea* that was first the first effector to be cloned and characterised (Staskawicz *et al.*, 1984). AvrA provided the first evidence of a protein that induces a specific response in the plant host through recognition by the corresponding resistance (R) gene product in the

plant, a theory that was proposed some years earlier (Flor, 1971; Ellingboe, 1982). AvrA induces a hypersensitive response (HR), a localised cell death preventing spread of infection in soybean cultivars expressing the corresponding R gene in non-hosts (Keen, 1990; Nürnberger, 2004). Many Avr proteins have been identified that function as virulence factors with a range of functions that aid colonisation of their plant host (reviewed by Alfano & Collmer, 2004).

The T3SS spans not only the entire cell envelope but also transverses the host cell membrane allowing delivery of effector proteins directly into the host cell cytoplasm. In order to achieve this T3SS are complex machineries composed of at least 20 different proteins (reviewed by Worrall *et al.*, 2011).

At the cytoplasmic membrane there are five conserved transmembrane proteins and several membrane associated proteins that includes the ATPase of the T3SS. Two of the transmembrane proteins contain large cytoplasmic domains that have been proposed to regulate entry of substrates the export channel (Bange *et al.*, 2010).

The basal body of the T3SS complex spans the bacterial cell envelope. Although this is a large structure it has been shown to be composed of three main proteins that form ring structures through homo-oligomerisation. Two of these proteins are present in the CM whereas the third, a member of the secretin family, is located in the OM. Recent evidence suggests that a conserved structural motif is important for the association of these three proteins (Spreter *et al.*, 2009). The secretin protein is also associated with pilotin, a small lipoprotein involved in its localisation to the OM (Lario *et al.*, 2005; Okon *et al.*, 2008).

In addition to these components there is a needle complex, composed of hundreds of subunits of the same protein, that protrudes from the basal body and is anchored at approximately the mid-point of the basal body by the inner rod protein (Marlovits *et al.*, 2006). At the end of the needle, at the point of contact with the host

cell membrane, are several proteins that form what is known as the 'tip complex' that allow a pore to be formed in the host cell membrane (Sani *et al.*, 2007; Mueller *et al.*, 2008).

Interestingly, in plant pathogenic bacteria there is currently no evidence for existence of the tip complex (Büttner & He, 2009) and the tip includes an appendage, known as the Hrp pilus that forms a longer structure presumed to be required for penetration of the thick plant cell wall (Roine *et al.*, 1997; Weber *et al.*, 2005; Kvito *et al.*, 2007).

Substrate proteins are targeted for secretion by means of an ill-defined signal sequence at the N-terminus that is thought to rely on biophysical properties rather than the specific amino acid sequence (Guttman *et al.*, 2002; Arnold *et al.*, 2009; Samudrala *et al.*, 2009). There is also some evidence to suggest that specific chaperones, encoded alongside the substrates, are required for their delivery to the T3SS apparatus (Parson *et al.*, 2003; Büttner *et al.*, 2006).

As the translocation channel, formed by the needle complex, has been shown to have a diameter of only 2.8 nm (Marlovits *et al.*, 2004, 2006; Galán & Wolf-Watz, 2006) this implies that proteins are translocated in an unfolded state. Genes for T3SS are thought to be expressed upon contact with a host cell (Pettersson *et al.*, 1996). Once the apparatus is assembled, which involves secretion of the needle components through the apparatus itself, there has been shown to be a switch to secretion of effector proteins (Cornelis *et al.*, 2006; Ferris & Minamino, 2006; Wood *et al.*, 2008).

1.3.1.4 The Type IV secretion system (T4SS)

The T4SS has been shown to bear extensive structural and functional features to the F plasmid conjugation system and as such both systems are ancestrally related (Lawley *et al.*, 2003). Substrates of the T4SS therefore consist not only of proteins but

also protein-DNA complexes. The system has been most widely studied in *Agrobacterium tumefaciens* where the delivery of effector proteins and protein-DNA complexes into the plant host cell and subsequent integration into host DNA manifests as crown gall disease (Vergunst *et al.*, 2000).

The T4SS generally secretes its substrates across the entire cell envelope without the formation of periplasmic intermediates. In the *A. tumefaciens* system the translocation machinery is composed of multimeric complexes of twelve components (reviewed by Alvarez-Martinez & Christie, 2009). At the inner face of the CM three ATPases have been described that are required for interactions with the substrate and subsequent delivery of the substrate into the channel (Chen *et al.*, 2008; Lu *et al.*, 2005; Middleton *et al.*, 2005; Mojica *et al.*, 2009).

Delivery of substrates to the secretion apparatus has been shown to be dependent on the presence of a C-terminal sequence on the substrate protein (or protein component of the protein-DNA complex) that contains hydrophobic or positively charged residues (Nagain *et al.*, 2005; Simone *et al.*, 2001; Vergunst *et al*., 2005). However, other substrate properties along with chaperones and accessory factors have also been shown to be important for substrate binding to the T4SS (Chen *et al.*, 2005; de Jong *et al.*, 2008; Lu *et al.*, 2005; Page & Parson, 2002; Parker & Meyer, 2007; Schulein *et al.*, 2005).

Also located in the CM are four transmembrane proteins that interact with each other as well as other components of the secretion apparatus. One CM component, VirB10, undergoes conformational changes following ATP hydrolysis that stabilises interaction of components in the CM and the OM (Cascales & Christie, 2004). Another CM protein, VirB6, has been shown to have a periplasmic domain that mediates DNA transfer (Jakubowski *et al.*, 2004).

The outer membrane pore complex is formed from the association of multimers of two proteins, VirB7 and VirB9 (Bayliss *et al.*, 2007), that interact with VirB10 forming a complex that is thought to span the entire cell envelope (Fronzes *et al.*, 2009). The channel is composed of multimers of a single pilin protein, VirB2, which protrudes from the cell surface and has been shown to make contact with host cell membranes and deliver substrates directly into the host cell (Lai & Kado, 1998; Lai *et al.*, 2002). Pilus formation is aided by the activity of a transglycosylase, VirB1, which degrades peptidoglycan in the cell wall (Zahrl *et al.*, 2005). Another protein, VirB5, has also been shown to localise to the tip of the pilus and probably assists attachment to the host cell (Aly & Baron, 2007).

1.3.1.5 The Type V secretion system (T5SS)

The T5SS was first discovered when IgA1, a protease produced by *Neisseria gonorrhoeae* was shown to have three functional domains required for secretion; an N-terminal signal sequence, the functional enzyme and a C-terminal helper domain (Pohlner *et al.*, 1987). Numerous substrates have since been discovered, such as the toxin EspP from *E. coli* and the YapH adhesion-like protein from *Xanthomonas oryzae* pv. *oryzae* (Das *et al.*, 2009). Although substrates may be very different, the C-terminal domains are highly homologous (Yen *et al.*, 2002a) and all substrates possess the necessary features required for their own secretion. The T5SS system is therefore also termed the autotransporter secretion pathway (Henderson *et al.*, 1998).

Substrate proteins of the T5SS are secreted in a two-step process that first of all depends on the Sec pathway for export of the substrate across the CM into the periplasm. Following translocation, the N-terminal sequence is cleaved and then the C-terminal domain inserts into the OM forming a pore with a β-barrel structure (Maurer *et al.*, 1999). A linker region in the protein then directs secretion of the functional domain,

whose folding on the outer surface is assisted by the autochaperone domain of the protein (Oliver *et al.*, 2003). Once secretion and folding of the protein is complete it either remains exposed on the surface of the cell or is cleaved by a protease and released into the extracellular environment.

1.3.1.6 The Type VI secretion system (T6SS)

The T6SS is the most recently discovered of the Gram-negative secretion systems and is therefore the least well characterised. The first indication of the existence of a new Gram-negative secretion system dates back to 1996 when secretion of the protein Hcp in *Vibrio cholerae* was observed despite the fact that it lacked a cleavable signal peptide (Williams *et al.*, 1996). However, the T6SS was only recognised ten years later due to its role in secretion of Hcp and the VgrG proteins, and its contribution to cytotoxicity of *V. cholerae* towards the amoebae *Dictyostelium* (Pukatzki *et al.*., 2006). Another substrate of the T6SS has since been identified, VasX, and shown to be important for virulence (Miyata *et al.*, 2011). The T6SS has since been recognised as important for the virulence of other organisms such as *Pseudomonas aeruginosa* (Mougous *et al.*, 2006) and *Burkholderia spp*. (Aubert *et al.*, 2008; Burtnick *et al.*, 2010; French *et al.*, 2011). Functions beyond pathogenesis have now been shown, for example mediating cooperative feeding between members of the same species (Konovalova *et al.*, 2010). Genes encoding components of the T6SS are also present in the genomes of symbiotic organisms (Roest *et al.*, 1997; Ortet *et al.*, 2011; Pedrosa *et al.*, 2011).

Of the effector proteins discovered to date the best characterised of these is VgrG-1 from *V. cholerae* that contains a C-terminal actin cross-linking domain (Pukatzki *et al.*, 2007). VgrG-1 has been shown to cross-link actin both *in vitro* and *in vivo* and is secreted by *V. cholerae* in a mouse intestine model of infection, where it

contributes to virulence (Ma *et al.*, 2009; Ma & Mekalanos, 2010). However, the C-terminal domains of other VgrG proteins have a range of predicted functions (Pukatzki *et al*, 2007).

In *P. aeruginosa* three effector proteins, Tse1-3, have recently been identified (Hood *et al.*, 2010). Each of these has been shown to be part of a toxin-antitoxin system that specifically targets other bacterial species (Hood *et al.*, 2010; Russell *et al.*, 2011). Tse2 was initially characterised and although it was shown to have cytotoxic effects against both prokaryotic and eukaryotic organisms *in vitro,* it was shown to be targeted specifically to prokaryotic cells *in vivo* (Hood *et al.*, 2010). Tse1 and Tse3 have also been shown to be delivered to bacterial cells. These proteins are targeted to the periplasm of host cells where they break down peptidoglycan thereby interfering with the integrity of the cell (Russell *et al.*, 2011). In this way the T6SS of *P. aeruginosa* is required for antagonism of other bacterial species.

The current knowledge on how these effectors are targeted for secretion and the mechanism of export is limited. However, several of the core components of the translocation apparatus have been shown to be related to phage tail proteins (Bönemann *et al.*, 2010; Cascales, 2008; Leiman *et al.*, 2009). Considering that bacteriophage are thought to deliver their DNA across only the outer membrane of Gram-negative bacteria (Rakhuba *et al.*, 2010) taken together with the recent report by Russell *et al.* (2011) showing that effectors are delivered directly into the periplasm of the host cell, a mechanism has been proposed whereby the secretion apparatus forms a channel spanning the entire cell envelope of the donor strain as well as the outer membrane of the recipient/host strain. This is strengthened by reports indicating that persistent contact with the host cell is required for delivery of effectors (Schwarz *et al.*, 2010; Hood *et al.*, 2010; Russell *et al.*, 2011).

The number of components that make up the T6SS apparatus varies between species but several genes are conserved. Of the available Gram-negative genome sequences at the time of writing this thesis, 13 genes appear to be completely conserved (Boyer et al. 2009; Zheng and Leung 2007). Based on this and the limited information available from protein-protein interaction studies, a proposed organisation for the components of the system is as follows: At the CM there are two transmembrane proteins, IcmF and IcmH, that associate with each other (Sexton *et al.*, 2004) as well as an AAA+ ATPase, ClpV, that is thought to provide energy necessary for secretion (Bönemann *et al.*, 2009; Mougous *et al.*, 2006). Also anchored in the CM is a tubular structure composed of two proteins, VipA and VipB (Bönemann et al. 2009). This extends into the periplasm where it is thought to associate with a protein, VCA0109, and a lipoprotein at the inner face of the OM (Aschtgen *et al.*, 2008).

Hcp and VgrG have been found in the culture supernatant but they are thought to be components of the secretion apparatus itself. Hcp has been shown to crystallize as a ring structure that can oligomerise to form a nanotube (Mougous *et al*, 2006; Ballister *et al.*, 2008). The nanotube formed by Hcp is thought to extend from the cytoplasm as well as being capable of, along with VgrG, penetrating the membrane of a target cell. Other conserved components include chaperone-like proteins (Boyer *et al.*, 2009).

1.3.2 Protein translocation systems present in both Gram-positive and Gram-negative bacteria

As mentioned above, in Gram-negative bacteria a number of protein secretion systems exist that allow proteins to be exported across the cell envelope by either a one or two step process. In the case of the T2SS and the T5SS, proteins must first of all be transported across the cytoplasmic membrane to the periplasm, before their secretion across the outer membrane. This first step is achieved by either of two routes, namely

the Sec or Tat pathways. Gram-negative bacteria also utilise these two general transport systems for the localisation of periplasmic proteins or the insertion of inner membrane proteins.

The Sec and Tat systems are also found in Gram-positive bacteria where, given the different architecture of their cell envelopes, proteins are delivered directly into the extracellular milieu or inserted into the membrane.

1.3.2.1 The Sec pathway

The Sec pathway is present in Gram positive and Gram negative bacteria, as well as archaea and eukaryotes, and is generally regarded as the major pathway of protein export. As such the components of the Sec system and mechanism of protein export are highly conserved between species, and substrate proteins are exported in an unfolded state. Due to the wide range of substrates exported by the Sec system as well as the diversity and importance of their function, the Sec system is essential for viability. Although the Sec system is found in all bacteria it has been best characterised in *E. coli* and as such much of the information has been gained through studies carried out in this organism.

1.3.2.1.1 Sec signal peptides

All proteins destined for translocation by the Sec system contain a signal sequence at their N-terminus. This follows a general set of rules ensuring that it is properly targeted to the Sec machinery. In *E. coli* Sec signal sequences are usually between 18 and 25 amino acids residues in length, and have three domains (Figure 1.7) (i) the n-domain contains one to three positively charged amino acids; (ii) the h-domain contains 10-15 hydrophobic amino acid residues that forms an α-helix; (iii) the

hydrophilic c-domain contains a cleavage site, a conserved motif with neutral amino acids such as alanine at the -1 and -3 positions, that is recognised and cleaved by a signal peptidase following export (Paetzel *et al.*, 1998; 2002). The C-terminus of lipoproteins contain an alternative cleaveage site, known as the lipobox, consisting of a general motif L-A/S/T-G/A-C starting at the -3 position which is recognised and cleaved by a lipoprotein signal peptidase (Lsp; Rahman *et al.*, 2008) It should be noted that Sec signal peptides in Gram-positive bacteria conform to these basic principles but have an average length of 29-31 amino acid residues (von Heijne & Abrahmsen, 1989).

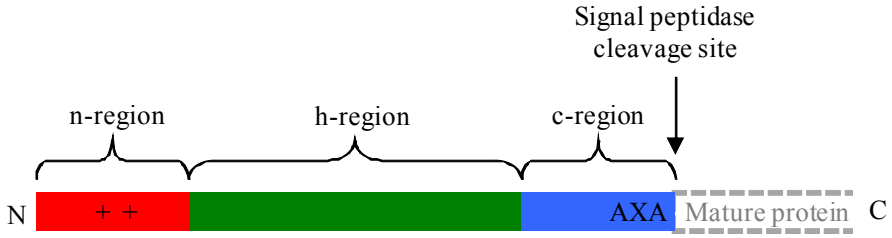

Figure 1.7: Schematic representation of the Sec signal peptide

1.3.2.1.2 The Sec Translocon

The three components of the Sec translocation machinery that have been shown to be absolutely essential in *E. coli* are SecA, SecE and SecY (Emr *et al.*, 1981; Oliver and Beckwith, 1982; Murphy *et al.*, 1995). Purification studies showed that two of these proteins, SecE and SecY, formed a complex with a third protein, SecG (Brundage *et al.*, 1990). Each component of the SecYEG complex is a transmembrane protein and together they form the protein conducting channel (van den Berg *et al.*, 2004). Although in *E. coli* SecG is not absolutely required for translocation of proteins it has been shown to increase transport efficiency *in vivo* (Nishiyama *et al.*, 1994), which also holds true for other organisms including *Streptomyces lividans* (Palomino & Mellado, 2008).

The essential protein, SecA, is a peripheral membrane protein having ATPase activity and makes contact with SecY (Eichler *et al.*, 1998) as well as SecB (Hartl *et al.*, 1990). SecA is a chaperone that binds to substrates, bringing them into contact with the translocation channel. Additionally SecA provides energy for protein transport through ATP hydrolysis - this process is described further below.

In *E. coli* a complex formed by three periplasmic-facing inner membrane proteins SecD, SecF and YajC is also required for maximal protein transport through the Sec translocase (Gardel *et al.*, 2000; Pogliano & Beckwith, 1994; Duong & Wickner, 1997a). YidC, whose role in insertion of membrane proteins is discussed below, also interacts with the SecDE/YajC complex (Nouwen & Driessen, 2002).

1.3.2.1.3 Targeting of proteins to the Sec Translocon

In the case of most soluble proteins, they are targeted for export following completion of translation by the ribosome. As the translocation apparatus can only accommodate unfolded polypeptides, the substrate proteins must be maintained in this state. All polypeptides that emerge from the ribosome are initially bound by a chaperone known as trigger factor (TF) (Valent *et al.*, 1997; Beck *et al.*, 2000). They are then bound by a number of chaperones, such as GroEL and DnaK (Bessinger & Buchner, 1998; Fink, 1999). However, these are non-specific chaperones in that they interact with many proteins and also assist in their folding (Hartl & Hayer-Hartl, 2002). In order to be maintained in an unfolded state the Sec signal peptide is recognised and bound by the Sec-specific chaperone SecB that interacts with multiple regions on the protein to prevent it folding into the mature structure (Park *et al.*, 1988; Randall *et al.*, 1998; Xu *et al.*, 2000).

SecB subsequently interacts with SecA dimer, which mediates transfer of the polypeptide to the membrane-bound translocation channel formed by the SecYEG

complex (Hartl *et al.*, 1990; Fekkes *et al.*, 1997). The protein is then transported through the SecYEG channel following several cycles of ATP hydrolysis by SecA (Driessen 1992, Duong & Wickner, 1997b; Economou *et al.*, 1995). A model has been proposed whereby binding of ATP leads to a conformational change in SecA that causes opening of the SecY channel and entry of the substrate. ATP hydrolysis leads to a further conformational change in SecY, driving transport of the polypeptide through the channel. Protein export is accomplished by further rounds of ATP hydrolysis (Mitra *et al.*, 2006). The proton motive force is also necessary for Sec-dependent transport however its role is currently not well understood (Schiebel *et al.*, 1991). A signal peptidase, whose active site is facing away from the external face of the membrane, cleaves the signal peptide resulting in release of the mature protein (Paetzel *et al.*, 1998; 2002). A schematic representation of protein export by the Sec translocase can be found below in Figure 1.8.

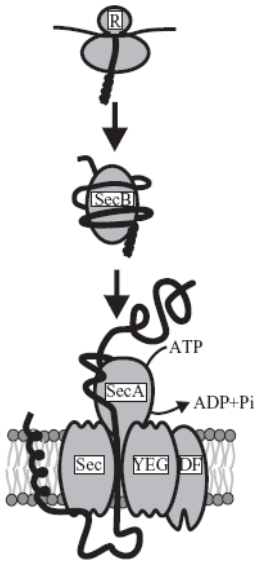

Figure 1.8: Schematic representation of protein export by the Sec translocase. Following ribosomal synthesis of a substrate, SecB binds the polypeptide. SecB then interacts with SecA and the substrate is delivered to the membrane bound SecYEG complex. Hydrolysis of ATP then results in protein translocation across the membrane. Courtesy of Bolhuis (2003).

The Sec pathway is bi-functional in that it is also required for insertion of proteins into the inner membrane (de Gier *et al.*, 1997). To achieve this, a second, SecB-independent pathway exists. In this pathway hydrophobic residues, such as those present in integral membrane proteins, are recognised and bound by the signal recognition particle (SRP) as the protein emerges from the ribosome (Valent *et al.*,

1997). SRP forms a complex with the ribosome and the nascent polypeptide chain (RNC) which then binds to FtsY in the cytoplasmic membrane (Bernstein 1989), which in turn associated with SecYEG (Angelini *et al*., 2005). GTP hydrolysis by FtsY leads to release of SRP and transfer of the RNC complex to SecYEG (Scotti *et al.*, 1999; Connolly *et al.*, 1991; Valent *et al.*, 1998). The protein is then thought to be exported in a co-translational manner with the energy for protein transport being provided by the translating ribosome (Menetret *et al.*, 2007). The transmembrane segments of these proteins are then inserted into the membrane laterally through periodic opening of the SecYEG channel (Valent *et al.*, 1998; Duong & Wickner, 1998). If the proteins contain large hydrophilic domains, SecA may also be required for translocation (Neumann-Haefelin *et al*., 2000).

1.3.2.1.4 Insertion of membrane proteins by YidC

YidC is an essential protein in *E. coli* that has been shown to be required for Sec-dependent as well as Sec-independent integration of membrane proteins (Samuelson *et al.*, 2000; Urbanus *et al.*, 2001). Evidence for interaction of YidC with the Sec system has also been found, since it co-purifies with SecYEG (Scotti *et al.*, 2000). Depletion of YidC has been shown to have little effect on exported proteins (Samuelson *et al.*, 2000). YidC therefore is thought to interact with the SecYEG/DFYajC complex to facilitate the lateral release of hydrophobic helices into the membrane.

YidC has also been shown to insert membrane proteins independently of the Sec translocon, for example the F_o subunit of the F_1F_o-ATP synthase (Yi *et al.*, 2004) as well as some small phage derived proteins (Kuhn, 1988; Chen *et al.*, 2002; Stiegler *et al.*, 2011). However, how YidC recognises these substrate proteins remains elusive.

1.3.2.1.5 SecA2 and virulence in Gram-positive pathogens

A number of Gram-positive bacteria encode a second homologue of SecA, which as discussed above is one of the main components of the secretion apparatus that is involved in substrate recognition and subsequent translocation (Hartl *et al.*, 1990). In these organisms it has been shown that the two SecA proteins do not recognise the same substrates and the second protein is known as SecA2 (reviewed by Rigel & Braunstein, 2008). Unlike SecA, SecA2 is generally not essential for viability (Braunstein *et al.,* 2001; Bensing & Sullam, 2002; Lenz & Portnoy, 2002; Braunstein *et al.*, 2003; Chen *et al.*, 2004). Although not confined to pathogens, SecA2 has been shown to be important for virulence in the pathogenic bacteria in which it is found, for example *M. tuberculosis* (Braunstein *et al.*, 2003; Kurtz *et al.*, 2006) and *Listeria monocytogenes* (Lenz *et al.*, 2003; Muraille *et al.*, 2007), as it specifically recognises and promotes secretion of virulence factors.

Analysis of the available streptomycete genomes at the time of writing this thesis indicates that SecA2 proteins are not present in *Streptomyces*.

1.3.2.2 The twin-arginine translocation (Tat) pathway

The Tat pathway was first described in the thylakoid membrane of plant chloroplasts (Mould & Robinson, 1991) and later proposed as an alternative route of protein transport for exported bacterial proteins containing cofactors (Berks, 1996). It was the first protein transport system found to be solely dependent on the transmembrane proton gradient for energisation. In contrast to the Sec pathway, substrates are exported by the Tat pathway as folded proteins that are also targeted for transport by means of an N-terminal signal sequence. However in this case signal peptides that target to the Tat pathway contain a conserved twin arginine motif

distinguishing them from Sec signal peptides (Berks, 1996; Santini *et al.*, 1998). To date, this protein transport system has only been found in plants, archaea and bacteria. It has been most widely characterised in the Gram negative bacterium *E. coli* (reviewed by Palmer *et al*., 2005) but is also found in Gram positive organisms such as *Bacillus subtilis* (Tjalsma *et al*., 2000) and *Streptomyces coelicolor* (Bentley *et al*., 2002).

1.3.2.2.1 The Tat signal peptide

Proteins are targeted to the Tat machinery by means of an N-terminal signal sequence that resembles the tripartite structure of the Sec signal sequence but with important differences, as shown in Figure 1.9. For example, by contrast with Sec signal peptides, the boundary between the n- and h-regions of the Tat signal peptide contains a conserved amino acid motif, (S/T)-R-R-x-F-L-K, where x represents any polar amino acid (Berks *et al.*, 1996; Stanley *et al*., 2000). The motif contains an almost invariant pair of arginine residues, although in some cases a mutation of one of these arginine residues to a lysine residue may be tolerated (Stanley *et al*., 2000; Hinsley *et al*., 2001). The n-region of the Tat signal peptide is generally much longer than that of a Sec signal peptide. The h-region, although hydrophobic, is generally more moderately so than that of a Sec signal peptide and ranges from 12 to 20 amino acids, and the c-region in addition to containing the conserved signal peptidase recognition site often contains basic amino acid residues that are thought to serve as a 'Sec-avoidance' motif (Bogsch *et al.*, 1997; Cristobal *et al*., 1999). The overall length of Tat signal peptides is generally greater than Sec signal peptides, ranging from 24-58 amino acid residues (Cristóbal *et al.,* 1999; Stanley *et al*., 2000).

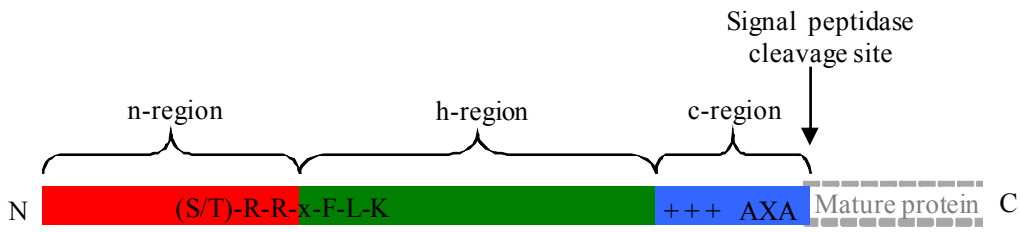

Figure 1.9: Schematic representation of the Tat signal peptide

Based on the differences between the Sec and Tat signal sequences two bioinformatics tools have been developed, TatP and TatFind, that have been shown to predict Tat substrate proteins based on their primary amino acid sequence (Rose *et al.*, 2002; Bendtsen *et al.*, 2005). These prediction programmes are often used in conjunction with fusion reporter assays such as agarase, described above, or maltose-binding protein to reliably confirm Tat-exported proteins (Widdick *et al*., 2006; 2008; Joshi *et al*., 2010; Tullman-Ercek *et al*., 2007).

1.3.2.2.2 Tat substrate proteins

Tat substrates carry out a variety of cellular functions so inactivation of the Tat system in *E. coli* is associated with a number of pleiotropic phenotypes such as aberrant cell division, sensitivity to bile salts and detergents, impaired biofilm formation and defects in anaerobic respiration (Ize *et al.*, 2003; Ize *et al.*, 2004; Stanley *et al.*, 2001).

In *E. coli*, most of the Tat substrate proteins, are respiratory enzymes (Sargent *et al.*, 2002) and contain cofactors that are oxygen sensitive and must be inserted in a reducing environment, so the Tat system was originally thought to be a specialised means of transporting these proteins (Berks *et al.*, 2003). However, one of the most striking effects of Tat pathway inactivation in *E. coli* is chaining of cells, that was later accounted for by mislocalisation of AmiA and AmiC, two cell wall amidases that are

Tat substrates (Bernhardt & de Boer, 2003; Ize *et al.*, 2003; Stanley *et al.*, 2001). These proteins are two examples of Tat substrate proteins that do not contain cofactors.

In addition, there are examples of proteins not in possession of an N-terminal signal sequence that are exported by the Tat pathway by virtue of the fact that they form a complex with a protein that does. In *E. coli* one such protein is the hydrogenase-2 complex formed by HybC that is exported by partnership with HybO, where only HybO has a signal peptide (Rodrigue *et al.*, 1999). Another example is *S. lividans* tyrosinase MelC2, which is exported in concert with its signal-peptide bearing chaperone MelC1 (Chen *et al.*, 1992; Schaerlaekens *et al.*, 2001).

In *E. coli* approximately 40 proteins are Tat substrates, constituting around 10 % of the exported proteome. However, in other organisms such as halophilic archaea the majority of secreted proteins are predicted to be exported by the Tat system (Rose *et al.*, 2002). An extremely high number (approximately 30 %) of the predicted secretome have also been identified and verified as Tat substrates in various streptomycetes (Schaerlaekens *et al.*, 2004; Widdick *et al.*, 2006; Joshi *et al.*, 2010). The role of the Tat system varies between organisms and is probably reflective of the physiological adaptions made by each to the conditions to which they are exposed in their natural environment. The role of the Tat system in *Streptomyces* is discussed in Section 1.3.2.2.5.

1.3.2.2.3 The Tat translocase

Early studies into the bacterial Tat system were conducted when it was noted that homologous genes to those encoding the protein translocation system from the thylakoid membrane of plants were also present in the genome of *E. coli* (Sargent *et al.*, 1998). Two loci, *tatABCD* and *tatE* were analysed and it was found that the TatA, TatB

and TatC proteins make up the major constituents of the Tat translocation machinery (Sargent *et al*., 1998; 1999; Bogsch *et al*., 1998). The *tatD* gene encodes a metal dependent nuclease that is not required for Tat-dependent transport (Wexler *et al.*, 2000). TatE is homologous to TatA but is produced in much lower levels and has only a minor role in Tat-dependent protein export (Sargent *et al.*, 1998; Sargent *et al.*, 2001; Jack *et al.*, 2001). Deletion of both *tatA* and *tatE* together abrogated Tat-dependent protein export (Sargent *et al.*, 1998).

In *E. coli*, TatA, TatB and TatC are each membrane spanning proteins. In archaea and the majority of Gram-positive bacteria only the TatA and TatC constituents are encoded (Dilks *et al.*, 2005; Jongbloed *et al.*, 2000; Jongbloed *et al.*, 2004). This can be explained by an early gene duplication event as *E. coli* TatA and TatB are homologous proteins (Yen *et al.*, 2002b). However, they are not functionally interchangeable so have clearly evolved separate functions (Sargent *et al.*, 1998; 1999; De Keersmaeker *et al.*, 2005). Interestingly, the streptomycetes (and related Actinobacteria such as *Mycobacterium tuberculosis*) encode TatA, B and C proteins but the genes are arranged separately in two operons, *tatAC* and *tatB* (Widdick *et al.*, 2006). However, the *E. coli* and *S. coelicolor* genes have been shown to be functionally interchangeable (Hicks *et al.*, 2006) and therefore the Tat machineries in these two organisms are expected to be fundamentally similar.

Biochemical studies on the components of the Tat system has resulted in the characterisation of two distinct complexes - a TatBC complex of about 450kD (Bolhuis *et al*., 2001; de Leeuw *et al*., 2002; McDevitt *et al.*, 2006) and a TatA complex of about 350-650 kDa (Porcelli *et al*., 2002; Sargent *et al*., 2001).

The TatBC complex contains each protein in a ratio of 1:1 (Bolhuis *et al.*, 2001), and each is present in multiple copies (Oates *et al.*, 2005) – probably 6-8 (Tarry *et al*., 2009; Maldonado *et al*., 2011b). The N-terminal 55 amino acid residues of TatB have

been shown to be important for formation of the TatBC complex (Maldonado *et al.*, 2011a). When taken together with results from cross-linking studies (Lee *et al.*, 2006; Punginelli *et al.*, 2007) it suggests that the TatBC complex is multimeric with TatC arranged on the outside and TatB on the inside of the complex.

The TatBC complex is required for recognition and binding of the Tat signal peptide, where TatC recognises the twin arginine signal motif on the substrate protein (Alami *et al*., 2003; Strauch & Georgiou, 2007). Several points throughout the TatC protein are required for substrate binding to take place indicating that a structural rather than a sequence motif is important for this interaction (Holzapfel *et al.*, 2007; Strauch & Georgiou, 2007). Binding of a folded substrate to TatB has also been shown to occur but this is dependent on the presence of TatC (Alami *et al.*, 2003; Maurer *et al*., 2010).

TatA forms a series of homooligomeric complexes of various sizes, ranging from 100 to over 700 kDa (Sargent *et al.*, 2001; deLeeuw *et al.*, 2002). Structural analysis of purified TatA complexes by electron microscopy shows that TatA forms channel-like structures with central cavities of differing sizes capable of accommodating even the largest known Tat substrate in *E. coli* (Gohlke *et al*., 2005). These observations suggest that TatA forms the channel in the membrane that can vary in size allowing the transport of substrate proteins of different sizes.

When TatA is overexpressed alongside TatB and TatC purification studies of the TatBC complex shows that a low level of TatA is also present (Bolhuis *et al.*, 2001; de Leeuw *et al.*, 2002; Porcelli *et al.*, 2002; Sargent *et al.*, 2001). However, it is thought that TatA only associates with the TatBC complex when it is bound to a substrate and the membrane is energised (Mori & Cline, 2002). Indeed, a recent study has shown, using a TatA-YFP fusion, that TatA forms foci in the membrane only when the TatBC complex is also present (Leake *et al.*, 2008).

The multimeric state and known interactions of these proteins supports the proposed model depicted in Figure 1.10 for the Tat translocation cycle. Following transport of the precursor protein, as with Sec translocation, the protein may either be inserted into the membrane or cleaved by a signal peptidase and released (Lüke *et al.*, 2009).

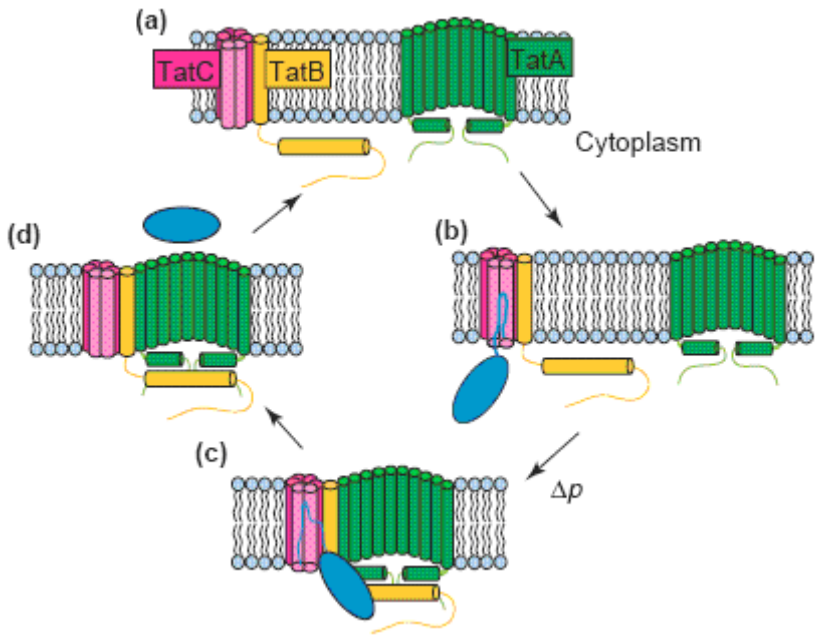

Figure 1.10: Model of the Tat translocation cycle. Under non-transporting conditions, two separate complexes of Tat components (TatA and TatBC) are found. (a) Binding of the twin arginine signal peptide of substrate protein to TatBC initiates the cycle (b). Signal peptide binding causes the TatBC-substrate complex to interact with TatA in a Δ*p*-dependent manner (c). The substrate protein is translocated across the membrane through a channel formed by multiple TatA protomers (d). The substrate protein is released at the periplasmic side of the membrane and the TatABC complex dissociates. Courtesy of Palmer *et al.* (2005).

1.3.2.2.4 Tat chaperones & proofreading

Perhaps the most striking feature of the Tat system is that the substrate proteins are translocated across the membrane in a folded state and that unfolded proteins are not able to be transported (Sanders *et al.*, 2001; DeLisa *et al.*, 2003). Proper folding and

insertion of cofactors into Tat substrate proteins is often mediated by cytoplasmic chaperones (Jack *et al*., 2004; Oresnik *et al.*, 2001) and has been referred to as a 'proofreading' system (reviewed by Palmer *et al*., 2005). Two examples of Tat-specific chaperones from *E. coli* are DmsD and TorD that bind to the Tat substrates DmsA and TorA, respectively (Jack *et al.*, 2004; Pommier *et al.*, 1998; Oresnik et al., 2001).

The first example of a proofreading chaperone is DmsD, which was first discovered when it co-purified with a reporter protein-DmsA signal peptide fusion (Oresnik *et al.*, 2001). DmsD was shown to be required for co-factor insertion into DmsA and given the fact that it binds to the DmsA signal peptide it has been proposed that it masks this from the Tat translocase until the protein is correctly folded (Oresnik *et al.*, 2001).

A second example of a proofreading chaperone is TorD, which interacts with the TorA signal peptide and also regions of the unfolded mature domain of TorA (Jack *et al.*, 2004; Pommier *et al.,* 1998). Increased expression of TorD leads to enhanced export of a TorA-GFP fusion protein (Li *et al.*, 2006). However, it should be noted that Tat chaperones belong to different families, for example NapD (the signal peptide binding chaperone for the nitrate reductase NapA; Maillard *et al*., 2007) and HyaE (the signal peptide binding chaperone for the hydrogenase HyaAC; Dubini & Sargent, 2003; Schubert *et al*., 2007) are unrelated to each other and to the TorD/DmsD family, and therefore they may be quite different in their mechanisms of action.

1.3.2.2.5 The Tat system in *Streptomyces*

As mentioned previously, in most bacteria the Tat pathway is utilised for the export of the minority of exported proteins. In *E. coli* many of these contain cofactors, fold quickly in the cytoplasm or form complexes prior to export, negating export by the Sec pathway. However it has recently been shown by Widdick *et al*. (2006) that the Tat

system functions as a major protein export pathway in *S. coelicolor* and that somewhere in the region of 30 % of all predicted secreted proteins are exported via the Tat pathway. Moreover, the vast majority of these proteins were predicted not to contain cofactors (Widdick *et al.*, 2006).

The physiological role of the Tat system has been examined in several streptomycetes, including *S. lividans, S. coelicolor* and *S. scabies* (Schaerlaekens *et al.*, 2001; Schaerlaekens *et al.*, 2004; De Keersmaeker *et al.*, 2007; Widdick *et al.*, 2006; Joshi *et al.*, 2010). In *S. lividans*, *S. coelicolor* and *S. scabies* deletion of *tatC* was shown to completely block secretion by the Tat pathway and in each case was shown to severely affect fitness and development (Schaerlaekens *et al.*, 2004; Widdick *et al.*, 2006; Joshi *et al.*, 2010; Mann, 2009). Inactivity of the system could be observed by performing a test for secreted xylanase activity in *S. lividans* (Schaerlaekens *et al.*, 2001), secreted agarase activity in *S. coelicolor* (Widdick *et al.,* 2006; Widdick *et al.*, 2008) or through visual assessment of melanin production in a culture containing tyrosine in *S. scabies* (Mann, 2009), as xylanase, agarase and the tyrosinase MelC1 are Tat substrates naturally produced by these organisms.

The study carried out by Widdick *et al.* (2006) used a combination of 2-dimensional electrophoresis and the agarase reporter assay (Widdick *et al.*, 2008) to identify and verify Tat substrate proteins in *S. coelicolor*. Confirmed Tat substrates were found to perform a broad range of functions including phosphate and carbohydrate metabolism, nutrient transport, peptidoglycan metabolism and some substrates were strongly predicted to be lipoproteins (Widdick *et al*., 2006). Subsequent studies focussed on lipoprotein biogenesis and demonstrated that around 25 % of the lipoproteome of *S. coelicolor* and *S. scabies* were probably Tat substrates (Thompson *et al.*, 2010; Widdick *et al.*, 2011).

Analysis of the *S. scabies* genome (www.sanger.ac.uk/Projects/S_scabies) also revealed that many putative Tat substrates were encoded in this organism, many of which have now been verified (Joshi *et al*., 2010; Mann, 2009). In addition to performing a wide range of physiological functions, several of these Tat substrates have been shown to function as virulence factors and many more are predicted to do so (Joshi *et al.*, 2010). Some of these Tat substrates have apparently been acquired through horizontal gene transfer as the closest homologues are found in unrelated species such as *Ralstonia solanacearum* and *Pectobacterium atrosepticum* (previously *Erwinia carotovora*), both Gram negative organisms that cause disease in potato, and many fungal plant pathogens. The role of the Tat system in the virulence of this and other organisms is mentioned briefly below.

1.3.2.2.6 Contribution of the Tat system to virulence

The Tat pathway has been shown to contribute to the virulence of a number of plant and animal pathogens. Given the different contributions made by the Tat system to the physiology of the different organisms in which it is found, it is likely that the reasons for the effects on virulence will also vary greatly between pathogenic species.

In *S. scabies* a *tatC* mutant strain was shown to be severely attenuated in virulence. However, the fitness of the organism was also seriously compromised. This led to an investigation of individual substrate proteins, showing that at least seven Tat substrates function as virulence factors. These virulence factors are thought to act in various ways including breakdown of various complex carbohydrates, amine oxidation and plant cell attachment (Joshi *et al.*, 2010).

The various functions of Tat substrate proteins are highlighted in the animal pathogen *Pseudomonas aeruginosa.* A Tat mutant strain was shown to be attenuated in virulence and substrate proteins included phospholipases, proteins involved in

pyoverdine-mediated iron-uptake, anaerobic respiration, osmotic stress defence, motility, and biofilm formation were identified (Ochsner *et al.*, 2002). Interference with any one of these processes could affect virulence. For example, biofilm formation has been shown to be important for the establishment of infection in numerous organisms, including *P .aeruginosa* (Høiby *et al.*, 2011) and the plant pathogen *Erwinia amylovora* (Koczan *et al.*, 2009).

The Tat pathway has also been shown to be important for virulence of plant pathogens such as *Agrobacterium tumefaciens* (Ding & Christie, 2003), *Pseudomonas syringae* (Caldelari *et al*., 2006), *Ralstonia solanacearum* (González *et al.*, 2007) and *Dickeya dadantii* (Rodríguez-Sanz *et al.*, 2010).

1.3.3 Protein translocation systems present in Gram-positive bacteria

Until recently it was thought that specialised protein export machineries had evolved in Gram-negative bacteria to achieve export of proteins across the double membrane and that the signal peptide-dependent Sec and Tat pathways were sufficient to achieve protein export in the Gram-positive bacteria. However, the recent discovery of a new protein export system in *M. tuberculosis* led to its identification in a wide range of Gram-positive organisms. This has been referred variably as the ESAT-6/CFP-10 pathway, WXG100 pathway, the Esx pathway and Snm pathway.

There is currently some debate in the literature about the naming of this protein secretion system in line with the Gram-negative secretion systems 1-6 given the differences in the cell envelope architecture, but for simplicity it will be referred to hereafter as the Type VII secretion system (T7SS).

1.3.3.1 The TypeVII secretion system (T7SS)

The T7SS was first discovered in *Mycobacterium tuberculosis* (Stanley *et al*., 2003). Many studies had previously shown that when the attenuated BCG strain of *M. bovis* (a member of the *M. tuberculosis* species complex used to immunise against tuberculosis) was grown in culture medium, many small proteins, including CFP-10 and ESAT-6, were missing that were found in medium of the wild type strain (Sonnenberg & Belisle, 1997). Despite being secreted proteins, they lacked the obvious signal peptides that are a prerequisite for secretion by the other known pathways in Gram positive bacteria. Recent genetic analysis of the BCG strain identified that the genes deleted in the loci termed 'RD1' were critical for virulence (Lewis *et al*., 2003; Pym *et al*., 2002). Multiple loci for homologues of the Esx secretion system were found within the genome and have since been found in many other Gram positive bacteria (Abdallah *et al*., 2007).

Very little is currently known about the operation of this system but it has been shown that all of the clusters contain homologues of six genes. The proteins produced by these are two members of the ESAT-6 family, an FtsK/SpoIIIE family protein, a subtilin-like protease, and two integral membrane proteins (reviewed by Abdallah *et al*., 2007). There may be other proteins present in the gene cluster that are thought to be individual protein substrates of the system or have a specific function in the transport process.

Proteins that are targeted to the Esx system display specificity for the secretion components that they cluster with in the genome. It is known that ESAT-6 and CFP-10 form a tight dimer through a large hydrophobic face on their surface, and dimerisation is required for stabilisation of these proteins (Renshaw *et al*., 2005). Other proteins thought to be secreted via this system also appear to form tight dimeric complexes (Strong *et al*., 2006). There is poor sequence similarity between Esx substrates but to

date it has been shown that they are small proteins of approximately 100 amino acids in length and contain a W–x-G amino acid sequence motif (Renshaw *et al*., 2005). The carboxy terminus of CFP-10 is unstructured and mutational studies of this region have shown it to be required for export of CFP-10 and ESAT-6, with ESAT-6 possibly being exported by virtue of binding to CFP-10 (Champion *et al*., 2006).

The arrangement and composition of the secretion machinery is unknown, but a channel is thought to be formed by a putative multi-transmembrane spanning protein, designated Rv3877 in *M. tuberculosis.* The association of two proteins, Rv3870 and Rv3871 in *M. tuberculosis*, are predicted to form an FtsK/SpoIIIE ATPase located in the cytoplasmic membrane. The channel and ATPase are thought to form the functional translocation machinery (Abdallah *et al*., 2007).

Homologues of this secretion machinery have been found in non-pathogenic Gram positive bacteria, but it has also been implicated in the virulence of various *Mycobacterium* spp (Stanley *et al*., 2003; Champion *et al*., 2006) as well as *Staphylococcus aureus* (Burts *et al*., 2005). The genome of *M. tuberculosis* encodes five orthologous system named Esx-1 to Esx-5. Both Esx-1 and Esx-5 have been implicated in virulence related to secretion of WXG100 proteins (Stanley *et al.*, 2003) and PE/PPE proteins (Daleke *et al.*, 2011) however the role of these proteins in pathogenesis is not yet understood. No function has been reported for either Esx-2 or Esx-4. However, Esx-3 has been shown to be important for iron homeostasis and acquisition (Serafini *et al.*, 2009; Siegrist *et al.*, 2009). Further investigation is required to understand the role of the T7SS secretion systems in the various organisms in which it is found. The T7SS is discussed in more detail in Chapter 3.

1.4 Aims of this thesis

The aims of this thesis were to examine whether the T7SSs that were apparently encoded in the genomes of *S. coelicolor* and *S. scabies* were functional, and what roles they contributed to the physiology of *Streptomyces* and to the virulence of *S. scabies*.

2. MATERIALS & METHODS

2.1 Strains & growth conditions

Bacterial strains used or constructed in this study are listed in Table 2.1. Growth media utilised in this study are listed in Table 2.2. *E. coli* strain DH5α was used for general transformation of plasmids and ligation reaction mixtures, as described in Section 2.1.2. *E. coli* strain BW25113 harbouring a *Streptomyces* cosmid clone was used for cosmid gene replacement by PCR-targeted homologous recombination of an antibiotic resistance-*oriT* cassette, as described in Section 2.3.1.2. *E. coli* strain ET12567 was used to carry out interspecies mating, as described in Section 2.3.1.3 for conjugal transfer of *oriT* containing plasmids and cosmids into *Streptomyces* strains. Antibiotics used to supplement growth media and the concentrations applied at are listed in Table 2.3.

Unless otherwise stated, *E. coli* strains were routinely grown in 5-10 ml volumes of Luria-Bertani (LB) medium and incubated at 37°C with shaking at 200 rpm (revolutions per minute). For solid cultures, *E. coli* strains were grown on LB medium containing 1.2 % agar and incubated at 37°C.

Streptomyces strains were routinely grown on solid media incubated at 30°C. *Streptomyces* cultures grown in liquid media were incubated at 30°C with shaking at 250 rpm. Stainless steel springs were inserted into 250 ml Erlenmeyer flasks to act as baffles to aerate cultures and promote dispersal of mycelia during growth in liquid

culture. Preparation of standard growth media was carried out by the Media Service at the College of Life Sciences, University of Dundee.

E. coli strains were stored for up to ten days on LB agar plates at 4ºC, or in LB medium diluted to a final concentration of 20 % glycerol (v/v) in Nalgene cryovials at -80 ºC. *Streptomyces* strains were stored for up to 30 days on agar plates at 4ºC or as spore suspensions in 20 % glycerol (v/v) in Nalgene cryovials at -20 ºC or -80 ºC.

Table 2.1: Bacterial strains used in this study

Strain	Genotype	Resistance	Source
E. coli DH5α	F$^-$, *endA1, glnV44, thi-1, recA1, relA1, gyrA96, deoR, nupG, Φ80dlacZΔM15,* Δ(*lacZYA-argF*)*U169, hsdR17*(r_K^- m_K^+), λ^-	N/A	Hanahan (1983)
E. coli BW25113	F$^-$, Δ(*araD-araB*)*567*, Δ*lacZ4787* (::*rrnB-3*), λ^-, *rpoS396*(*Am*), *rph-1*, Δ(*rhaD-rhaB*)*568*, *hsdR514*	N/A	Datsenko & Wanner (2000)
E. coli ET12567	*dam13*::Tn9, *dcm6*, *hsdM*, *hsdR*, *rec143F*, *zjj201*::Tn10, *galK2*, *galT22*, *ara14*, *lacY1*, *leuB1*, *thi1*, *tonA31*, *rpsL136*, *hisG4*, *tsx78*, *mtli*, *glnV44*, F$^-$	Cml^R/Tet^R	McNeil *et al*. (1992)
S. scabies 87.22	Wild-type strain	N/A	Prof Rosemary Loria
S. scabies Δ*tatC*	*S. scabies* 87.22, Δ*tatC*	Apra^R	Joshi *et al*. (2010)
JKFSCAB1	*S. scabies* 87.22, Δ*ssc58621*	Apra^R	This study
JKFSCAB2	*S. scabies* 87.22, Δ*ssc58651*	Apra^R	This study
JKFSCAB3	*S. scabies* 87.22, Δ*ssc58671*	Apra^R	This study
JKFSCAB4	*S. scabies* 87.22, Δ*ssc58681*	Apra^R	This study
S. coelicolor M145	Wild-type derivative of *S. coelicolor* A(3)2 lacking plasmids	N/A	Bentley *et al*. (2002)
JKFSCO1	*S. coelicolor* M145, Δ*sco4508*	Apra^R	This study
JKFSCO2	*S. coelicolor* M145, Δ*sco5734*	Apra^R	This study
JKFSCO3	*S. coelicolor* M145, Δ*sco4508*, Δ*sco5734*	Apra^R/Hyg^R	This study

Table 2.2: Composition of bacterial and plant growth media used in this study

Medium	Components:	
DNA	Difco nutrient agar	2.3 % (w/v)
DNB	Difco nutrient broth powder	0.8 % (w/v)
Instant potato mash medium (IPM)*	Instant mashed potato	5 % (w/v)
	Agar	1.2 % (w/v)
Luria-Bertani medium (LB) pH 7.0	Tryptone	1 % (w/v)
	Yeast extract	0.5 % (w/v)
	NaCl	1 % (w/v)
Minimal Medium (MM)	*L*-asparagine	0.05 % (w/v)
	K_2HPO_4	0.05 % (w/v)
	$MgSO_4.7H_2O$	0.02 % (w/v)
	$MgSO_4.7H_2O$	0.001 % (w/v)
	Glucose or mannitol (added after autoclaving)	1 % (w/v)
	Agar	1 % (w/v)
MM-C	$(NH_4)_2SO_4$	0.05 % (w/v)
	K_2HPO_4	0.05 % (w/v)
	$MgSO_4.7H_2O$	0.02 % (w/v)
	$MgSO_4.7H_2O$	0.001 % (w/v)
	Agar	1 % (w/v)
Minor Elements Solution	$FeSO_4.7H_2O$	0.0001 % (w/v)
	$ZnSO_4.7H_2O$	0.0001 % (w/v)
	$MnCl_2.4H_2O$	0.0001 % (w/v)
	$CaCl_2$	0.0001 % (w/v)
Murashige & Skoog (MS) pH 5.8	MS basal salts	0.44 % (w/v)
Oat Bran Broth (OBB) pH 7.2	Oat bran (boiled 20 minutes in water then filtered through cheesecloth)	2 % (w/v)
	Trace Element solution	0.2 % (v/v)
Oat Bran Medium (OBM)*	Oat bran	4 % (w/v)
	Agar	1.2 % (w/v)
Potato sub-culture media pH 5.8	MS basal salts	0.44 % (w/v)
	Sucrose	1 % (w/v)
	NaH_2PO_4	0.017 % (w/v)
	Phytagel	0.25 %(w/v)

R2	Sucrose	10.3 % (w/v)
	K_2SO_4	0.025 % (w/v)
	$MgCl_2.6H_2O$	1.012 % (w/v)
	Glucose	1 % (w/v)
	Difco casaminoacids	0.01 % (w/v)
	Agar	2.2 %
	The following components were added after autoclaving:	
	KH_2PO_4	0.005 % (w/v)
	$CaCl_2.2H_2O$	0.3 % (w/v)
	L-proline	0.3 % (w/v)
	TES buffer pH 7.2	0.573 % (w/v)
	Trace elements solution	0.002 % (v/v)
	NaOH	5 mM
R2YE	Prepared as R2 with the addition of:	
	Yeast extract	0.5 % (w/v)
Soft Nutrient Agar (SNA)	Difco nutrient broth powder	0.8 % (w/v)
	Agar	0.5 % (w/v)
Soya flour mannitol agar (SFM)*	Soya flour	2 % (w/v)
	Mannitol	2 % (w/v)
	Agar	2 % (w/v)
Thaxtomin defined media (TDM) pH 7.2	$(NH_4)_2SO_4$	0.2 % (w/v)
	L-Sorbose	1 % (w/v)
	$MgSO_4.7H_2O$	0.06 % (w/v)
	K_2HPO_4	0.26 % (w/v)
	Cellobiose	0.7 % (w/v)
	Minor Element Solution	0.1 % (v/v)
Trace Elements Solution	$ZnCl_2$	40 mg/L
	$FeCl_3.6H_2O$	200 mg/L
	$CuCl_2.2H_2O$	10 mg/L
	$MnCl_2.4H_2O$	10 mg/L
	$Na_2B_4O_7.10H_2O$	10 mg/L
	$(NH_4)_6Mo_7O_{24}.4H_2O$	10 mg/L
Tryptone soya broth (TSB)	Oxoid Tryptone Soya Broth powder (CM129)	3 % (w/v)
Yeast Malt Extract Agar (YME)*	Glucose	0.4 % (w/v)
	Yeast extract	0.4 % (w/v)
	Malt extract	1 % (w/v)
	Agar	1.5 % (w/v)
2xYT	Tryptone	1.6 % (w/v)
	Yeast extract	1 % (w/v)
	NaCl	0.5 % (w/v)

Media were autoclaved at 121°C for 20 minutes prior to use. Element solutions were filter sterilised prior to their addition to media. All media were prepared using $_{dd}H_2O$ unless marked with an asterisk, denoting that they were prepared using tap water.

Table 2.3: Antibiotics used in this study and their working concentrations

Antibiotic	**Stock Solultion (mg/ml)**	**Final Concentration (µg/ml)**
Ampicillin (Amp)	125	125
Apramycin (Apra)	50	50 (for *E. coli* and for the *S. scabies* Δ*tatC* strain) 100 (for all other *Streptomyces* strains)
Chloramphenicol (Cml)	25 (in 80 % ethanol)	25
Hygromycin B (Hyg)	50	50 (for *E. coli*) 100 (for *Streptomyces* strains)
Kanamycin (Kan)	50	50
Nalidixic Acid	25 (in 0.2 M NaOH)	25
Unless otherwise stated all antibiotics were prepared in ultra pure H_2O, sterilized by filtration through a 0.25 µm filter and stored at -20°C		

2.1.1 Preparation of *Streptomyces* spore stocks

S. coelicolor spores were spread onto SFM (soya flour mannitol) and *S. scabies* spores were spread onto IPM (instant potato mash) media supplemented with 20 mM $CaCl_2$. The plates were incubated at 30 ºC for up to ten days to allow formation of a confluent lawn of spores on the surface of the plate. Spores were then harvested by adding 25 ml of sterile water to the surface and scraping with a thick, sterile inoculation loop. The crude spore suspension was then transferred to a 50 ml sterile tube and vortexed for one minute to break up spore chains. These were then filtered through cotton wool in a 20 ml syringe to remove mycelial and agar fragments. The resulting spore suspension was centrifuged at 2,773 x*g* for 10 minutes and the supernatant discarded. Spore pellets were re-suspended in 20 % (v/v) glycerol, transferred to a 2 ml Nalgene cryovial and stored at -20 ºC or -80 ºC.

2.1.2 Preparation of chemically competent *E. coli* cells

Chemically competent *E. coli* cells, which were to be transformed with plasmid DNA or ligation reactions, were prepared by inoculating 50 ml LB medium with 500 µl of a stationary phase culture. This was incubated until an OD_{600} of 0.4-0.5 was reached, after which time cells were pelleted by centrifugation of the culture at 2,773 x*g* for 5 minutes. Cells were re-suspended in 5 ml of ice cold 100 mM $CaCl_2$ and incubated on ice for 10 minutes. Subsequent steps were carried out on ice or at 4°C. A cell pellet was obtained by centrifugation at 2,773 x*g* for 5 minutes which was re-suspended in 1 ml of fresh 100 mM $CaCl_2$. Cells were stored on ice overnight after which ice cold glycerol was added to a final concentration of 20 %. 100-200 µl aliquots were flash frozen using liquid nitrogen and subsequently stored at -80°C. Prior to use, chemically competent cells were thawed on ice.

DNA or ligation reaction mixtures (1-10 µl) were incubated on ice with 50 µl of competent cells for 30 minutes. Cells were then heat shocked either by incubation at 37°C for 5 minutes (for temperature sensitive strains) or 42°C for 90 seconds and then placed on ice for 5 minutes. Cells were then grown with 1 ml LB medium for 1 hour either at 30°C or 37°C. Cells were subsequently pelleted by centrifugation at 16,100 x*g* for 1 minute, the supernatant discarded and the cell pellet re-suspended in the remaining 50-100 µl of liquid. This was spread onto LB plates containing the appropriate antibiotics and incubated at 30°C or 37°C overnight.

2.1.3 Preparation of electro-competent *E. coli* cells

Electro-competent *E. coli* cells, for transformation with linear or cosmid DNA, were prepared by inoculating 10 ml LB medium with 100 µl of a stationary phase culture and grown until an OD_{600} of 0.5-0.6 was reached. A cell pellet was obtained by centrifugation at 2,773 x*g* for 5 minutes. Subsequent steps were carried out on ice or at

4°C. Cells were washed twice in 10 ml of 10 % glycerol then with 5 ml of 10 % glycerol (v/v), with cells pelleted by centrifugation at 2,773 x*g* for 5 minutes in between. The final cell pellet was re-suspended in 100-200 µl of 10 % glycerol and the cell suspension was used immediately.

Cosmid or linear DNA (1-5 µl) was mixed with 50 µl of electro-competent cells and transferred to a 2 mm chilled electroporation cuvette. Electroporation was carried out with a BioRad GenePulser II set to 200 Ω resistance, 25 µF capacity and 2.5 kV. Following electroporation, 1 ml of ice cold LB medium was added to the cells and then transferred to a 1.5 ml Eppendorf tube for incubation at 30°C or 37°C for 1 hour. A cell pellet was obtained by centrifugation at 16,100 x*g* for 1 minute which was re-suspended in the 50-100 µl of remaining liquid and spread onto LB plates containing the appropriate antibiotics and incubated at 30°C or 37°C overnight.

2.1.4 Analysis of *Streptomyces* growth

For growth analysis of *Streptomyces* strains, 100 ml of TSB medium in a spring baffled Erlenmeyer flask was inoculated from a pre-enumerated frozen spore stock to a concentration of $1x10^6$ spores per ml of TSB. Flasks were incubated at 30°C at 250 rpm and three separate 1 ml samples were withdrawn every 2-3 hours over a period of up to 45 hours. Each sample was centrifuged at 16,100 x*g* for 2 minutes, the supernatant discarded and protein extracts prepared from the mycelial pellet.

2.2 DNA techniques

Solutions and buffers used in this thesis can be found in Table 2.4.

Table 2.4: Composition of buffers and solutions used in this study

Solution/Buffer	Components:	
Blocking buffer (western blot)	Tris/HCl (pH 7.6)	20 mM
	NaCl	137 mM
	Tween 20	0.1 % (v/v)
	Skimmed milk powder	5 % (w/v)
Denaturation solution	NaOH	500 mM
	NaCl	1M
Detection buffer	Tris/HCl (pH 9.5)	0.1 M
	NaCl	0.1 M
Equilibriation buffer 1	SDS	2 % (w/v)
	Tris/HCl (pH 8.8)	50 mM
	Urea	6 M
	Glycerol	30 % (v/v)
	Bromophenol Blue	0.002 % (w/v)
	DTT	1 % (w/v)
Equilibriation buffer 2	SDS	2 % (w/v)
	Tris/HCl (pH 8.8)	50 mM
	Urea	6 M
	Glycerol	30 % (v/v)
	Bromophenol Blue	0.002 % (w/v)
	Iodoacetamide	2.5 % (w/v)
Fixation solution	Ethanol	40 % (v/v)
	Acetic acid	10 % (v/v)
Glucose-Tris-EDTA (GTE) solution	Glucose	50 mM
	Tris	25 mM
	EDTA	10 mM
Neutralisation solution	NaCl	3 M
	Tris/HCl (pH 7.5)	500 mM
Phosphate buffered saline (PBS) pH 7.0	Na_2HPO_4	57.5 mM
	NaH_2PO_4	42.3 mM
	NaCl	154 mM
SDS PAGE running buffer	Tris	25 mM
	Glycine	192 mM
	SDS	0.1 % (w/v)
Staining solution	Ammonium sulphate	10 % (w/v)
	Phosphoric acid	2.08 % (v/v)
	Methanol	20 % (v/v)
	Coomassie Blue G	0.1 % (w/v)
Stop solution	Ethanol	95 % (v/v)
	Acid phenol	5 % (v/v)
TAE Buffer	Tris	40 mM
	Acetic acid	1.142 % (v/v)
	EDTA	1 mM
TBS/Tween	Tris/HCl (pH 7.6)	20 mM
	NaCl	137 mM
	Tween 20	0.1 % (v/v)

TE	Tris (pH 8.0) EDTA	30 mM 1 mM
Transfer buffer	Tris Glycine Methanol	25 mM 192 mM 10 % (v/v)
Wash solution I	NaCl Sodium citrate (pH 7.0) SDS	195 mM 30 mM 0.1 % (w/v)
Wash solution II	NaCl Sodium citrate (pH 7.0) SDS	19.5 mM 3 mM 0.1 % (w/v)
Washing buffer	Maleic acid NaCl (pH 7.5) Tween 20	0.1M 0.15 M 0.3 % (v/v)
10xLoading Dye	Orange G Glycerol	0.5 % (w/v) 50 % (v/v)
1x Blocking buffer (Southern blot)	Blocking reagent Maleic acid (pH 7.5) NaCl	1 % (v/v) 0.1 M 0.15 M
20xSSC	NaCl Sodium citrate (pH 7.0)	1.95 M 300 mM
2D Sample buffer	Urea CHAPS DTT	8 M 4 % (w/v) 60 mM
2x Laemmli buffer	Tris/HCl pH 6.8 SDS β-mercaptoethanol glycerol bromophenol blue	62.5 mM 2 % (w/v) 15 % (v/v) 25 % (v/v) 0.01 % (w/v)

2.2.1 Plasmid DNA preparation

Preparation of plasmid DNA from *E. coli* cells was carried out using a QIAprep spin miniprep kit (Qiagen) according to the manufacturer's instruction. This procedure is based on the alkaline lysis method of Birnboim and Doly (1979). Cells from a 5 ml or 10 ml stationary phase culture harbouring the plasmid of interest were harvested by centrifugation at 2,773 x*g* for 10 minutes and re-suspended in 250 µl of re-suspension buffer. Cells were then lysed by the addition of 250 µl of an alkaline lysis buffer and neutralisation was carried out by the addition of 350 µl of neutralisation buffer. Cell debris and denatured protein was removed by centrifugation at 16,100 x*g* for 10 minutes

and the supernatant applied to a column. The plasmid DNA remained bound to the silica membrane contained within the column following centrifugation at 16,100 x*g* for 1 minute. The DNA was washed whilst bound to the column using a buffer containing ethanol, then eluted from the membrane using 30-50 µl of the manufacturer's elution buffer or ultra pure water and stored at -20°C. The plasmids used in this study are described in Table 2.5.

Table 2.5: Plasmids and cosmids used or constructed in this study

Plasmid/Cosmid	Vector	Description	Antibiotic resistance	Reference
pBluescript (KS^+)	-	Cloning vector	$Amp^R/Carb^R$	Stratagene
pDWU5	pBluescript (KS^+)	Kanamycin resistant derivative of pBluescript (KS^+)	$Amp^R/Carb^R/Kan^R$	D. Widdick
pDrive+Apra	pDrive	pDrive cloning vector with FTR- *oriT*-*aac*(3)*IV*- FRT cassette cloned into the multiple cloning site	$Amp^R/Carb^R/Kan^R/Apra^R$	D. Widdick
pSET152	-	Cloning vector for conjugal transfer and chromosomal integration at the φC31 attachment site in *Streptomyces*	$Apra^R$	Bierman *et al*. (1992)
pSET152-Hyg	pSET152	Derivative of pSET152 where the apramycin resistance marker has been replaced with hygromycin resistance marker by homologous recombination	Hyg^R	Bierman *et al*. (1992)
pIJ773	pBluescript (KS^+)	Template containing the apramycin resistance gene and *oriT* from plasmid RP4, flanked by FRT sites.	$Amp^R/Apra^R$	Gust *et al.* (2003)
pIJ790	pKD20	Modified λRED recombination plasmid pKD20	Cml^R	Gust *et al.* (2003)
pIJ10700	pBluescript (KS^+)	Template containing the hygromycin resistance gene and *oriT* from plasmid RP4, flanked by FRT sites.	Amp^R/Hyg^R	Gust *et al.* (2004)
pIJ8660	pSET152	Vector containing the promoterless *EGFP* gene	$Apra^R$	Sun *et al*. (1999)
pIJ8641	pIJ8660	Vector containing the *EGFP* gene downstream of the strong constitutive promoter for *ermE* from *S. erythraea*	$Apra^R$	Coombs & Franco (2003)
pRFSRL16	pIJ8641	Derivative of pIJ8641 with the kanamycin resistance gene inserted	$Apra^R/Kan^R$	R.F. Seipke
pUZ8002	-	Non-transmissible *oriT*-mobilizing plasmid.	Kan^R	Paget *et al.* (1999)
SuperCos I	-	Cosmid vector	$Kan^R/Amp^R/Carb^R$	Stratagene

p5734KO	pBluescript (KS^+)	Kanamycin resistant derivative of pBluescript (KS^+) containing a construct to allow introduction into *S. coelicolor* via conjugation and homologous recombination replacing *sco5734* with an antibiotic resistance marker flanked with FRT sites	Kan^R/$Apra^R$	This study
Cosmid StD35	SuperCos I	Part of the *S. coelicolor* ordered cosmid library containing the chromosomal region 4914231-4950624, encoding genes *sco4495* to *sco4529*	Kan^R/Amp^R/$Carb^R$	Redenbach *et al*. (1996)
Cosmid 212	SuperCos I	Part of the *S. scabies* ordered cosmid library containing the chromosomal region 6503242 to 6543663, encoding genes *ssc58421* to *ssc58800*	Kan^R/Amp^R/$Carb^R$	Loria, unpublished
212_KO_ssc58621	Cosmid 212	Cosmid 212 derivative where *ssc58621* has been replaced with FTR- *oriT-aac*(3)*IV*- FRT cassette by homologous recombination	Kan^R/Amp^R/$Carb^R$/$Apra^R$	This study
212_KO_ssc58651	Cosmid 212	Cosmid 212 derivative where *ssc58651* has been replaced with FTR- *oriT-aac*(3)*IV*- FRT cassette by homologous recombination	Kan^R/Amp^R/$Carb^R$/$Apra^R$	This study
212_KO_ssc58671	Cosmid 212	Cosmid 212 derivative where *ssc58671* has been replaced with FTR- *oriT-aac*(3)*IV*- FRT cassette by homologous recombination	Kan^R/Amp^R/$Carb^R$/$Apra^R$	This study
212_KO_ssc58681	Cosmid 212	Cosmid 212 derivative where *ssc58681* has been replaced with FTR- *oriT-aac*(3)*IV*- FRT cassette by homologous recombination	Kan^R/Amp^R/$Carb^R$/$Apra^R$	This study
D35_KO_sco4508-Apra	Cosmid StD35	Cosmid D35 derivative where *sco4508* has been replaced with FTR- *oriT-aac*(3)*IV*- FRT cassette by homologous recombination	Kan^R/Amp^R/$Carb^R$/$Apra^R$	This study

D35_KO_sco4508-Hyg	Cosmid StD35	Cosmid 212 derivative where *sco4508* has been replaced with , FTR-*oriT-hyg*-FRT by homologous recombination	$Kan^R/Amp^R/Carb^R/Hyg^R$	This study
pScab_WxG_operon	pSET152-Hyg	*S. scabies* chromosomal region spanning genes *ssc58671-ssc58681* and surrounding region cloned into the MCS for complementation of mutant strains	Hyg^R	This study
pSsc58671_6H	pRFSRL16	*S. scabies* gene *ssc58671* containing a C-terminal his-tag coding sequence for expression from the strong constitutive *ermE* promoter from *S. erythraea*	$Apra^R/Kan^R$	This study
pSsc58681_6H	pRFSRL16	*S. scabies* gene *ssc58681* containing a C-terminal his-tag coding sequence for expression from the strong constitutive *ermE* promoter from *S. erythraea*	$Apra^R/Kan^R$	This study
pDagA_EGFP_1	pSET152-Hyg	Transcriptional fusion plasmid coding for agarase and GFP expressed from the constitutive *hrdB* promoter of *S. coelicolor*	Hyg^R	This study
pDagA_EGFP_2	pSET152-Hyg	Translational fusion plasmid coding for agarase and GFP expressed from the constitutive *hrdB* promoter of *S. coelicolor*	Hyg^R	This study
pDagAss_EGFP	pRFSRL16	Plasmid encoding for a fusion of the agarase signal peptide to EGFP expressed from the strong constitutive *ermE* promoter of *S. erythraea*	$Apra^R/Kan^R$	This study

2.2.2 Cosmid CCC (covalently closed circular) DNA preparation

Preparation of cosmid DNA from *E. coli* cells was carried out according to the protocol described in REDIRECT© technology by Gust *et al.* (2002). Cells from a 1 ml stationary phase culture harbouring the cosmid of interest were harvested by centrifugation at 2,773 x*g* for 10 minutes and re-suspended in 100 µl of Solution I. Cells were then lysed by the addition of 200 µl of Solution II and neutralisation was carried out by the addition of 150 µl of Solution III. Cell debris and denatured protein was removed by centrifugation at 16,100 x*g* for 5 minutes and the supernatant transferred to a fresh Eppendorf tube. Extraction of the supernatant was carried out by the addition of 400 µl of phenol/chloroform followed by centrifugation at 16,100 x*g* for 5 minutes. The upper phase was transferred to a fresh tube, mixed with 600 µl of isopropanol and incubated on ice for 10 minutes. A DNA pellet was obtained by centrifugation at 16,100 x*g* for 5 minutes which was subsequently washed with 70 % ethanol by centrifugation at 16,100 x*g* for 5 minutes. The supernatant was discarded and the DNA air dried at room temperature prior to re-suspension in 50 µl of 10 mM Tris/HCl (pH 8.0) or ultra pure water and stored at -20°C. The cosmids used in this study are described in Table 2.5.

2.2.3 Chromosomal DNA preparation from *Streptomyces*

Mycelia from a 10 ml culture of *Streptomyces* that had been incubated in TSB medium for 24-48 hours were harvested by centrifugation at 2,773 x*g* for 10 minutes and re-suspended in 500 µl of glucose-Tris-EDTA (GTE) solution. Lysis was carried out by the addition of 10 µl of each of a 30 mg/ml lysozyme solution and a 5 mg/ml RNase solution and incubated at 37°C for 1 hour. Following this, 10 µl of a 10 % SDS solution was added, the sample mixed by inversion and then extracted by the addition of 250 µl of phenol/chloroform followed by centrifugation at 16,100 x*g* for 10 minutes.

The upper phase was transferred to a fresh tube and extracted with a further 250 µl of phenol/chloroform followed by centrifugation at 16,100 x*g* for 10 minutes. The upper phase was again transferred to a fresh tube, mixed with 1 ml of ethanol and incubated at room temperature for 10 minutes. A DNA pellet was obtained by centrifugation at 16,100 x*g* for 10 minutes which was subsequently washed with 80 % ethanol by centrifugation at 16,100 x*g* for 5 minutes. The supernatant was discarded and the DNA air dried at room temperature prior to re-suspension in 100 µl of 10 mM Tris/HCl (pH 8.0) or ultra pure water and stored at -20°C.

2.2.4 DNA quantification

A NanoDrop ND-1000 system (Thermo Scientific) was used for quantification of nucleic acids in DNA preparations. Samples were applied to the sample pedestal and the absorbance peak read at 260 nm. The software calculated and provided the DNA concentration in the sample.

2.2.5 DNA amplification by Polymerase Chain Reaction (PCR)

DNA fragments were amplified by PCR using *Taq* DNA polymerase (Roche) for general PCR reactions whereas Herculase II (Agilent Technologies Inc.) was utilised for reactions requiring high-fidelity. Oligonucleotide primer pairs included in the reaction mix were complementary to the base sequence surrounding the region of DNA to be amplified. These were synthesised by Sigma-Genosys and are listed in Table 2.6. Template for inclusion in the reaction mix was either 0.5 µl of plasmid DNA, cosmid DNA, or chromosomal DNA (pre-diluted to an appropriate concentration) or 0.5 µl of cell suspension from an overnight culture of *E. coli* or *Streptomyces*. dNTPs were also included in the reaction mixture at a final concentration of 200 µM. A typical reaction mixture for a single PCR reaction can be found in Table 2.7. Reactions were carried out

in a thermocycler (Eppendorf Mastercycler personal) using a standard programme outlined in Table 2.8. The annealing temperature chosen varied between 54°C and 63°C depending on the melting temperature (T_m) of the specific primers used. The time allowed for elongation was 1 minute per kilobase of DNA to be amplified.

Table 2.6: Oligonucleotide primers used in this study

Primer	Sequence 5'→ 3'	Restriction sites	Comments
Sco4508 KO Fwd	GAAGCGGCCGCAGGGGCTCGCCTGGGGAG GACAAGCGTGATTCCGGGGATCCGTCGACC	-	Redirect primer pair to knockout *sco4508*
Sco4508 KO Rev	GCGCAGCGGGGCCGGGGCCCCGCCCCTTCC ACCGGCTCATGTAGGCTGGAGCTGCTTC	-	
Ssc58621 KO Fwd	GAGCGACCACCGGTACGAAGCAGAAGGGG TCCGTCCGTGATTCCGGGGATCCGTCGACC	-	Redirect primer pair to knockout *ssc58621*
Ssc58621 KO Rev	CGGGCATCTTCGGGGTGAGCGCGATGACGT CCCTGGTCATGTAGGCTGGAGCTGCTTC	-	
Ssc58651 KO Fwd	GGTGATAGGTTCGATCGCGTGATGTCTACG GGGACGTTGATTCCGGGGATCCGTCGACC	-	Redirect primer pair to knockout *ssc58651*
Ssc58651 KO Rev	CAGTCCCCTGCCTGCGCCATGTCCGGCCCCC AGCCCTTATGTAGGCTGGAGCTGCTTC	-	
Ssc58671 KO Fwd	TAACCAGCGACGAGCAGGAACGAGGGGGA AGTACTCATGATTCCGGGGATCCGTCGACC	-	Redirect primer pair to knockout *ssc58671*
Ssc58671 KO Rev	TCACTCGATTCACCGGCCGCCGGCCGGCCG CCCGGTCATGTAGGCTGGAGCTGCTTC	-	
Ssc58681 KO Fwd	GACGTAGGTGAACCGACACACGGGGGTGG ACACGACGTGATTCCGGGGATCCGTCGACC	-	Redirect primer pair to knockout *ssc58681*
Ssc58681 KO Rev	ATGAGTACTTCCCCCTCGTTCCTGCTCGTCG CTGGTTATGTAGGCTGGAGCTGCTTC	-	
Apra500check_up	ATGACATCAGTCGATCATAG	-	Primes 500 bp inside of apramycin resistance cassette, to check knockouts in conjunction with "gene#_up" primer
Apra500check_down	ATCCATTGCCCCTGCCACCT	-	Primes 500 bp inside of apramycin resistance cassette, to check knockouts in conjunction with "gene#_down" primer

Hyg1000check_up	GCCGGCCGAGCACCCGGCCG	-	Primes 1000 bp inside of hygromycin resistance cassette, to check knockouts in conjunction with "gene#_up" primer
Hyg500check_down	GCGGCGACCGTCGAGGACCA	-	Primes 500 bp inside of hygromycin resistance cassette, to check knockouts in conjunction with "gene#_down" primer
Sco4508_520up	GGTAGTCGCCCATGCCGTCG	-	Primes 520 bp upstream of *sco4508,* to check replacement of gene on cosmid by PCR
Sco4508_520down	GACGGTGGGGTCGGTGTTCG	-	Primes 520 bp downstream of *sco5734,* to check replacement of gene on cosmid by PCR
Sco5734_520up	GCGCAGCAGGTCGTCGCTCC	-	Primes 520 bp upstream of *sco5734,* to check replacement of gene on cosmid by PCR
Sco5734_520down	CCGCCGCGTCGGTCGGCATA	-	Primes 520 bp downstream of *sco5734,* to check replacement of gene on cosmid by PCR
Ssc58621_520up	GGCGGCTGGCTGCTGCCCGG	-	Primes 520 bp upstream of *ssc58621,* to check replacement of gene on cosmid by PCR
Ssc58621_520down	ATCTGCAGGGACCTCCGGGA	-	Primes 520 bp downstream of ssc58621, to check replacement of gene on cosmid by PCR
Ssc58651_500up	GCGAGATCATCCGCCTGGTA	-	Primes 500 bp upstream of *ssc58651,* to check replacement of gene on cosmid by PCR
Ssc58651_500down	GTACAGGCGGCTGCGCGGTG	-	Primes 500 bp downstream of *ssc58651,* to check replacement of gene on cosmid by PCR
Ssc58671_520up	GGGGCGGCAGCACGGCCATT	-	Primes 520 bp upstream of *ssc58671,* to check replacement of gene on cosmid by PCR
Ssc58671_520down	TCTCCGGCAGCAGCAGCCCG	-	Primes 520 bp downstream of *ssc58671,* to check replacement of gene on cosmid by PCR
Ssc58681_520up	CCGCTTCCCCGACCTGGAAC	-	Primes 520 bp upstream of *ssc58681,* to check replacement of gene on cosmid by PCR

Ssc58681_520down	TCGTCCACCGAGCGCCGCTG	-	Primes 520 bp downstream of *ssc58681,* to check replacement of gene on cosmid by PCR
Sco4508inside_fwd	ACATGACCTTCGTCCTCGTC	-	Primer pair to check for presence of *sco4508* gene Gives product of 496 bp if present
Sco4508 inside_rev	GGGGTGTTCTTGGAGATGTG	-	
Sco5734 inside_fwd	GGCTCTCAAGGACCAGATCA	-	Primer pair to check for presence of *sco5734* gene Gives product of 502 bp if present
Sco5734 inside_rev	GTAGTCACCGACGACGATCC	-	
Ssc58621 inside_fwd	CTCCTCCCGCTCGTTCAGCAT	-	Primer pair to check for presence of *ssc58621* gene Gives product of 477 bp if present
Ssc58621 inside_rev	TCGGTGATGGTGGACCAACTCG	-	
Ssc58651 inside_fwd	GAGCAGCACCACGACCTC	-	Primer pair to check for presence of *ssc586521* gene Gives product of 1033 bp if present
Ssc58651 inside_rev	GCCCTCGCGTGAAGAAGT	-	
Ssc58671 inside_fwd	TTCTGTTCGAGCGTGGTGCGT	-	Primer pair to check for presence of *ssc58671* gene Gives product of 244 bp if present
Ssc58671 inside_rev	AGAACCAGGACCGCCGTTCGTA	-	
Ssc58681 inside_fwd	TCGAAGTTGGCCAGGCCCAT	-	Primer pair to check for presence of *ssc58681* gene Gives product of 271 bp if present
Ssc58681 inside_rev	TCGGCAAGAGCACGCTCGGAT	-	
Sco5724H6_fwd	GCGCGGATCCCATCATCATCATCATCATGTGACAGCC ACCAGTGCTTCAGAGGAAGG	-	Primer pair amplifies *sco5724-sco5725* region Gives product of 760 bp
Sco5725H6_rev	GCGCGATATCTCAGCCCATCAGCATGCGGA	-	
ssc58621 South Fwd	GATGCAGCTCACCGATGTC	-	For production of 561 bp Southen blot probe to confirm deletion of *ssc58621* from the chromosome of *S. scabies* 87.22. Anneals to restriction fragment of 2036 bp if wild-type *ssc58621* present or 741 bp if mutant
ssc58621 South Rev	GACCCCTTCTGCTTCGTACC	-	

ssc58651 South Fwd	CGGTGAATCGAGTGACAGAG	-	For production of 372 bp Southen blot probe to confirm deletion of *ssc58651* from the chromosome of *S. scabies* 87.22. Anneals to restriction fragment of 2736 bp if wild-type *ssc58651* present or 430 bp if mutant
ssc58651 South Rev	GTAGACATCACGCGATCGAA	-	
ssc58671 South Fwd	ACCTCGGAACCCTGATCC	-	For production of 299 bp Southen blot probe to confirm deletion of *ssc58671* from the chromosome of *S. scabies* 87.22. Anneals to restriction fragment of 643 bp if wild-type *ssc58671* present or 362 bp if mutant
ssc58671 South Rev	GAGTACTTCCCCCTCGTTCC	-	
ssc58681 South Fwd	GTCGAGGCGCTCTGAAAC	-	For production of 670 bp Southen blot probe to confirm deletion of *ssc58681* from the chromosome of *S. scabies* 87.22. Anneals to restriction fragment of 1076 bp if wild-type *ssc58681* present or 1894 bp if mutant
ssc58681 South Rev	ACGTCTCGAACATGCTCTCC	-	
ssc58671_RTPCR_fwd	GACTACCACGGCAAGGAAGA	-	To detect co-transcription of *ssc58671* and *ssc58661* during RT-PCR (Product = 486 bp)
ssc58661_RTPCR_rev	AGACCGCTGTGGAGACAGA	-	
ssc58661_RTPCR_fwd	CAGTTGCTGGAGTCGGTGT	-	To detect co-transcription of *ssc58661* and *ssc58651* during RT-PCR (Product = 532 bp)
ssc58651_RTPCR_rev	CTCCAGCGTGCTGTCGTG	-	
ssc58651_RTPCR_fwd	GAGGTCGTGGTGCTGCTC	-	To detect co-transcription of *ssc58651* and *ssc58631* during RT-PCR (Product = 801 bp)
ssc58631_RTPCR_rev	ATCGTTTGCGCCTGCTGTA	-	
ssc58631_RTPCR_fwd	CGTGGTGGACTGCGAGAC	-	To detect co-transcription of *ssc58631* and *ssc58621* during RT-PCR (Product = 739 bp)
ssc58621_RTPCR_rev	CGCAGCTCCTCCAGATACTC	-	
ssc58621_RTPCR_fwd	CGACGACTACGACATCCTCA	-	To detect co-transcription of *ssc58621* and *ssc58611* during RT-PCR (Product = 750 bp)
ssc58611_RTPCR_rev	CTCGGTCACCACGTCCAC	-	
DRB21	GTCTGGCAGTTCCAGGAGTC	-	To detect transcription of the housekeeping gene *murX*, a positive control for RT-PCR
DRB22	AGGTGTTCCACCACAGGAAG	-	

Scab_WxGop_fwd	GCGCGAATTCGCAACTGGCTGTCGTAATCC	*EcoR*I	For amplification of the *S. scabies* genes *ssc58671-ssc58681* and surrounding region for cloning into pSET152-Hyg
Scab_WxGop_rev	GCGCGATATCGATGCCACTTACGACACTCG	*EcoR*V	
ssc58671_fwd	GCGCCATATGGCTCAGAACCAGGACCGCCG TT	*Nde*I	For amplification of *ssc58671* with a C-terminal his-tag coding sequence for cloning into pRFSRL16 replacing the *egfp* gene downstream of the *ermE* promoter
ssc58671H6_rev	GCGCGCGGCCGCTCAGTGGTGGTGGTGGTG GTGGCCGATCTGGTCGACCGCCGCGCGGGC CTT	*Not*I	
ssc58681_fwd	GCGCCATATGAAGTTCGACATGGGGTCGA	*Nde*I	For amplification of *ssc58681* with a C-terminal his-tag coding sequence for cloning into pRFSRL16 replacing the *egfp* gene downstream of the *ermE* promoter
ssc58681H6_rev	GCGCGCGGCCGCTCAGTGGTGGTGGTGGTG GTGTCGGGCGCCGAAACGGGCCGCGT	*Not*I	
hrdB_prom_fwd	GCGCTCTAGAACCGCCTTCCGCCGGAACGG	*Xba*I	For amplification of the *hrdB* promoter from *S. coelicolor* to clone into pBluescript (KS^+)
hrdB_prom_rev	GCGCGGATCCCATATGGAACAACCTCTCGG AACGTT	*BamH*I & *Nde*I	
dagA::GFP Fwd	GCGCCATATGGTGGTCAACCGACGTGATCT	*Nde*I	Forward primer for amplification of *dagA* from *S. coelicolor* to clone downstream of *hrdB* promoter in pBluescript (KS^+)
dagA::GFP Transcriptional Rev	GCGCGATATCGGTACCCTACACGGCCTGAT ACGTCCTGACC	*EcoR*V & *Kpn*I	Reverse primer for amplification of *dagA* from *S. coelicolor* to clone downstream of *hrdB* promoter in pBluescript (KS^+). Stop codon included in *dagA* sequence for transcriptional fusion to *gfp*
dagA::GFP Translational Rev	GCGCGATATCGGTACCCACGGCCTGATACG TCCTGACCCAG	*EcoR*V & *Kpn*I	Reverse primer for amplification of *dagA* from *S. coelicolor* to clone downstream of *hrdB* promoter in pBluescript (KS^+). Stop codon omitted from *dagA* sequence for transcriptional fusion to *gfp*

dagAss_Fwd_GFP	GCGCCATATGGTCAACCGACGTGATCTCATCAA	*Nde*I	For amplification of the coding sequence for the *dagA* signal sequence for cloning into pRFSRL16
dagAss_Rev	GCGCCCATGGCGTCTGCGGCATGAGCGGCG GGT	*Nco*I	

Table 2.7: PCR reaction mixture for a 50 µl reaction

Component	**Volume (µl)**	**Final concentration**
10x Enzyme Buffer	5	1x
DMSO	2.5	5 % (v/v)
dNTP mix (20 mM)	0.5	200 µM
Forward primer (100 µM)	0.5	1 µM
Reverse primer (100 µM)	0.5	1 µM
Template	1	50-100 ng
Polymerase (1-5 U/µl)	0.5	0.5-2.5 U
H_2O	39.5	-

Table 2.8: Standard PCR conditions

Step	**Temperature (°C)**	**Time (min:sec)**	**Cycles**
Initial denaturation	96	2:00	1
Denaturation	94	00:30	30
Annealing	54-63	00:30	
Elongation	72	1:00 per kb	
Final elongation	72	5:00	1

2.2.6 Agarose gel electrophoresis

PCR products and restriction digests for analytical or cloning purposes were resolved by agarose gel electrophoresis. DNA fragments in the range of 0.5-12 kb were resolved using a 1 % (w/v) agarose gel whereas DNA fragments below 0.5 kb were resolved using a 2 % (w/v) agarose gel. Gels were prepared in TAE buffer and

ethidium bromide was added to a final concentration of 0.5 µg/ml to allow for DNA visualisation. Samples were prepared by mixing with 10x loading dye and loaded into the wells of a gel placed in TAE buffer alongside a DNA standard (100 bp or 1 kb ladder, Roche). 80-120 V was applied across the gel for up to 1 hour. DNA migration in the gel was visualised by exposure to UV light and displayed using a Bio-Rad gel doc XR. The size of the DNA of interest could then be extrapolated by comparison to the DNA standard.

2.2.7 Extraction of DNA from agarose gels

DNA fragments were cut from an agarose gel during visualisation under UV light. The DNA was then isolated using a QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The gel slice was incubated at 50°C with buffer QG until solubilised, mixed with isopropanol (where appropriate), and applied to a column. DNA became bound to the silica membrane in the column following centrifugation at 16,100 x*g* for 1 minute. The DNA was washed whilst bound to the column with buffer containing ethanol, then eluted from the membrane using 30 µl of the manufacturer's elution buffer or ultra pure water and stored at -20°C.

2.2.8 Digestion of DNA with restriction endonucleases and preparation for cloning

Digests of DNA using restriction endonuclease enzymes were carried out in suitable buffers according to the manufacturer's recommendations (New England Biolabs; Roche). Reactions were carried out in a 10 µl volume with an enzyme concentration of 2 U/µg of DNA and incubated at 37ºC for 2 hours or overnight.

Restriction enzymes were removed from digested PCR products using StrataClean Resin (Stratagene) according to the manufacturer's instructions. The resin was added to the reaction mix, at a volume equal to the square root of the reaction mix, vortexed and incubated at room temperature for 1 minute. Resin was pelleted by centrifugation at 16,100 x*g* for 1 minute and supernatant transferred to a fresh 1.5 ml Eppendorf tube.

To prevent self-ligation, digested vector DNA was treated with alkaline phosphatase (Roche) according to the manufacturer's instructions. Reactions were carried out in a 50 µl volume at 37°C for 2 hours.

Enzymes were removed from insert DNA (released following digestion of a plasmid) or dephosphorylated vectors by resolution on an agarose gel and the DNA was extracted using a QIAquick gel extraction kit.

2.2.9 Ligation of DNA

The concentration of vector and insert DNA fragments was quantified allowing the volume required for a minimum insert to vector ratio of 3:1 to be determined. This was calculated using the equation:

$$\text{mass}_{\text{insert}}[\text{ng}] = \frac{\text{mass}_{\text{vector}}[\text{ng}] \,.\, \text{size}_{\text{insert}}[\text{bp}] \,.\, 3}{\text{size}_{\text{vector}}[\text{bp}]}$$

Ligation reactions were set up in a 10 µl total mixture containing 1 µl T4 DNA ligase (Roche) and 1 µl 10x buffer. Reactions were incubated at 16°C overnight prior to transformation, using chemically competent *E. coli* cells.

2.2.10 DNA Sequencing

DNA sequencing was used to confirm insertion of target DNA into plasmids after cloning. Reactions were carried out by the *DNA Sequencing & Services* at the College of Life Sciences, University of Dundee. One sequencing reaction was carried out in a total volume of 30 µl containing 200-300 ng of DNA as a template and 1 µl of the appropriate oligonucleotide primer (from a 3.2 µM stock). Results were provided as a trace file which were analysed using Chromas Lite 2.01 (Technelysium Pty Ltd).

2.2.11 Southern blotting

2.2.11.1 Preparation of DNA and agarose gel electrophoresis

Chromosomal DNA isolated from the strain of interest was digested with an appropriate restriction endonuclease. Reactions were carried out at 37°C overnight in a total volume of 30 µl containing 5µg of DNA and 3 µl of restriction enzyme. The digested chromosomal DNA was then resolved by application of 80 V across a large (15 cm) 1 % agarose gel for 4 hours. The DNA bands in the gel were visualised by exposure to UV light prior to transfer of the DNA from the gel to a nylon membrane for Southern blotting.

2.2.11.2 Transfer of DNA to nylon membrane

The agarose gel was soaked for 30 minutes in distilled H_2O and then soaked twice, for 10 minutes each time, in 0.25 M HCl to promote partial depurination of the DNA and ease transfer of larger fragments. The gel was rinsed briefly in distilled H_2O and then soaked twice with gentle agitation, for 15 minutes, in Denaturing Solution. The gel was rinsed briefly three times in H_2O and then soaked for 20 minutes with gentle agitation in Neutralization solution.

The transfer apparatus was then set up. A reservoir at the base was filled with 20xSSC and a glass plate placed above this. A sheet of filter paper was placed across the glass plate with each end contacting the 20xSSC to act as a wick. A second piece of Whatmann filter paper no. 2 was soaked in 20xSSC and placed on top of this. The orientation of the gel was noted then placed on top of the filter paper. The entire apparatus was then covered in cling film leaving only the surface of the gel exposed. A piece of nylon membrane (Hybond N^+) cut to equal size was placed upon the gel carefully so as to allow good contact between the gel and the membrane. Two pieces of Whatmann paper were then placed on top of the membrane. A 3-4 cm stack of paper towels, pre-soaked in 20xSSC, were placed on top and weighted down. Transfer of DNA was allowed to run overnight after which time DNA was cross-linked to the nylon membrane by exposure to UV light.

2.2.11.3 Preparation of a DIG-labelled probe

Probes were prepared by PCR, resolved by agarose gel electrophoresis and isolated using a QIAquick gel extraction kit. Probes were denatured by boiling for 10 minutes then labelled using a digoxigenin (DIG) DNA labelling kit (Roche) according to the manufacturer's instructions. Immediately prior to use the DIG-labelled probe was denatured by boiling for 5 minutes then stored on ice for 1 minute.

2.2.11.4 Hybridisation and detection of the DIG-labelled probe

The nylon membrane was pre-hybridised in DIG Easy Hyb Solution (Roche) by incubating for 4 hours with rotation at 55°C in hybridisation tubes. The DIG-labelled probe was then added to the tube and incubation continued overnight.

The hybridisation solution was decanted and the membrane washed briefly in Wash Solution I. The membrane was washed twice with Wash Solution I at room temperature for 5 minutes. The membrane was then washed twice with pre-warmed Wash Solution II at 65°C for 15 minutes.

The membrane was transferred to a washing dish and subsequent steps carried out at room temperature with agitation. The membrane was rinsed briefly in Washing Buffer then incubated for 1 hour in 1x Blocking buffer. The membrane was then incubated with 1x Blocking buffer containing an alkaline phosphatase labelled α-DIG antibody at a concentration of 1:10,000 for 30 minutes.

The membrane was washed twice with Washing Buffer for 15 minutes then equilibrated in Detection buffer for 5 minutes. The membrane was then transferred to a hybridisation bag with the DNA side facing up and the surface flooded with 1 ml of CSPD (disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro)tricyclo [3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate) ready-to-use solution (Roche). The bag was sealed and incubated for 5 minutes at room temperature. The bag was opened, surplus liquid squeezed out then resealed prior to incubation for 10 minutes at 37°C to enhance the luminescent reaction.

For detection of bands corresponding to regions where the probe had hybridised to DNA the nylon membrane was exposed to BioMax MR scientific imaging film (Kodak) and developed.

2.3 Genetic manipulation of *Streptomyces*

2.3.1 Disruption of *Streptomyces* genes by the PCR-targeting system

The methods in this Section are as described by Gust *et al*. (2002) in the REDIRECT© technology manual, or a derivative thereof.

2.3.1.1 PCR amplification and isolation of the disruption cassette

Primers were designed (Table 2.6) allowing amplification of the OriT-apramycin resistance cassette from pIJ773 or OriT –hygromycin resistance cassette from pIJ10700 by PCR as described in Section 2.2.5. The 5' end of each primer consisted of a sequence homologous to the region of the chromosome immediately surrounding the gene to be disrupted. PCR products were resolved by agarose gel electrophoresis and isolated using a QIAquick gel extraction kit as described in Section 2.2.7 to ensure no contamination of the PCR product with the template plasmid.

2.3.1.2 Generation of mutated cosmid in *E. coli* BW25113/pIJ790

E. coli BW25113/pIJ790 cells were transformed with appropriate clones from a *Streptomyces* cosmid library (Table 2.5) by electroporation, as described in Section 2.1.3, with the exception that cells were grown at 30°C in media supplemented with chloramphenicol, as pIJ790,which encodes the *cat* resistance gene, has a temperature sensitive origin of replication.

These cells were then grown at 30°C in media containing amp, cml and kan to maintain selection for pIJ790 as well as the Supercos-derived *Streptomyces* cosmid clone. Media was also supplemented with 10 mM *L*-arabinose for induction of the λ-red recombinase genes encoded on pIJ790. Electrocompetent cells were prepared as described in Section 2.1.3, transformed with 5 μl of the linear PCR-amplified disruption cassette and spread onto LB plates supplemented with the appropriate antibiotics. Difco Nutrient Agar was used for Hygromycin selection as it promoted clean selection of resistant colonies. Mutated cosmids were confirmed by diagnostic

PCR using primer pairs that bind outside the gene targeted for replacement and within the resistance cassette giving product of a specified size (Table 2.6).

2.3.1.3 Transfer of the mutated cosmid into *Streptomyces*

Mutated cosmids were isolated from *E. coli* BW25113 as described in Section 2.2.2 and introduced into *E. coli* ET12567/pUZ8002 by electroporation, as described in Section 2.1.3. As the *Streptomyces* strains used in this study have a methyl-sensing restriction system passage through this *E. coli* host is necessary as it does not methylate DNA.

E. coli ET12567/pUZ8002 cells containing the mutated cosmid were prepared for conjugation with *S. coelicolor* or *S. scabies* by inoculating 10 ml or 50 ml LB medium, respectively, with a stationary phase culture at a ratio of 1:100. This was grown until an OD_{600} of 0.4-0.5 was reached and then cells were pelleted and washed twice by centrifugation at 2,773 x*g* for 5 minutes in an equal volume of LB medium. Cells were re-suspended in 1 ml of LB medium and mixed with *Streptomyces* spores prepared as follows.

S. coelicolor: 5 µl of a spore stock was mixed with 500 µl of 2xYT medium and subjected to heat shock at 50°C for 10 minutes. This was allowed to cool at room temperature and then mixed with 500 µl of the *E. coli* cell suspension. A pellet was obtained by centrifugation of this cell/spore mix at 5,900 x*g* for 1 minute. The supernatant was decanted and the cell/spore mix re-suspended in the remaining liquid. Serial dilutions from 10^{-1} to 10^{-4} of the mixture were prepared in sterile H_2O then 100 µl of each dilution spread onto MS agar plates supplemented with 10 mM $MgCl_2$.

S. scabies: Spores were freshly harvested from a well-sporulating surface-grown culture by scraping into 2 ml of 2xYT medium. 1 ml of this spore suspension

was mixed with 1 ml of the *E. coli* cell suspension. A pellet was obtained by centrifugation of this cell/spore mix at 5,900 x*g* for 1 minute. The supernatant was decanted and the cell/spore mix re-suspended in the remaining liquid. This was divided evenly and spread onto MS agar plates supplemented with 10 mM $MgCl_2$.

Conjugation plates for both *S. coelicolor* and *S. scabies* were incubated at 30°C for 20 or 24 hours, respectively, prior to being overlaid with 1 ml of an antibiotic solution containing appropriate concentrations of nalidixic acid and either apramycin or hygromycin, depending on the marker used for gene disruption. After drying in a laminar flow hood plates were further incubated at 30°C for up to three weeks.

2.3.1.4 Identification of double-crossover mutants in *Streptomyces*

Exconjugant colonies that grew after overlay solution was applied were replica plated onto Difco nutrient agar plates supplemented with nalidixic acid and apramycin / hygromycin and onto plates containing nalidixic acid, apramycin / hygromycin and kanamycin. Following two days incubation at 30°C, kanamycin sensitive and apramycin or hygromycin resistant colonies, corresponding to double crossover exconjugants, were picked and streaked for single colonies on SFM or IPM for *S. coelicolor* and *S. scabies*, respectively. A further round of replica plating was used to confirm kanamycin sensitivity and exconjugants were spread onto SFM or IPM plates and allowed to sporulate. Spore stocks were prepared as described in Section 2.1.1 and gene disruption confirmed by PCR and/or Southern blot analysis, as described in Sections 2.2.5 and 2.2.11, respectively.

2.3.2 Integration of plasmid DNA into the chromosome at the φC31 attachment site

Several plasmids were constructed in this study for expression of genes *in trans* using pSET152-based vectors as a backbone. These vectors encode the *attP* and *int* genes allowing them to attach and integrate site-specifically at the φC31 attachment site on the chromosome, as well as the *oriT* gene for transfer into *Streptomyces* by conjugation. These plasmids were used to transform *E. coli* ET12567/pUZ8002 as described in Section 2.1.2 and conjugation was carried out as described for *S. coelicolor* in Section 2.3.1.3. One round of replica plating was carried out using Difco nutrient agar plates supplemented with nalidixic acid and the appropriate antibiotic, prior to spreading onto SFM or IPM. The strains were grown to sporulation and spore stocks were prepared as described in Section 2.1.1.

2.4 RNA Techniques

2.4.1 Isolation of total RNA from *Streptomyces*

RNA was extracted from *Streptomyces* using reagents of the RNeasy mini kit (Qiagen) and a modification of the manufacturer's protocol as follows.

Streptomyces strains were grown in liquid culture using appropriate media and for the desired period after which time stop solution was added to the culture at a ratio of 5:1 culture to stop solution. Samples were centrifuged at 16,100 x*g* for 1 minute and the supernatant discarded. The mycelial pellet was then re-suspended in 90 µl of TE buffer to which 10 µl of 50 mg/ml lysozyme stock solution was added, and incubated for 10 minutes at 37°C. A solution of 10 µl of β-mercaptoethanol per ml of RLT buffer was prepared and 350 µl was added to the sample and vortexed to mix. Insoluble material was removed at this stage by centrifugation at 16,100 x*g* for 2

minutes and the supernatant transferred to a fresh tube containing 250 µl of ethanol and mixed by pipetting. This was applied to a column and RNA bound to the membrane in the column by centrifugation at 16,100 x*g* for 30seconds. The flow-through was discarded then the 350 µl of RW1 added to the column and washed through by centrifugation at 16,100 x*g* for 1 minute. The flow-through was discarded and 80 µl of RDD/DNase (at a 7:1 ratio) added and incubated for 15 minutes at room temperature after which time 350 µl of RW1 was added to the column and the column washed by centrifugation at 16,100 x*g* for 1 minute. The column was transferred to a fresh collection tube and washed with 500 µl of RPE buffer by centrifugation at 16,100 x*g* for 30seconds. This wash step was repeated and the flow-through discarded. The column was dried by centrifugation at 16,100 x*g* for 1 minute and then transferred to an RNase-free Eppendorf tube. RNA was eluted from the column in 30 µl of RNase-free water by centrifugation at 16,100 x*g* for 1 minute. This was repeated by collection in the same Eppendorf tube to improve RNA yield. DNA was removed from RNA by further treatment with DNase - this was achieved by the addition of 6.8 µl of 10x DNase buffer (Qiagen) and 1.2 µl DNase (Qiagen) and incubating at 37°C for 30 minutes. DNase was removed using StrataClean Resin (Stratagene) according to the manufacturer's instructions.

2.4.2 Synthesis of cDNA from *Streptomyces* RNA

Synthesis of cDNA from RNA was carried out using SuperScript III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. RNA concentration was estimated using a NanoDrop ND-1000 system (Thermo Scientific) then diluted in RNase-free water to allow 2 ng of RNA per reaction in a total volume of 8 µl. Random hexamers were added to a final concentration of 5

ng/µl and dNTP mix to a final concentration of 1 mM and the sample was incubated at 65°C for 5 minutes followed by 1 minute on ice.

The cDNA synthesis mix was then prepared in the following order for one reaction, or multiples thereof for multiple reactions:

1. 2 µl of 10xRT buffer
2. 4 µl of $MgCl_2$ (25 mM)
3. 2 µl DTT (100 mM)
4. 1 µl RNaseOUT (400 U/ µl)
5. 1 µl SuperScript III RT (200 U/ µl)

This mix was then added to the RNA/primer mix and the cDNA synthesis reaction carried out by incubation at 25°C for 10 minutes followed by 50°C for 50 minutes then 85°C for 5 minutes. RNA was then removed by the addition of 1 µl of RNase H and incubation at 37°C for 20 minutes. Samples of cDNA were stored at -20°C prior to use. PCR was carried out as described in Section 2.2.5 using cDNA as a template for assessment of gene expression.

2.5 Protein Techniques

2.5.1 Preparation of crude total cytosolic protein and production of *Streptomyces* growth curves

Mycelial pellets obtained as described in Section 2.1.4 were re-suspended in 1 ml of 1 M NaOH and boiled for 10 minutes. Mycelial debris was removed by centrifugation at 16,100 x*g* for 5 minutes. The supernatant was transferred to a fresh tube, diluted with water to a ratio of 1:1 and 5 µl of this was used for estimation of protein concentration in the sample using the microplate assay protocol from the BioRad DC protein assay kit, according to the manufacturer's instructions. The

absorbance was read at 750 nm by an ELx808 absorbance microplate reader (BioTek). A standard curve was produced concurrently using serial dilutions, in 0.5 M NaOH, of bovine serum albumin (BSA) in the range of 0 to 1.5 mg/ml. Each measurement was carried out in triplicate. Calculations were then undertaken to determine the protein concentration in the samples (which correlates directly to growth) allowing growth curves to be produced for each strain.

2.5.2 Preparation of whole cell protein extracts from *Streptomyces* for SDS PAGE and western blotting

Streptomyces were grown for a specified period of time in liquid media and a mycelial pellet obtained by centrifugation at 2,773 x*g* for 10 minutes. The pellet was re-suspended in 1 ml of PBS containing Complete Mini, EDTA free, Protease Inhibitor (Roche) prepared according to the manufacturer's instructions. Mycelia were incubated on ice during disruption by sonication using an S-250D DIGITAL Sonifier (Branson) with a 3 mm tapered tip. The sonifier was set at a power input of 10 % and 30 seconds total sonication time, with 5 second bursts followed by 5 second intervals. Following sonication, cell debris was removed by centrifugation at 4°C at 16,100 x*g* for 1 minute. The supernatant was transferred to a fresh tube and stored at -20°C. Prior to resolution of samples by SDS PAGE, samples were thawed on ice and protein concentration estimated using the BioRad DC protein assay kit, according to the manufacturer's instructions.

2.5.3 Isolation of extracellular proteins from *Streptomyces* strains grown in liquid media

Streptomyces were grown for a specified period of time in liquid media inoculated from fresh or frozen spores, after which a mycelial pellet was obtained by centrifugation at 2,773 x*g* for 15 minutes. The culture supernatant was filtered through a 0.45 µm syringe filter to remove any remaining traces of bacteria and then trichloroacetic acid (TCA) added to a final concentration of 10 %. This was incubated on ice for 30 minutes after which time the precipitated protein pellet was obtained by centrifugation at 2,773 x*g* for 15 minutes. The supernatant was decanted and the protein pellet re-suspended in 2 ml of acetone. This was transferred to a 2 ml Eppendorf tube and washed by centrifugation at 16,100 x*g* for 2 minutes. This wash step was repeated ten times then the protein pellet chilled to -20°C and air dried in a laminar flow hood. Prior to resolution of protein in each sample by SDS PAGE, samples were solubilised in 2D sample buffer and protein concentration estimated using the 2D Quant kit (GE Healthcare) according to the manufacturer's instructions.

2.5.4 Isolation of extracellular proteins from *Streptomyces* strains grown on solid media

Autoclaved cellophane discs of 8 cm diameter (Focus Packaging & Design Ltd.) were placed on the surface of agar plates and inoculated with equal number of spores of *Streptomyces* wild-type or mutant strains to be compared. For strains to be grown for a 48 hours incubation period, a pre-enumerated stock was used, allowing 10^6 spores to be spread per plate. For incubation periods of up to 24 hours a whole plate of fresh spores were harvested and filtered through a cotton wool plug in a syringe. Spore numbers in the resulting suspension was then estimated by

measurement of the OD_{600} and diluted appropriately so that inocula were of equal OD_{600}. Ten plates were prepared for each strain examined. After incubation at 30°C for a set period of time, biomass was harvested from the surface of the cellophane by scraping with a clean spatula, and dispersed in 30 ml of 5 M LiCl in a 50 ml Falcon tube. This was incubated on ice for 30 minutes and then vortexed for 3 minutes after which the biomass was removed by centrifugation at 2,773 x*g* for 10 minutes. The supernatant was filtered through a 0.45 µm syringe filter to remove any remaining traces of bacterial hyphae or spores and then TCA added to a final concentration of 10 %. This was incubated on ice for 30 minutes after which time the precipitated protein was obtained as a pellet by centrifugation at 2,773 x*g* for 15 minutes. The supernatant was carefully decanted leaving behind a gel-like protein pellet which was mixed with 30 ml of ultra-pure H_2O, giving a cloudy suspension. The protein pellet was obtained by centrifugation at 2,773 x*g* for 10 minutes and re-suspended in 2 ml of acetone. This was transferred to a 2 ml Eppendorf tube and washed by centrifugation at 16,100 x*g* for 2 minutes. This wash step was repeated ten times then the protein pellet chilled to -20°C and air dried in a laminar flow hood.

2.5.5 SDS PAGE

SDS polyacrylamide gel electrophoresis (SDS-PAGE) gels were prepared using the Mini-PROTEAN II System (Bio-Rad). A spacer plate of desired thickness and short plate were assembled as instructed by the manufacturer. Resolving gel of the required acrylamide percentage was prepared (Table 2.9) and poured between the two plates so that adequate space was left for addition of the stacking layer and comb insertion (approximately 2 cm). Isopropanol was overlaid on the resolving layer immediately after pouring to ensure a straight edge. After polymerisation of the

resolving layer the isopropanol overlay was poured off and the top edge of the resolving gel rinsed with distilled H_2O. The stacking gel was then prepared (Table 2.9) and poured on top of the resolving gel until it reached the top edge of the short plate and a comb carefully inserted into the stacking layer. After polymerisation of the stacking layer the comb was removed leaving wells into which protein samples were loaded. The gel and glass plate assembly were then removed from the casting apparatus and placed with the electrode assembly into the buffer tank containing SDS PAGE running buffer.

Protein samples, whose concentration had been previously estimated, were diluted in their original buffer so that each contained equal amounts of protein, generally 10 µg or 20 µg. Samples were then diluted in an equal volume of 2x Laemmli buffer and boiled for 5 minutes prior to loading into the wells of an SDS-PAGE gel alongside a pre-stained molecular weight standard (Precision Plus, Bio-Rad).

A constant voltage of 100 V was applied across the gel until the samples had entered the resolving gel, after which time the voltage was increased to 200 V until the bromophenol blue dye front had reached the bottom of the resolving gel.

The gels were then removed from the buffer tank and the proteins visualised by Coomassie Brilliant Blue staining with InstantBlue (Novexin) or used for western blotting.

Table 2.9: Composition of SDS-PAGE resolving and stacking gel

	Resolving gel	**Stacking gel**
Acrylamide/bisacrylamide (37.5:1)	12 -17 % (v/v)	4 % (v/v)
Tris/HCl	375 mM (pH 8.8)	125 mM (pH 6.8)
SDS	0.1 % (w/v)	0.1 % (w/v)
APS	0.1 % (w/v)	0.1 % (w/v)
TEMED	0.1 % (v/v)	0.1 % (v/v)

2.5.6 Western Blotting

Following resolution of proteins by SDS-PAGE, the gel and an equally sized piece of nitrocellulose membrane (Amersham Hybond-ECL, GE healthcare) was soaked separately in Transfer buffer for 5 minutes. Four pieces of filter paper were cut to the size of the gel and soaked briefly in Transfer buffer. The transfer "sandwich" was then assembled by firstly placing two layers of filter paper on the positive electrode of a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell, followed by the nitrocellulose membrane, then the gel and finally the additional two layers of filter paper. Each layer was placed carefully so as to allow good contact between each layer. The apparatus was assembled and the transfer carried out by applying 10 V across the sandwich for 45 minutes.

After the transfer was complete the membrane was removed from the apparatus and incubated in blocking buffer overnight at 4°C.

The membrane was incubated with the primary antibody (Table 2.10) diluted in blocking buffer for 1 hour at room temperature with shaking. The membrane was washed 3 times with TBS/0.1 % Tween for 10 minutes and then incubated with the secondary antibody (Table 2.10) diluted in blocking buffer for 1 hour with shaking. The membrane was washed three times with TBS/0.1 % Tween and dried by touching the edge to a paper towel. The surface of the membrane was then flooded with Immobilon Western Chemiluminescent HRP Substrate (Millipore) and incubated for 5 minutes at room temperature.

For detection of bands corresponding to the presence of the protein of interest the membrane was exposed, for a given time, to BioMax MR scientific imaging film (Kodak) which was developed using a SRX-101A (Konica Minolta).

Table 2.10: Antibodies used in this study

Antibody	Dilution	Raised in	Source
Monoclonal α-GFP	1:10,000	mouse	Sigma-Aldrich (Cat. No. G1546)
Monoclonal α-His_4	1:2,000	mouse	QIAgen (Cat. No. 4670)
Polyclonal α-Sco4509	1:10,000	rabbit	Seqlab (Göttingen, Germany)
Polyclonal α-Sco5724/Sco5725	1:5,000	rabbit	Seqlab (Göttingen, Germany)
α-mouse IgG HRP conjugate	1:10,000	goat	Bio-Rad (Cat. No.170-6516)
α-rabbit IgG HRP conjugate	1:10,000	goat	Bio-Rad (Cat. No. 170-6515)

2.5.7 2D gel electrophoresis

All steps and solution preparation were carried out whilst wearing gloves to minimise contamination from skin keratin. Solutions and buffers used can be found in Table 2.4.

2.5.7.1 Sample preparation and running of IPG (immobilised pH gradient) strips – 1st dimension

Precipitated protein pellets were re-suspended in 2D sample buffer and insoluble protein particles removed by centrifugation at 16,100 x*g* for 1 minute. The supernatant was transferred to a fresh tube and protein concentration estimated using a 2D Quant Kit (GE Healthcare) according to the manufacturer's instructions. Proteins were diluted in 2D sample buffer so that each sample contained a total of 0.9 mg in 340 µl total volume. Bromophenol blue was added to the sample at a final

concentration of 0.002 % and IPG buffer (GE Healthcare), of the appropriate pH range, to a final concentration of 0.5 %.

The sample was then applied to the middle of the strip holder and an Immobiline DryStrip IPG strip (pH 4-7, 18 cm) oriented so that the positively marked end was aligned to the pointed end of the strip holder. The IPG strip had the protective plastic removed and was placed carefully, to avoid the introduction of air bubbles, gel side down into the sample until the entire strip was wet with the sample and laid flat. The strip was then overlaid with Imobiline DryStrip cover fluid (GE Healthcare) to prevent dehydration of samples during running of the first dimension.

The strip holders were then aligned in the correct orientation in the Ettan IPGphor II focusing unit. The current on the focusing unit was set at 50 μA per strip and the following program applied across the strips:

1. 11 hours rehydration
2. 1 hour at 500 V
3. 1 hour at 1 kV
4. 14 hours at 8 kV

Immediately after the first dimension run was completed, the IPG strips were removed from the strip holder, by grasping one end in a pair of forceps, and rinsed gently with ultra pure water to remove traces of the cover fluid. The IPG strips were then stored in petri dishes by pushing the plastic backing against the curvature of the inside of the dish. Petri dishes were sealed with parafilm and stored at -80°C until running the second dimension.

2.5.7.2 Preparation of IPG strips and running of the 2nd dimension SDS-PAGE gel

The second dimension was run in an Ettan DALT twelve electrophoresis unit (GE Healthcare) using pre-cast Ettan DALT 12.5 % gels and DALT buffer kit (GE Healthcare). Gels and buffers were prepared as instructed by the manufacturer. The power pack was set to a constant voltage of 1 V and constant temperature 25°C.
IPG strips were removed from the freezer and allowed to thaw at room temperature.

Equilibration buffer 1 (15 ml) was added to the petri dish and incubated with the IPG strip at room temperature for 15 minutes with shaking. This was decanted and then Equilibration buffer 2 added and again incubated with the IPG strip at room temperature for 15 minutes with shaking. The IPG strips were then dipped into cathode buffer to remove traces of the Equilibration buffer and oriented so that the positive end of the strip was at the left hand side of the 2nd dimension gel. The plastic backing of the IPG strip was placed flat against the glass plate of the cassette that contained the 2nd dimension gel and then carefully pushed down with a spatula, touching only the plastic backing, until the IPG strip formed contact with the 2nd dimension gel along its entire length. The IPG strip was then sealed in place using agarose sealing solution provided with the buffer kit.

Once prepared, all gels were loaded into the electrophoresis unit containing anode buffer. If less than 12 gels were to be run, any spaces were filled with a plastic spacer. The top chamber was then filled with cathode buffer to a set point marked on the unit and the lid closed. The gels were run at 5 W per gel for 30 minutes and then the power increased to 200 W and ran until the bromophenol blue dye front had reached the bottom of the gel (approximately 5-7 hours). Gels were then removed

from the cassettes and placed into staining trays in batches of 3. Fixation solution was added and allowed to shake for 2 hours or overnight prior to staining.

2.5.7.3 Visualisation of proteins in the 2D-SDS-PAGE gel by colloidal Coomassie staining

The Fixation solution was decanted and 400 ml of Staining solution added to each tray and allowed to shake overnight. The gels were then destained in 1 % acetic acid until background staining of gels was removed and protein spots could be clearly distinguished. Images were taken of the gels and then they were stored in clean staining trays immersed in ultra pure water at 4°C.

2.6 Scanning Electron Microscopy (SEM)

Spores were inoculated onto solid media and incubated at 30°C for ten days to allow confluent sporulation. After this time a square section was cut from the surface of the media with a scalpel and the excised layer of biomass was mounted on the sample holder using Tissue-Tech OCT Compound (BDH Laboratory Supplies). This was rapidly frozen in liquid N_2 slush then the cryo-preserved sample was then transferred under vacuum to the cryo-preparation stage and warmed to -95°C for 5 minutes to remove surface water. After sublimation the samples were then cooled to −115°C and coated with approximately 5 nm Au/Pd. Samples were examined using a Philips XL 30 emission scanning electron microscope (ESEM) operating at an accelerating voltage of 15 kV.

2.7 Phage plaque assays

Serial dilutions of phage were prepared in Difco nutrient broth (DNB), covering the range 10^0 to 10^{-8}. 100 µl aliquots of each dilution were transferred to the surface of DNA media in a 55 mm petri dish supplemented with 0.5 % glucose, 10 mM MgSO4 and appropriate concentration of calcium ions (Table 2.11). Molten soft nutrient agar (SNA) was cooled to 45°C prior to the addition of spores, from a pre-enumerated stock, to a concentration of 10^7-10^8 per ml. A 1 ml aliquot of this spore overlay suspension was then transferred to the surface of the media containing the phage dilution. The petri dish was swirled to ensure the surface of the media was completely covered then left undisturbed for 15 minutes to allow the agar to set. The plates were then inverted and incubated at 30°C for 18-20 hours after which time plaque formation was assessed. The plates containing phage dilutions at which plaques could clearly be distinguished were then photographed.

Table 2.11: Phage used in this study

Phage	Concentration of $Ca(NO_3)_2$	Source
φC31	8 mM	P. Herron & E. Bell, University of Strathclyde
φHau3AI	4 mM	
φR4	25 mM	

2.8 Virulence assays

2.8.1 Radish seedling virulence assay

Radish seeds were surface sterilised for 5 minutes in 70 % ethanol followed by 10 minutes in 15 % bleach. The seeds were washed ten times in 50 ml of sterile H_2O and transferred to the base of a petri dish lined with sterile filter paper that was pre-

wet with 2 ml of sterile H_2O. The petri dish was sealed with parafilm and incubated in the dark at room temperature for 36-40 hours. Deep petri dishes were prepared that contained 50 ml of 1.5 % water agar. Six wells of approximately 1 cm in diameter each were cut from the agar using a sterile corer. The agar plugs were transferred to a sterile bottle, melted and 200 µl used to seal the base of the well. Seedlings were chosen that displayed a similar level of germination and transferred to the wells.

Spores of the inoculation strains were scraped from the surface of a plate showing confluent sporulation, placed into 10 ml of sterile H_2O and filtered through cotton wool in a 20 ml syringe and collected in a sterile tube. A 1:20 dilution in H_2O was prepared from this spore suspension, for measurement of the OD_{600}. Based on this, spores from all strains to be compared were diluted to give an OD_{600} of 0.5 and 200 µl of this was used to inoculate the seedlings in each well an agar plate. One plate/six seedlings were used per strain to be tested. Petri dishes were sealed with parafilm and incubated at 21(+/-2) °C with a 16 hour photoperiod for six days. Roots were carefully cut from the agar using a scalpel, assessed for disease symptoms and photographed.

2.8.2 *Arabidopsis* virulence assay

Arabidopsis seeds in a 1.5 ml Eppendorf tube were surface sterilised with chlorine gas generated by the addition of 2 ml of 37 % HCl to 48 ml bleach in a 100 ml beaker. Immediately following addition of the HCl to the beaker, an open Eppendorf tube containing the seeds were sealed inside a bell jar in the fume hood and left for 3 hours. Following removal of the bell jar lid, the Eppendorf tube was briskly closed and transferred to the laminar flow cabinet where it was left open for 10

minutes for dispersal of residual chlorine gas. 1 ml of 0.1 % phytagar (Sigma) was mixed with the sterile seeds and stored at 4°C in the dark for 2-5 days.

Seeds were then transferred onto MS/1 %sucrose/0.8 % agar in square petri dishes by pipetting. Fourteen seeds were allowed per plate in two rows of seven. Plates were then sealed with parafilm and incubated in an upright position at 21(+/-2) °C with a 16 hour photoperiod for four days. Fresh spores were prepared for inoculation as described in Section 2.8.1 and 10 µl of spore suspension was used to inoculate the root tip of the seedlings. Plates were again sealed with parafilm and incubated at 21(+/-2) °C with a 16 hour photoperiod. Observations were made on a regular basis and after approximately 4 weeks plants were photographed.

2.8.3 *Nicotiana* virulence assay

Nicotiana seeds were surface sterilised and stored as described for *Arabidopsis* seeds in Section 2.8.2. Seeds were then transferred onto MS/1 %sucrose/0.8 % agar in sterile magenta boxes, allowing for six of eight seeds per box. These were incubated at 21(+/-2) °C with a 16 hour photoperiod for 7-10 days.

A pre-enumerated spore stock was used for inoculation with approximately $5x10^7$ spores in 1 ml of sterile H_2O used to flood the surface of the agar in each box. Plants were incubated as before and observations made on a regular basis. After 9 weeks plants were assessed for disease symptoms and photographed.

2.8.4 Potato plant virulence assay

Potato plants of the Chippewa variety, that had been maintained as sterile micro-propagated plants were used to carry out virulence assays. During standard propagation cuttings were taken at the site of axilliary bud formation. Prior to

planting of these into glass tubes containing fresh potato sub-culture medium (Table 2.2), the cutting was dipped in a spore suspension of each inoculation strain, prepared as described in Section 2.8.1. One cutting was planted per tube and incubated at 21(+/-2) °C with a 16 hour photoperiod for 21 days.

2.8.5 Potato tuber slice disc assay

Potato tubers were peeled then surface sterilised by soaking in a 15 % bleach solution for 10 minutes. Each potato was then transferred to a large beaker containing 1L of sterile H_2O and washed for 1 minute. A second wash step was repeated using a fresh beaker of sterile H_2O. Potatoes were then transferred to a surface covered with sterile paper towels. An autoclaved corer was then used to aseptically cut cores of approximately 1 cm in diameter from the tuber. The core was pushed out using a sterile glass rod and cut into discs of 5 mm thickness using a sharp, autoclaved knife. These discs were transferred to a petri dish lined with sterile filter paper pre-wet with 2 ml of sterile H_2O. Each potato tuber slice was then inoculated with equally sized agar plugs excised from plates of the inoculation strains showing confluent sporulation. Agar plugs were placed spore side down onto the potato tissue and the petri dishes sealed with parafilm. The discs were incubated in the dark at room temperature for 5-7 days.

2.8.6 Thaxtomin extraction and quantification

For the extraction and quantification of thaxtomin, 50 ml cultures of OBB medium (Table 2.2) were set up in 250 ml Erlenmeyer flasks by inoculating with an equal number of spores, as determined by measurement of their OD_{600}. Cultures were incubated for one week at 28°C with shaking at 280 rpm and then transferred to 50 ml

Falcon tubes. Biomass and particulates were removed by centrifugation at 2,773 x*g* for 10 minutes and 3 ml of the supernatant applied to conditioned C-18 extraction cartridge (Dionex). The cartridge was conditioned by applying 3 ml of methanol then 3 ml of distilled H_2O, each drawn through using a vacuum manifold. The supernatant was then applied to the cartridge which turned yellow as thaxtomin bound to the matrix. The cartridge was then washed with 3 ml of distilled H_2O followed by 3 ml of 25 % methanol. The thaxtomin was eluted in 3 ml of 50 % methanol and collected in a glass vial for storage at -20°C prior to analysis.

Samples were analysed by HPLC on a Phenomenex Prodigy Column (5µm ODS3, 4.6 x 250 mm) at a flow rate of 1 ml/minute of an isocratic acetonitrile:water (40:60) mobile phase. Thaxtomin was detected by absorbance between 215 and 400 nm and quantified by measuring absorbance at 380 nm and comparing to a pure thaxtomin standard.

2.8.7 Colonisation of *Arabidopsis* roots by *S. scabies* strains

Plasmids pIJ8641 and pRFSRL16 were integrated into the chromosomes of *S. scabies* 87.22 and the isogenic Δ*tatC* strain, respectively, as described in Section 2.3.2. These plasmids resulted in expression of *egfp* under the control of the strong constitutive promoter for the erythromycin resistance gene from *S. erythraea*, *ermE**. Hyphae could then be visualised during infection of *Arabidopsis* roots as GFP can be excited at 450-490 nm and emits light at 500-550 nm which can be detected by fluorescence microscopy.

A. thaliana seeds were surface sterilised and stored as described in Section 2.8.2 prior to transfer to 3 ml of MS medium with 2 % sucrose in a 6 well culture plate (Corning). Seedlings were incubated with shaking at 100 rpm and a 16 hour

photoperiod for 7 days then transferred to fresh MS medium with 2 % sucrose in a culture plate and inoculated with $1x10^6$ c.f.u. of a spore suspension of the *egfp* expressing 87.22 and Δ*tatC* strains. Plants were incubated as before and visualised at 24 hour intervals by laser scanning confocal microscopy to observe colonisation of the *Arabidopsis* roots. Roots were removed from the media, rinsed gently in distilled water, mounted in water and observed using a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany), with a 10x and 63x water immersion objective lens. EGFP was visualized using a 4-line argon laser with an excitation wavelength of 488 nm and an emission wavelength of 500 to 550 nm. Differential interference contrast (DIC) images were collected simultaneously with the fluorescence images using the transmitted light detector and processed using Leica LAS-AF software (version 1.8.2).

3. CHARACTERISATION OF THE PUTATIVE TYPE VII PROTEIN EXPORT SYSTEM FROM STREPTOMYCES COELICOLOR

3.1 Introduction

As discussed in chapter one, genome analysis coupled with experimental studies have revealed that there are three known systems for protein secretion in Gram-positive bacteria. The Sec and Tat pathways, which are active in both Gram-positive and Gram-negative bacteria, are the best characterised of these. The more recently discovered T7SS, which appears to be unique to Gram-positive bacteria, is much more poorly understood. The current knowledge has been largely gleaned from studies carried out using *Mycobacterium tuberculosis*, as this was first organism in which this secretion system was experimentally described.

3.1.1 T7SS in *Mycobacteria*

Many studies have shown that when the attenuated Bacillus Calmette-Guérin (BCG) strain of *M. bovis* (a member of the *M. tuberculosis* species complex used to immunise against tuberculosis) was grown in culture medium, many small proteins, including CFP-10 and ESAT-6, that were found in the growth medium of the wild type *M. bovis* strain were missing (Sonnenberg & Belisle, 1997). Despite being secreted, they lacked the obvious N-terminal signal peptides that are a prerequisite for secretion by the other known pathways in Gram positive bacteria. Genetic analysis of the BCG strain identified that genes deleted in the locus termed RD1 (for region of difference 1; Figure 3.1) were not only critical for virulence but also for the secretion of these proteins into the culture supernatant (Mahairas *et al.*, 1996; Lewis *et al.*, 2003; Pym *et al*., 2002).

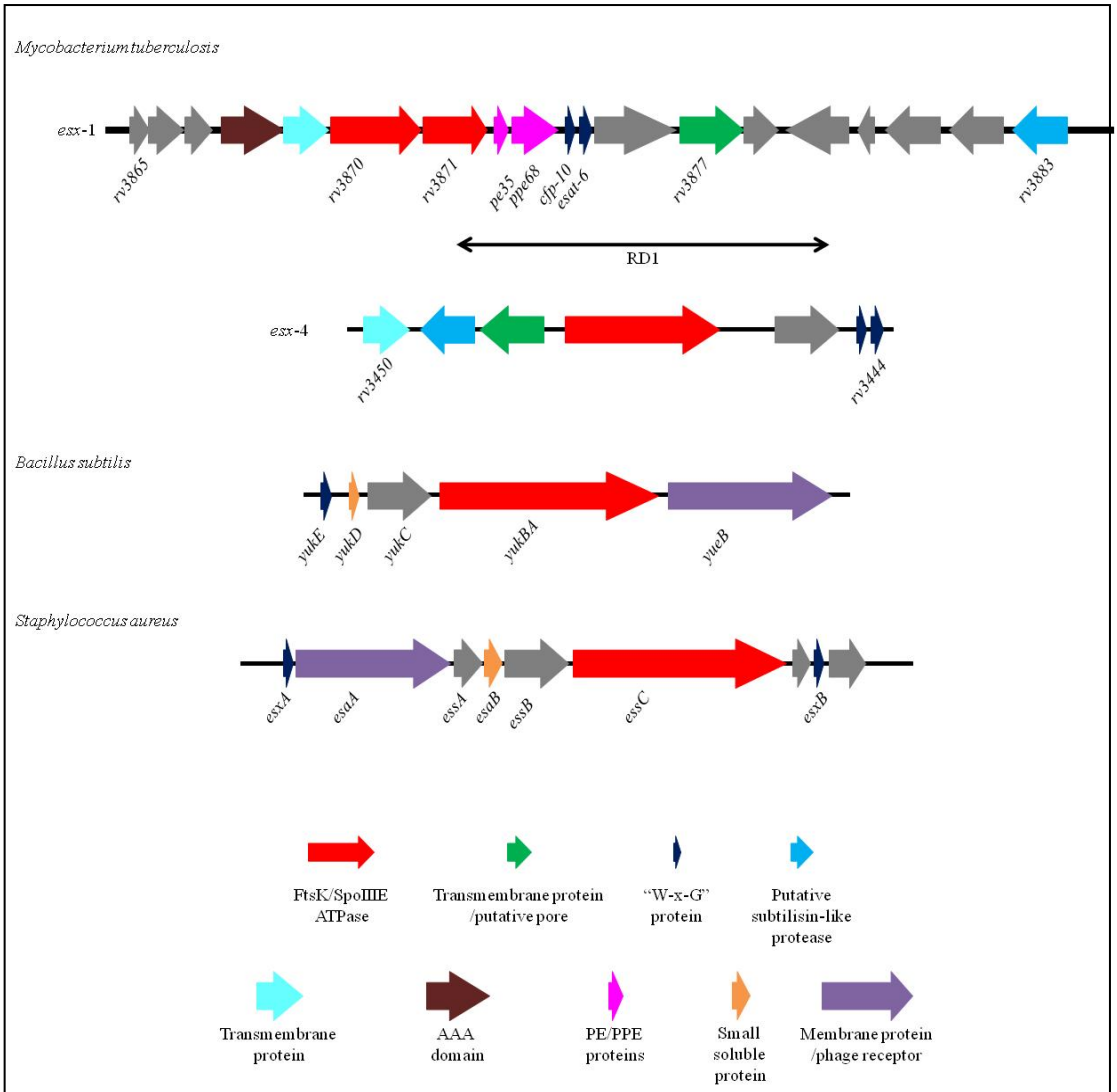

Figure 3.1: Arrangement of T7SS genes on the chromosome of various Gram-positive bacteria. Orthologous genes are shaded using the same colour. The double headed arrow below the *esx*-1 region of *M. tuberculosi*s indicates RD1, a syntenous region covering nine genes deleted from the chromosome of *M. bovis* in the attenuated BCG strain used as a vaccine against tuberculosis.

Genetic analysis of this and the surrounding region revealed that the individual genes *rv3870* and *rv3871* are required for secretion of ESAT-6 and CFP-10 (Stanley et al., 2003; Guinn et al., 2004). Since they have been shown to interact with each other Rv3870 and Rv3871 are thought to form a functional ATPase providing energy for secretion of the substrate proteins (Stanley *et al.*, 2003) and are both predicted by bioinformatic analysis to be FtsK/SpoIIIE domain (FSD) proteins. This class of proteins are well known for the role they play during the processes of cell division and sporulation where they are responsible for chromosomal segregation and transport of DNA into the appropriate daughter compartment (Ausmees *et al.*, 2007; Begg *et al.*, 1995; Burton *et al.*, 2007; Dedrick *et al.*, 2009; Wu & Errington, 1994; Wang *et al.*, 2006). It has since been shown that numerous genes in the vicinity of RD1 are also essential for secretion of both ESAT-6 and CFP-10. This region has become known as Esx-1 (Figure 3.1).

Proteins belonging to the ESAT-6/CFP-10 family (hereafter referred to WXG100 proteins owing to the presence of a characteristic WXG amino acid motif) are canonical substrate proteins of the system. Genes encoding such proteins in the vicinity of a gene encoding an FSD protein is indicative of the likely presence of a T7SS (Tekaia *et al.*, 1999; Gey van Pittius *et al.*, 2001; Pallen, 2002).

By far the most studied T7SS is Esx-1 of *M. tuberculosis*, owing to the massive impact this system has on pathogenesis. However, it has been noted that the genome of *M. tuberculosis* encodes 23 putative WXG100 proteins (Cole *et al.*, 1998) and a further four T7SS systems, named Esx-2 to Esx-5. Each of the five *esx* loci have genes coding for two putative WXG100 proteins genetically associated with genes coding for other components of the secretion apparatus. However, non-WXG100 substrate proteins of the T7SS have been identified that are encoded outside

of the Esx-1 locus but depend upon the machinery for secretion, for example EspA, encoded by *rv3616* (Fortune *et al.*, 2005). Interestingly, secretion of EspA is mutually dependent on the production and secretion of ESAT-6 and CFP-10 (Fortune *et al.*, 2005; MacGurn *et al.*, 2005). All but two of the additional WXG100 proteins that are encoded outside of *esx-1-5* have been functionally associated with one of the five Esx systems (reviewed by Bitter *et al.*, 2009).

Of the five T7SS loci of *M. tuberculosis*, *esx-1* encompasses 19 genes, the majority of which are required for secretion. By contrast, *esx-4* encodes the fewest components, with only seven genes (Figure 3.1). It has been proposed that based on the rather low number of genes in *esx-4* and on the order in which gene duplication events are thought to have arisen that the *esx-4*-encoded system is the most ancestral (Gey van Pittius *et al.*, 2001).

In addition to encoding genes for a pair of WXG100 proteins (shaded in dark blue, Figure 3.1), all of the *M. tuberculosis esx* loci code for a multi-transmembrane spanning protein that is thought to form a conduit through the membrane allowing passage of the substrate proteins (shaded in green, Figure 3.1), a subtilisin-like serine protease (shaded in light blue in Figure 3.1) and a membrane associated protein (shaded in turquoise, Figure 3.1). As mentioned previously, each system also comprises an FSD protein (shaded red, Figure 3.1). The FSD proteins of Esx-1 and Esx-5 are each encoded by two genes whereas in the case of Esx-2, Esx-3 and Esx-4 they are encoded by single genes.

Although commonly associated with the various *esx* loci in *M. tuberculosis*, genes coding for the PE/PPE proteins (named because of the conserved proline and glutamic acid residues in these proteins), and AAA+ ATPases as well as various transmembrane and soluble proteins appear to be region specific.

Early studies carried out in *M. tuberculosis* showed that deletion of *rv3870* and *rv3871* prevented secretion of ESAT-6 or CFP-10 (Stanley *et al*., 2003; Guinn *et al*., 2004; Brodin *et al*., 2006). Deletion of *rv3877* also abolished ESAT-6 and CFP-10 secretion (Stanley *et al*., 2003; Brodin *et al*., 2006), as did deletion of *rv3868* and *rv3869* (Brodin *et al*., 2006). The *rv3873* gene (encoding PPE68) and *rv3876* are not essential for secretion of ESAT-6 and CFP-10 (Brodin *et al*., 2006). Deletion of *rv3872* (PE35) led to reduced production of ESAT-6 and CFP-10 (Brodin *et al*., 2006).

ESAT-6 and CFP-10 are mutually dependent on each other for secretion (Guinn *et al*., 2004) which is in keeping with the fact that ESAT-6 and CFP-10 form a tight 1:1 complex (Renshaw *et al*., 2002). This was later explained through structural analysis of the ESAT-6/CFP-10 dimer, whereby interaction was found to take place through large hydrophobic faces on each of their surfaces, with dimerisation being necessary for stability of these proteins (Renshaw *et al*., 2005). The carboxy terminus of CFP-10 is unstructured and mutational studies of this region have shown that it is required for interaction with Rv3871, thus acting as an uncleaved C-terminal signal sequence. The signal sequence on CFP-10 was shown to direct both CFP-10 **and** ESAT-6 for export by the machinery, suggesting that they may be exported as a complex (and therefore as folded rather than unfolded proteins). Interestingly the seven amino acid stretch at the C-terminus of CFP-10 could be fused to the C-terminus of a heterologous protein which was subsequently secreted by the T7SS (Champion *et al*., 2006) and as such was the first targeting sequence for a T7SS substrate identified.

Comparison of the C-terminal 25 amino acid residues of CFP-10 to all predicted proteins produced by *M. tuberculosis* showed that EspC is the most similar

(DiGiuseppe Champion *et al.*, 2009). Like CFP-10 the last seven amino acid residues of this protein are required for secretion by the *esx*-1 system, however, these differ from the last seven amino acids of CFP-10 and direct interaction with an alternative AAA+ ATPase encoded in the region, Rv3868 (DiGiuseppe Champion *et al.*, 2009). Futhermore, multiple CFP-10 paralogues encoded in the genome of *M. tuberculosis* share only weak homology at their C-termini, differing in critical residues required for secretion of CFP-10 and are thus targeted for secretion independently of *esx-1* (Champion *et al.*, 2006). Low homology between substrate proteins of the T7SS coupled with the fact that the sequences required for recognition by an ATPase and subsequent secretion are very short (seven amino acids) has precluded efforts to define a consensus sequence required for secretion by this system.

Two studies, published almost simultaneously, described the first proteins encoded outside of the *esx-1* locus to be required for secretion of ESAT-6 and CFP-10 (MacGurn *et al*., 2005; Fortune *et al*., 2005). The three genes, *rv3616*, *rv3615* and *rv3614*, which were found to lie in an operon, are homologous to genes at the 3' end of the *esx-1* locus (*rv3864*, rv3865 and *rv3867*). Fortune *et al*. (2005) reported that a mutant in *rv3616* failed to secrete ESAT-6 and CFP-10 and that the gene product, EspA (Esx-1 secretion-associated protein A), is a substrate of the Esx-1 system that is mutually dependent on ESAT-6 and CFP-10 for secretion. A second study reported that Rv3614 and Rv3615 (later renamed EspD and EspC, respectively) are required for the secretion of ESAT-6 and CFP-10 through the Esx-1 T7S machinery (MacGurn *et al*., 2005). Although no evidence exists to indicate that EspD (encoded by *rv3614*) is itself a substrate of the Esx-1 system, EspC (encoded by *rv3615*) has since been shown to be secreted in an Esx-1-dependent manner (Millington *et al.*, 2011). Strains with mutations in each of the three genes showed attenuation in virulence similar to

strains with mutations in genes encoded at *esx-1* (MacGurn *et al*., 2005; Fortune *et al*., 2005). Interestingly, however, strains lacking either of the two proteins Rv3865 (EspF) and Rv3866 ($EspG_1$) encoded in the 3' region of *esx-1* that shows homology to the *rv3616-3615-3614* operon, were not affected for secretion of ESAT-6 and CFP-10 despite being severely attenuated in virulence, (Bottai *et al*., 2011).

Another study identified EspB (encoded by *rv3881*) as a substrate protein of the Esx-1 systems of both *M. tuberculosis* and *M. marinum* (McLaughlin *et al*., 2007). Although the ATPase Rv3871 is essential for secretion of EspB, in contrast to the other known substrates of the system CFP-10 is not required for EspB secretion (McLaughlin *et al*., 2007).

EspR (encoded by *rv3849*) is the first protein secreted by Esx-1 for which a function has been assigned. It has been shown that the protein functions as a transcriptional regulator of the *rv3616-rv3614* operon (Raghavan *et al*., 2008). Presumably the secretion of this transcription factor is a mechanism to regulate its activity, in an analogous manner to the secretion of the FlgM anti-sigma factor through the flagellar basal body controlling the final stages of flagella biosynthesis in *Salmonella* (Karlinsey *et al*., 2000). Indeed, given that at least one product of the *rv3616-rv3614* operon is a substrate of the Esx-1 system that is required for secretion of the other components (Fortune *et al*., 2005) this has been shown to be a means by which activity of the Esx-1 system is directly controlled by a negative feedback mechanism (Raghavan *et al*., 2008).

In summary, six proteins (ESAT-6, CFP-10, EspA, EspB, EspC and EspR) have been identified so far as being secreted in an Esx-1-dependent manner. Many of the genes encoded in the *esx-1* region are required for secretion and most of the substrates have been shown to be mutually dependent on each other for secretion.

Additionally, where examined, loss of secretion generally results in a decrease in virulence, consistent with loss of pathogenicity seen for an RD1 mutant. Two models proposed for the operation of the T7SS, based on conserved components across each of the Esx systems encoded by *M. tuberculosis* and protein-protein interaction studies, are shown in Figure 3.2.

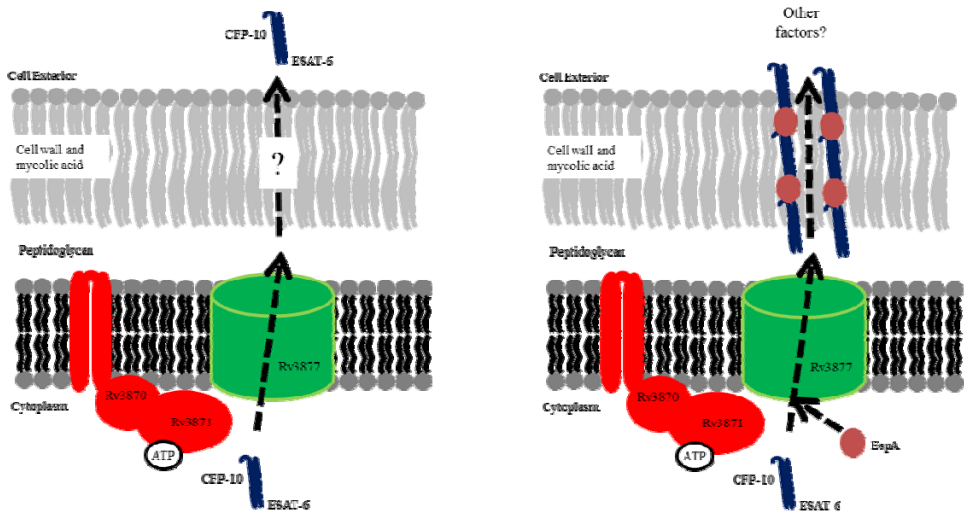

Figure 3.2: Proposed models for operation of the T7SS in *M. tuberculosis* (adapted from Ize and Palmer, 2006).
The model on the left proposes that the ESAT-6/CFP-10 dimer (dark blue) interacts with the ATPase formed by Rv3870 & Rv3871 (in red) and is transported through a pore thought to be formed by Rv3877 (in green) in an ATP-dependent manner. These are then transported through the cell wall to the exterior by an unknown mechanism.
The alternative model on the right proposes that ESAT-6 and CFP-10 are secreted alongside other known substrate proteins e.g. EspA, and form a conduit through the cell wall. In this model ESAT-6/CFP-10/EspA are considered as external structural components of the secretion apparatus whose correct localization is required for the secretion of unknown substrate proteins.

3.1.1.1 A role for the Mycobacterial T7SS in DNA transfer?

Two studies carried out in *M. smegmatis* have shown, through random transposon insertion mutagenesis, that multiple genes coding for components of the

Esx-1 system in this organism play a role in conjugal DNA transfer. This was first observed when insertions that resulted in a hyperconjugative phenotype of the donor strain mapped to multiple genes syntenous to those from the RD1 of *M. tuberculosis* (Flint et al., 2004). The authors were able to show that a plasmid containing the *esx-1* region of *M. tuberculosis* could complement the mutants, indicating that the genes in these two organisms perform similar functions. This has given rise to the notion that perhaps the Esx-1 system is directly involved in translocation of DNA. Given that a central component of the secretion apparatus is an ATPase belonging to the FtsK/SpoIIIE family of proteins, well known for their roles in DNA translocation (Burton *et al*., 2007; Sherratt *et al.*, 2010; Possoz *et al*., 2001), this would not be surprising. However, a plausible explanation, supported by the fact that the mixture of wild-type donors with hyperconjugative *esx-1* mutant donors successfully reduces transfer frequency from the mutant strain (Flint *et al*., 2004), is that the Esx-1 system secretes a protein that is involved in suppressing conjugation.

The second study, carried out by the same group, employed a similar transposon mutagenesis screen in a recipient strain. In this study the authors found that mutations also mapped to many regions within the *esx-1* locus but that these mutations abolished transfer of DNA to the recipient strain (Coros *et al*., 2008). Some of these mutations mapped to the same genes as the previous study, however, several were also found to map to new genes. These mutations were transferred into the donor strain and found to perform recipient–specific functions. The authors also examined secretion of ESAT-6 in the transfer-deficient mutant strains and found that, as expected, secretion was abolished in many of the mutants. However, three mutations were found that prevent DNA transfer but not ESAT-6 secretion. One of these genes is a homologue of EspB, a substrate of the Esx-1 system that is secreted

independently of other substrates (McLaughlin et al., 2007). This suggests a dual role for the Esx-1 system, with one pathway being required solely for secretion of proteins, such as ESAT-6/CFP-10 and a second pathway required for DNA transfer.

3.1.2 T7SS in the *Firmicutes*

Although it has been shown that the genomes of many Gram positive bacteria, including those from the *Firmicutes* phylum such as *Bacillus subtilis*, *Bacillus anthracis*, *Listeria monocytogenes*, and *Staphylococcus aureus*, code for FSD proteins in close proximity with ESAT-6-like proteins, indicating the presence of a T7SS (Pallen, 2002), relatively few studies have been carried out on T7SS in these organisms. The arrangement of genes coding for the *S. aureus* T7SS can be seen in Figure 3.1. Although the T7SS-encoding loci of *Mycobacteria* and *Staphyloccus* code for an FSD protein (shaded red, Figure 3.1), and two WXG100 proteins (shaded dark blue, Figure 3.1), it is evident that other than this, little similarity exists between the genes coded in the *esx* loci of *M. tuberculosis* and *S. aureus*. .

However, it can be seen that there are a number of apparently *Firmicutes*–specific genes encoded in the T7SS loci. Comparing these genomic regions of *S. aureus* and *B. subtilis*, it can be seen that besides encoding an FSD protein and WXG100 proteins, both loci encode a membrane protein (shaded lilac, Figure 3.1), that, in the case of *Bacillus subtilis,* has been shown to be a phage receptor (São-José *et al.*, 2004). A small soluble protein of unknown function is also conserved between the two species (shaded orange, Figure 3.1). In *B. subtilis* only one WXG100 protein, YukE, appears to be encoded in the region. Given that these proteins have been shown to form heterodimers (Renshaw *et al.*, 2005), it could be that this protein forms a homodimer, as has been reported for some WXG100 family proteins (Tanaka *et al.*,

2007; Shukla *et al.*, 2010). Indeed the EsxA protein from *S. aureus* crystallises as a homodimer (Sundaramoorthy *et al*., 2008).

The first study into any T7SS in the low-GC Gram-positive bacteria was carried out in the important human pathogen *S. aureus* (Burts *et al.*, 2005). In this study several insertion mutant strains were constructed in genes thought to encode components of a T7SS (Figure 3.1). The results showed that both of the WXG100 proteins (EsxA and EsxB) were secreted, and that this was dependent on the presence of EssC (the FSD protein) and on two other membrane proteins, EssA and EssB. Secretion of EsxA and EsxB was found to be mutually dependent, which is similar to observations made in *M. tuberculosis*. However, two genes in the region, *esaA* and *esaB*, encoding a multi-transmembrane spanning protein and a small soluble protein, respectively, were not required for secretion of EsxA and EsxB. The system was also reported to be required for full virulence, with *esxA*, *esxB* and *essC* mutant strains showing a reduction in abscess formation in a murine model for abscess formation (Burts *et al*., 2005). Later studies identified further substrate proteins, EsaC and EsaD, that contribute to virulence and which are encoded in the same locus (Burts *et al*., 2008; Anderson *et al*., 2011). EsaD was also shown to be required for secretion of EsxA (Anderson *et al*., 2011).

One gene that is conserved in the T7SS loci of many *Firmicutes* codes for a membrane protein that in *B. subtilis* acts as a phage receptor (São-José *et al*., 2004). This gene is termed *yueB* in *B. subtilis* (*esaA* in *S. aureus*), and the genes encoding putative components of the *B. subtilis* T7SS (Figure 3.1; Pallen, 2002) are found upstream of *yueB* and have been shown to form an operon with it (São-José *et al*., 2004). YueB forms fibers that span the peptidoglycan layer and the protein has been shown to trigger phage DNA ejection *in vitro* (São-José *et al*., 2006). It is possible

that the function of the putative T7SS in *B. subtilis* is not related to the role of YueB as a phage receptor. However, it is tempting to speculate that the T7SS in the *Firmicutes* plays a role in phage recognition and infection, perhaps through mediating translocation of phage DNA into the bacterial cytoplasm.

3.1.3 Aims of this chapter

In 2001, Gey van Pittius and colleagues (Gey van Pittius *et al*., 2001) identified a gene cluster in the genome sequence of *S. coelicolor* that contained orthologues of conserved T7SS genes from *M. tuberculosis* (Figure 3.1). This observation strongly indicates that a T7SS should be present in *S. coelicolor*, but at the time this study was initiated no role for the T7SS in any streptomycete had been assigned. Therefore the aim of the work in this chapter was to characterise Type VII protein secretion in *S. coelicolor*, specifically to determine whether the system was functional, its role in *S. coelicolor* physiology and to identify substrate proteins of the machinery. During the course of the work carried out here, a paper was published describing a partially overlapping study on T7S in *S. coelicolor* (Akpe san Roman *et al*., 2010). This overlapping study will be discussed in detail in Section 3.7 of this chapter.

RESULTS

3.2 Bioinformatic analysis of T7SS genes of *S. coelicolor*

It has previously been reported that the genome of *S. coelicolor* encodes components of a single T7SS (Gey van Pittius *et al.*, 2001). The authors noted that the genes in this region are most similar to those of the *esx*-4 region of *M .tuberculosis* in that fewer components are encoded than in the *esx*-1, -2, -3 or -5 systems of *M.*

tuberculosis and that these share the highest percentage of similarity to those encoded by the *esx*-4 locus. The arrangement of these genes encoding components of a T7SS on the chromosome of *S. coelicolor* can be seen immediately below the genes encoding components of the Esx-4 system from *M. tuberculosis* in Figure 3.3. Each of these loci encodes an FtsK/SpoIIIE ATPase (FSD) protein (*sco5734;* shaded red, Figure 3.3), a transmembrane protein that is the putative pore protein of the T7SS (*sco5735*; shaded green, Figure 3.3), a membrane protein *(sco5721*; shaded turquoise, Figure 3.3) and two WXG100 proteins (*sco5724* and *sco5725*; shaded dark blue, Figure 3.3). In addition, the T7SS locus of *S. coelicolor* encodes two putative subtilisin-like proteases, whereas *esx*-4 encodes only one (*sco5722* and *sco5731*; shaded light blue, Figure 3.3).

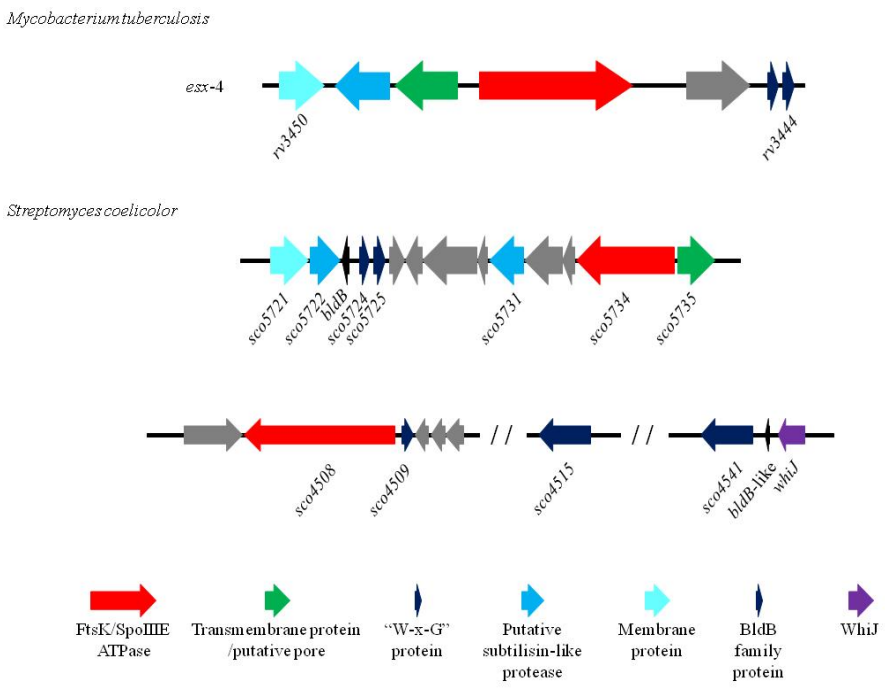

Figure 3.3: Arrangement of genes encoding the Esx-4 T7SS on the chromosome of *M. tuberculosis* and the T7SS genes on the chromosome of *S. coelicolor*. Orthologous genes are shaded using the same colour.

Given that the chromosome of *M. tuberculosis,* as well as other mycobacterial species, encode five separate T7SS it was deemed plausible that the genome of *S. coelicolor* encodes components of more than one T7SS. With a view to identifying these, PSI-BLAST analysis was performed (default settings were used for this and subsequent BLAST analyses described in this thesis) using the amino acid sequence of Sco5734, the FSD protein previously identified by Gey van Pittius *et al.* (2001), as a query. The closest paralogue is Sco4508, sharing 30 % identity and 46 % similarity over 57% of the protein, which is annotated as being similar to YukA from *B. subtilis*. Interestingly, YukA has previously been suggested as a component of a T7SS (Pallen, 2002) which infers that Sco4508 may also be a component of a second T7SS encoded by the genome of *S. coelicolor*. PSI-BLAST analysis was carried out using other previously identified T7SS proteins encoded in the *sco5734* locus (Figure 3.3) but failed to uncover any further paralogues encoded in the region of *sco4508*. Likewise, when T7SS components from *M. tuberculosis* and *S. aureus* were used for PSI-BLAST analysis this also failed to uncover any homologous proteins encoded in this region.

Genes encoding WXG100 proteins often cluster with those encoding components of the secretion apparatus, such as the FSD protein (Pallen, 2002). The WXG100 proteins are so named as they are approximately 100 aa residues in length and contain a distinctive W-X-G motif (or a structurally analogous alternative) near the mid-point of the protein. This causes a turn in the protein that is otherwise largely α-helical in structure. Based on these features, proteins belonging to the PFAM 06013 can be identified during BLASTP analysis. However, previous reports have highlighted that primary sequence similarity of the WXG100 proteins is very low, so visual inspection of the genetic region surrounding *sco4508* was carried out in order

to identify genes of approximately 250-400 nucleotides that may encode proteins of the WXG100 family. Translated protein sequences of candidate genes were analysed using BLASTP, leading to identification of a protein of 124 aa belonging to the WXG100 family, Sco4509.

As WXG100 proteins are also often encoded in adjacent pairs on the chromosome, the translated protein sequences of further candidate genes from the region surrounding *sco4509* were analysed using PHYRE (Kelley and Sternberg, 2009), a web-based program that is able to predict tertiary protein structure based on the primary sequence. However, no further WXG100 proteins were identified. The arrangement of the newly discovered T7SS gene cluster on the chromosome of *S. coelicolor*, comprising *sco4508* and *sco4509*, can be seen in Figure 3.3.

In an effort to further identify WXG100 proteins encoded in the *S. coelicolor* genome PSI-BLAST analysis was performed using YukE from *B. subtilis,* a WXG100 protein encoded in the same genetic locus as YukA (Figure 3.1). Although several hits were obtained, these were only weakly homologous to YukE so were initially disregarded, with the exception of Sco5724 that was already known to be a member of the WXG100 family of proteins (Gey van Pittius *et al.*, 2001). However, it was later discovered that Sco4541, a protein of 526 amino acid residues, has a WXG100 domain at its N-terminus. Although this protein exceeds the original criteria pertaining to mass of proteins belonging to the WXG100 family, it should be noted that WXG100 proteins of a similar size have been reported in *B. anthracis* (Garufi *et al.*, 2008). Interestingly, the WXG100 proteins Sco5724 and Sco5725 are encoded next to the *bldB* gene, whilst Sco4541 is encoded next to a *bldB* homologue (*sco4542;* shaded black, Figure 3.3) and WhiJ (*sco4543*; shaded purple, Figure 3.3). Using the sequence of Sco4541 a PSI-BLAST analysis was performed leading to the discovery

of Sco4697 (a protein of 604 aa) and Sco4515 (a protein of 401 aa) both containing possible WXG100 domains at their N-terminus. Given that two of these proteins are encoded outside of T7SS loci it raises the possibility that the genome of *S. coelicolor* may encodes further proteins containing WXG100 domains.

The original PSI-BLAST analysis that was performed using Sco5734 also identified FtsK homologues that had lower sequence identity than Sco4508. Of these, Sco5750 has been assigned a role in genetic stability of the chromosome during sporulation (Wang *et al*., 2007) and analysis of other proteins encoded in the region did not support the presence of a T7SS. Similarly, analysis of the genomic context of genes coding for other homologues with weaker sequence identity did not reveal genes for any further components of a T7SS.

3.3 Genetic inactivation of the putative T7SSs of *S. coelicolor*

In order to achieve targeted gene deletion in the streptomycetes, a number of obstacles posed by various physical characteristics of this genus must be overcome. The cell walls of many Gram-positive bacteria are composed of a thick peptidoglycan layer which hinders DNA introduction into cells by chemical means or electroporation. Digestion of the cell wall using lysozyme results in the formation of protoplasts which can then be transformed with DNA. However, protoplasting of *Streptomyces* cells is a harsh process which can trigger chromosomal DNA rearrangements and other methods for introducing DNA are preferable (Dr David Widdick, personal communication). Probably the most efficient and preferred method of introducing DNA into the streptomycetes relies on conjugal DNA transfer from an *E. coli* donor strain.

The REDIRECT© Technology, developed by Gust and colleagues (2002), takes advantage of the λRED method pioneered by Datsenko and Wanner (2000) to replace chromosomal genes with a selectable marker which is generated by PCR. In this approach, production of the λRED recombinase protein in the strain of interest results in a greatly increased frequency of homologous recombination and allows recombination to occur even if only short stretches of homology (>38 consecutive nucleotides) exist between the PCR product and the flanking regions of the gene to be disrupted.

For this procedure, the *S. coelicolor* gene of interest is initially disrupted on a cosmid clone from an ordered cosmid library which was constructed to assist with genome sequencing of *S. coelicolor* (each cosmid generally carries around 46 kB of contiguous genomic DNA; Redenbach *et al.*, 1996). This procedure is carried out in an *E. coli* strain harbouring both the cosmid encoding the gene to be replaced as well as a plasmid encoding the λRED recombinase enzyme that promotes recombination of linear DNA. The disruption cassette, which is introduced into the strain as a linear PCR product, encodes an antibiotic resistance marker that can be selected in both *E. coli* and *Streptomyces*, and also includes *oriT* (RK2) for introduction of the mutated cosmid into *Streptomyces* by conjugation.

The mutated cosmid is introduced into an *E. coli* strain harbouring the non-transmissible plasmid pUZ8002, which allows *oriT*-containing vectors to be mobilised *in trans*. As most streptomycetes possess a methyl-sensitive restriction system, which serves to efficiently degrade any methylated DNA that is introduced, the *dam*$^-$ and *dcm*$^-$ *E. coli* strain ET12567 was used as the donor strain for conjugal DNA transfer.

This methodology was used to construct a chromosomal *sco4508* mutant and adapted for construction of a *sco5734* mutant in *S. coelicolor*, as described below.

3.3.1 Chromosomal deletion of *sco4508*

Initially *sco4508* was selected for disruption, and the deletion marked with an apramycin resistance cassette. To this end, PCR amplification of the disruption cassette, FRT-*oriT*-*aac*(3)*IV*- FRT, (using pIJ773 as a template) was carried out using primers "Sco4508 KO Fwd" and "Sco4508 KO Rev" (Table 2.6) and the PCR product was purified away from the template DNA by agarose gel electrophoresis as described in Section 2.3.1.1.

The *S. coelicolor* chromosomal region encoding *sco4508* is present on cosmid clone StD35, which was subjected to PCR-targeted gene replacement as described in Section 2.3.1.2. The mutated cosmid was then transferred from *E. coli* to *S. coelicolor* M145 by conjugation, as described in Section 2.3.1.3, after which it integrated into the chromosome at the *sco4508* genomic region by homologous recombination. Double-crossover mutants were identified by replica plating, selecting for apramycin resistance (specified by the disruption cassette) and kanamycin sensitivity (from the cosmid backbone) as described in Section 2.3.1.4. A schematic representation of this procedure can be seen in Figure 3.4. The resultant strain was designated JKFSCO1.

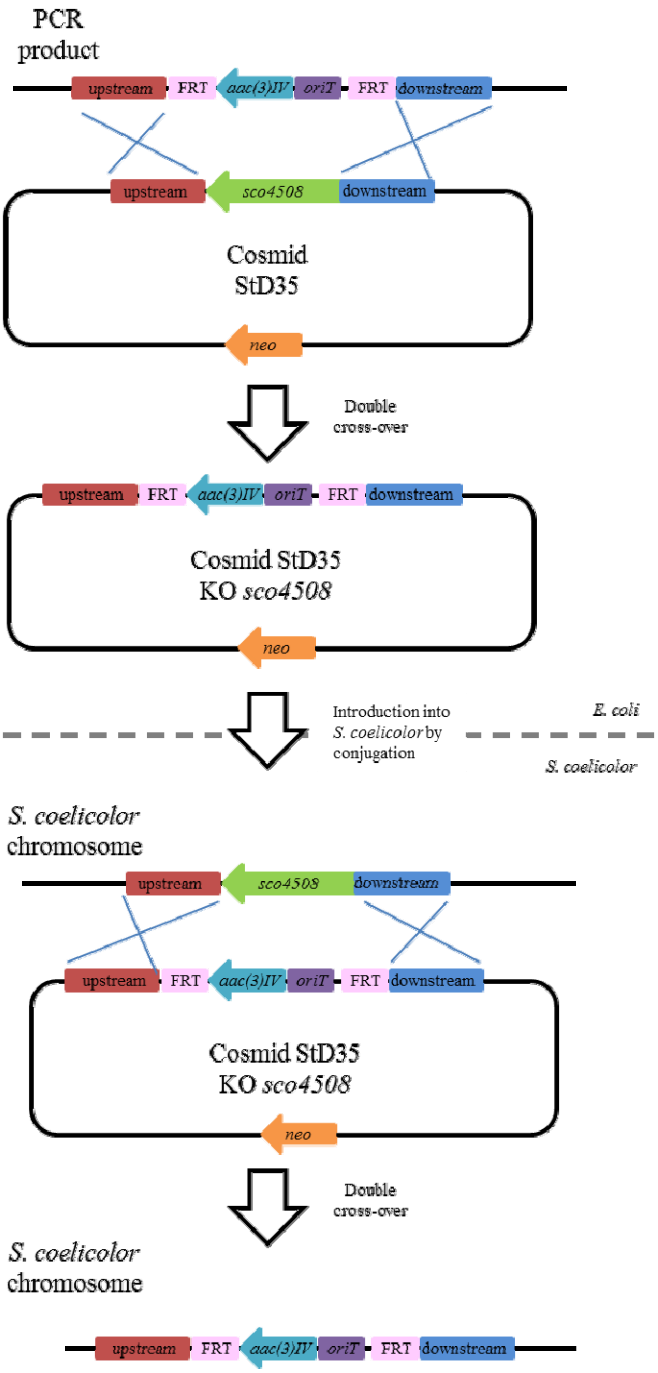

Figure 3.4: Schematic representation showing deletion of the gene *sco4508* from the chromosome of *S. coelicolor*. A construct was prepared in *E. coli* replacing the cosmid-encoded *sco4508* sequence with the PCR-amplified disruption cassette, FRT-*oriT*-*aac*(3)*IV*- FRT by PCR-targeted mutagenesis. This construct was then transferred into *S. coelicolor* by conjugation where the chromosomal copy of *sco4508* was replaced with FRT-*oriT*-*aac*(3)*IV*- FRT following homologous recombination. Screening was carried out for kanamycin sensitivity confirming that the disruption cosmid had been lost.

3.3.2 Chromosomal deletion of *sco5734*

Although the ordered *S. coelicolor* cosmid library originally contained clones covering the entire genome, some of these have since been lost, and unfortunately cosmid St3C3, covering the *S. coelicolor* chromosomal region encoding *sco5734* was one of these. Therefore, in order to construct a *sco5734* deletion strain it was initially necessary to construct, *de novo,* a vector containing regions flanking *sco5734* around a selectable marker.

Two synthetic constructs were purchased (Biomatic corporation) that contained 1.5 kb of sequence from the chromosomal region either immediately upstream or downstream of *sco5734*, and these were named pSCO5734UF and pSCO5734DF, respectively. These constructs were designed to allow the downstream region of *sco5734* to be cloned into pDWU5 (Table 2.5) using the *Bam*HI and *Eco*RI sites, followed by the upstream region of *sco5734* using the *Eco*RI and *Hind*III sites. Finally the FRT- *oriT*-*aac*(3)*IV*- FRT disruption cassette was released from pDrive+Apra (Table 2.5) by digestion with *Nde*I and *Eco*RI and cloned between the *sco5734* upstream and downstream regions present in pDWU5 using the same restriction sites.

The construct was then transferred to from *E. coli* to *S. coelicolor* M145 by conjugation, as described in Section 2.3.1.3, after which it integrated into the chromosome at the *sco5734* genomic region by homologous recombination. Double-crossover mutants were identified by replica plating as described in Section 2.3.1.4. The resultant strain was designated JKFSCO2.

3.3.3 Combined chromosomal deletion of *sco4508* and *sco5734*

In order to construct a strain that was deleted for genes encoding both of the putative ATPases of the *S. coelicolor* T7SS, it was necessary to use a second antibiotic resistance marker. Therefore a strategy was devised whereby the deletion of *sco4508* could be marked with a hygromycin resistance cassette. To this end PCR amplification of the disruption cassette, FRT-*oriT*-*hyg*-FRT (specifying hygromycin resistance and using plasmid pIJ10700 as a template) was carried out using primers "Sco4508 KO Fwd" and "Sco4508 KO Rev" (Table 2.6) and purified away from the template DNA by agarose gel electrophoresis as described in Section 2.3.1.1.

As mentioned above, the *S. coelicolor* chromosomal region encoding *sco4508* is present on the cosmid clone StD35, and this was subjected to PCR-targeted gene replacement with the hygromycin resistance cassette as described in Section 2.3.1.2. The mutated cosmid was then transferred from *E. coli* to strain JKFSCO2 by conjugation, as described in Section 2.3.1.3, after which it integrated into the chromosome at the *sco4508* genomic region by homologous recombination. Double-crossover mutants were identified by replica plating as described in Section 2.3.1.4, selecting for hygromycin resistance and kanamycin sensitivity (to ensure the vector backbone was lost). This strain was designated JKFSCO3.

3.3.4 Verification of *sco4508* and *sco5734* mutant strains by PCR analysis

To verify chromosomal deletion of *sco4508* and *sco5734*, chromosomal DNA was prepared from each of the strains, along with the parental strain, as described in Section 2.2.3, and this was used as a template in PCR reactions, using oligonucleotides that prime within the deleted genes (thus ensuring deletion of the targeted gene).

The presence of *sco4508* on the chromosome of the wild-type strain, *S. coelicolor* M145, was confirmed using oligonucleotide primers Sco4508inside_fwd and Sco4508inside_rev, which generates a product of 496 bp (Figure 3.5, Lane 2). Likewise, the presence of *sco5734* on the chromosome of the wild-type strain was confirmed using oligonucleotide primers Sco5734inside_fwd and Sco5734inside_rev, generating a product of 502 bp (Figure 3.5, Lane 3)

The absence of *sco4508* from the chromosome of strain JKFSCO1, was confirmed using oligonucleotide primers Sco4508inside_fwd and Sco4508inside_rev, which, as expected, generated no product (as the primer internal to the gene cannot prime in the deletion strain; Figure 3.5, Lane 4). To ensure that the PCR reaction failed due to the absence of the gene in the tested strain, a control reaction was also carried out using oligonucleotide primers Sco5734inside_fwd and Sco5734inside_rev, which, as expected, generated a product of 502 bp (Figure 3.5, Lane 5).

The absence of *sco5734* from the chromosome of strain JKFSCO2 was confirmed using oligonucleotide primers Sco5734inside_fwd and Sco5734inside_rev. This primer pair, as expected if the gene is deleted, generated no product (Figure 3.5, Lane 7). Again to ensure that the reaction failed due to the absence of the gene in the tested strain, a control reaction was also carried out using oligonucleotide primers Sco4508inside_fwd and Sco4508inside_rev, which, as expected, gave a product of 496 bp (Figure 3.5, Lane 6).

Finally, the absence of *sco4508* from the chromosome of strain JKFSCO3 was confirmed using oligonucleotide primers Sco4508inside_fwd and Sco5734inside_rev, which as expected generated product (Figure 3.5, Lane 8). Likewise, absence of *sco5734* from the chromosome of strain JKFSCO3 was confirmed using oligonucleotide primers Sco5734inside_fwd and Sco5734inside_rev, again yielding

no product (Figure 3.5, Lane 9). To ensure that the reaction failed due to the absence of the tested genes in this strain a control reaction was also carried out using oligonucleotide primers Sco5724H6_fwd and Sco5725H6_rev, which as expected generated a product of 760 bp (Figure 3.5, Lane 10).

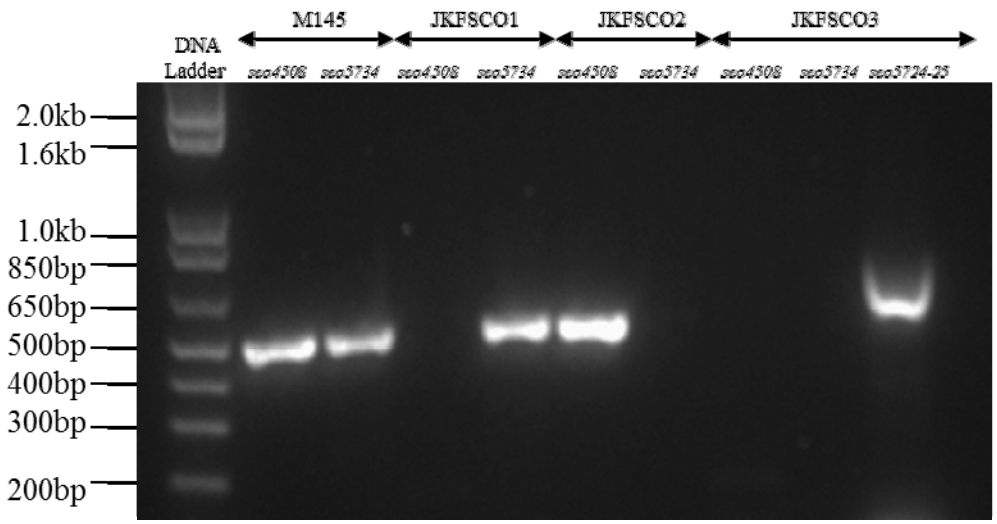

Figure 3.5: Confirmation of *S. coelicolor sco4508* and *sco5734* chromosomal deletion strains using diagnostic PCR. The legend above the agarose gel indicates the samples loaded in each lane. The top line denotes the strain from which chromosomal DNA was derived for use as a template in each PCR reaction. The second line denotes the gene(s) detected by each primer pair.

3.4 Phenotypic analysis of *S. coelicolor* T7SS mutant strains

A number of phenotypic changes have been associated with loss of secretion systems in bacteria. Of particular relevance to this study deletion of genes encoding components of the Tat translocase in the streptomycetes has been shown to have drastic phenotypic effects such as a reduced growth rate, delayed sporulation and

small colony morphology (Schaerlaekens *et al.*, 2004; Widdick *et al.*, 2006; Joshi *et al.*, 2010).

Deletion of Esx loci from *M. tuberculosis* has also been associated with phenotypic changes. The original BCG strain was infact isolated due to differences in colony morphology that can be directly attributed to a loss of T7S activity as complementation with the Esx-1 region has been shown to restore wild-type morphology (Pym *et al.*, 2002). Futhermore, loss of the Esx-3 T7SS from *M. tuberculosis* resulted in a strain that is defective in iron acquisition (Siegrist *et al.*, 2009). As the function(s) of the T7SSs of *S. coelicolor* are unknown it is possible that phenotypic changes will be evident following deletion of genes encoding components of the system.

3.4.1 Growth analysis of *S. coelicolor* T7SS mutant strains on solid media

S. coelicolor T7SS mutant strains were cultured alongside the parental strain M145 on nine different types of solid media and inspected periodically over the course of one week in order to visually assess their growth rate, morphology, production of pigmented antibiotics and sporulation pattern.

Three of the media utilised in this analysis (SFM - soya flour mannitol agar, OBM - oat bran medium and IPM - instant potato mash agar) containing plant-derived materials and are routinely used in our laboratory to achieve sporulation of *Streptomyces* strains. Difco nutrient agar (DNA) is a rich medium that is widely used in laboratories and, in the case of *S. coelicolor*, induces fast, non-sporulating growth. YME (yeast extract malt extract agar) is also a rich medium that is routinely used in laboratories working with *Streptomyces*.

R2 and R2YE are minimal media commonly used to assess production of the blue pigmented actinorhodin (Act) and red pigmented undecylprodigiosin (Red), two antibiotics produced by *S. coelicolor* whose production is growth phase-dependent. Minimal medium (MM), supplemented with either mannitol or glucose, was also utilised to assess either sporulation or antibiotic production, respectively.

An equal number of spores derived from each strain were applied to the surface of the growth media and streaked out with an inoculation loop to cover each section of the plate. The plates were then incubated at 30°C and observed daily over the course of a week to look for phenotypic differences between the wild-type and mutant strains cultured on each media. Pictures were then taken of growth on the surface of the agar following seven days incubation, which can be seen in Figure 3.6.

It is clear that on any of SFM (Figure 3.6A), OBM (Figure 3.6B), IPM (Figure 3.6C), MM+1 % mannitol (Figure 3.6F) and YME (Figure 3.6I) media each of the mutant strains showed a growth and sporulation pattern that was indistinguishable from the wild type. Furthermore, on R2YE (Figure 3.6D), R2 (Figure 3.6E), MM+1 % glucose (Figure 3.6G) and YME (Figure 3.6I) media, production of the pigmented antibiotics were also apparently unaffected by deletion of the *sco4508* and/or *sco5734* genes. On DNA medium (Figure 3.6H) a wild-type pattern of growth was also displayed by each of the mutant strains.

These observations clearly demonstrate that the growth pattern, colony morphology, production of pigmented antibiotics and sporulation pattern of the *S. coelicolor* strains harbouring mutations in genes coding for the putative T7SS are indistinguishable from the wild-type strain, at least on the growth media and conditions tested here.

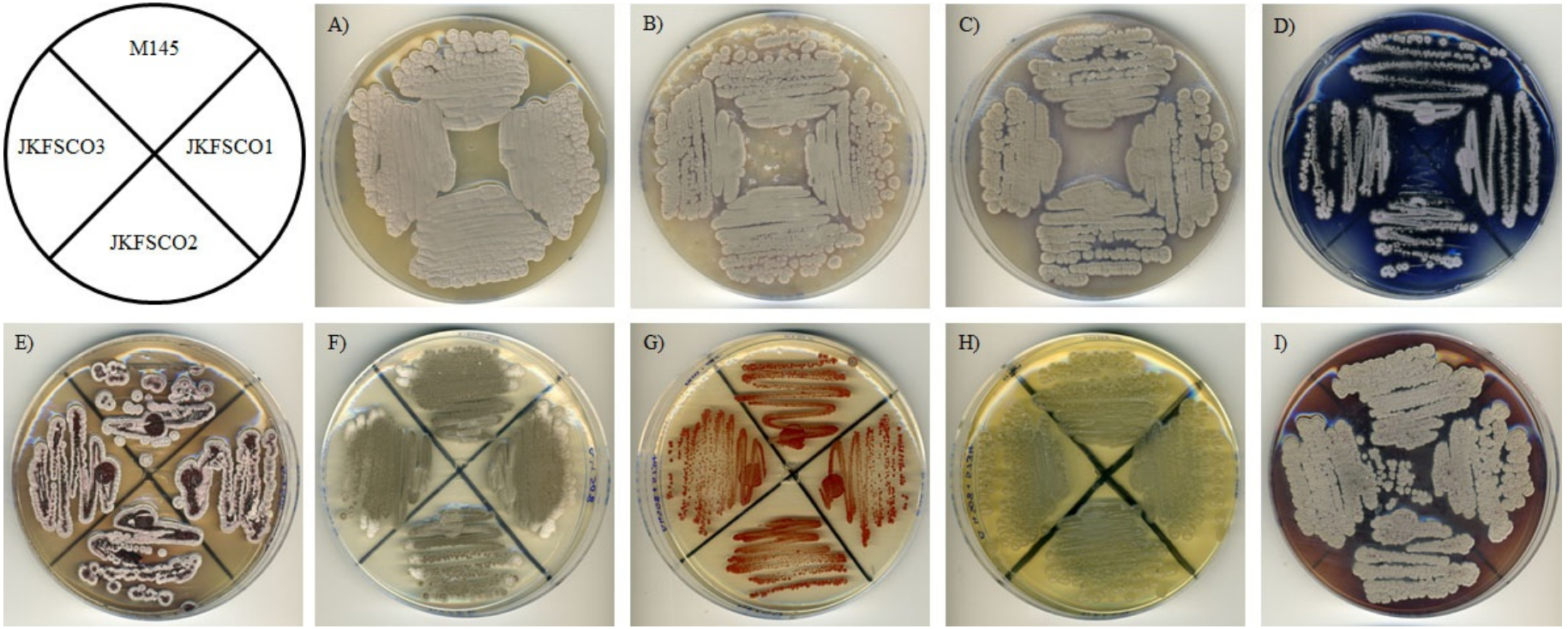

Figure 3.6: Growth analysis of *S. coelicolor* M145, JKFSCO1, JKFSCO2 and JKFSCO3 strains cultured on solid media and incubated at 30°C for one week. Equal numbers of spores were streaked from glycerol stock and grown on the different types of solid media: SFM (A), OBM (B), IPM (C), R2 (D), R2YE (E), MM+1 % mannitol (F), MM+1 % glucose (G), DNA (H) and YME (I). The pictures shown represent five independent experiments.

3.4.2 Growth analysis of *S. coelicolor* T7SS mutant strains in liquid culture

It has been reported previously that mutations in genes coding for components of protein secretion systems in bacteria lead to, either directly or indirectly, growth defects and membrane instability (Schaerlaekens *et al*., 2001; Schaerlaekens *et al*., 2004; Sibbald *et al*., 2010; Sikora, *et al.*, 2007; Söderberg, *et al.*, 2004; Widdick *et al*., 2006; Joshi *et al*., 2010; Garces *et al*., 2010). I therefore sought to determine whether mutants in the putative T7SS of *S. coelicolor* displayed a difference in growth rate when compared to the parental strain M145.

One method commonly used to assess bacterial growth rate is to culture the strain in liquid medium and measure the absorbance of the culture, using a spectrophotometer set at 600 nm, at various time intervals following inoculation. In the case of most bacteria, absorption readings correlate directly with cell density allowing production of growth curves from the data collected over the time-course. However, *S. coelicolor* displays non-dispersed growth as mycelial pellets in liquid culture that undergo a process of hyphal death leaving the cell wall intact (Miguélez *et al*., 1999). Both of these features negate the usefulness of absorbance readings in growth rate analysis for many streptomycetes.

In order to circumvent these factors, measurement of total cytosolic protein was used to assess the growth rate of *S. coelicolor* strains grown in liquid culture. TSB medium was inoculated with a spore stock and incubated and sampled as described in Section 2.1.4. At regular time intervals, samples were withdrawn, total cytosolic protein was prepared and its concentration assessed, as described in Section 2.5.1. Total cytosolic protein concentration values correlate with growth of *S. coelicolor* allowing production of growth curves from the data collected over the

time-course. The results of growth rate analysis of the *S. coelicolor* strains M145, JKFSCO1, JKFSCO2 and JKFSCO3 are presented in Figure 3.7.

It can clearly be seen that each mutant strain displayed a similar growth pattern to the wild-type. Growth became detectable at 15 hours post inoculation, marking entry into logarithmic growth phase, and reached a peak at 30 hours post inoculation after which a decrease in total cytosolic protein levels was observed. The reason for this is unclear although it could be speculated that exhaustion of nutrients in the culture may result in lysis of hyphae to release nutrients to sustain growth (Miguélez *et al*., 1999).

It can be concluded from the results presented here that the presence of the FSD protein homologues, Sco4508 and/or Sco5734, of the putative T7SS are not essential for normal laboratory growth of *S. coelicolor*.

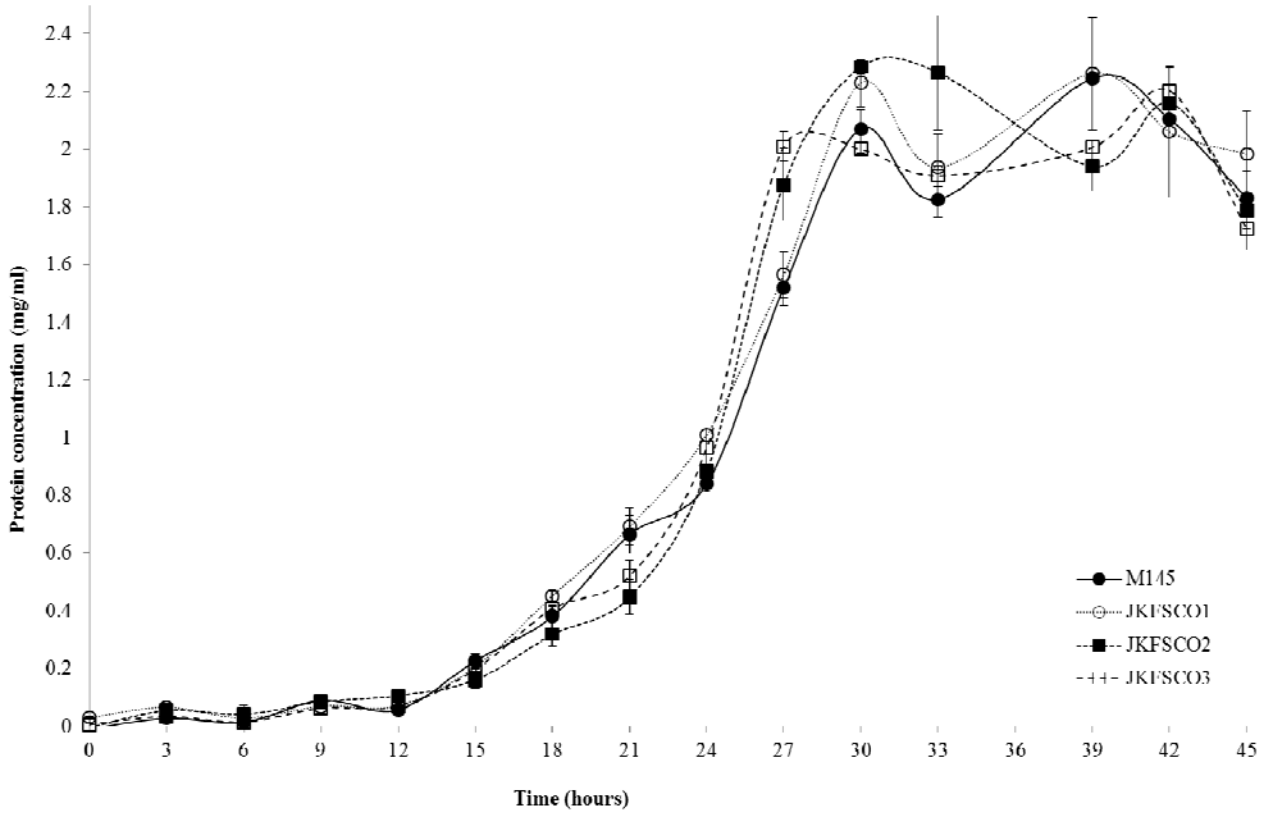

Figure 3.7: Growth rate analysis of *S. coelicolor* strains M145, JKFSCO1, JKFSCO2 and JKFSCO3 in liquid TSB medium. 100 ml aliquots of TSB medium was inoculated with spores of the strain of interest, to a density of $1x10^6$ per ml of culture medium, and incubated at 30°C with shaking. 1 ml samples were withdrawn from the cutures every three hours and total cytosolic protein prepared as described in Section 2.5.1. Protein concentration was assessed using the BioRad DC protein assay. Error bars represent the standard error of the mean, where n=3.

3.4.3 Analysis of the effect of SDS on the growth of *S. coelicolor* T7SS mutant strains

Treatment of cells with the ionic detergent sodium dodecyl sulphate (SDS) is an effective way of disrupting cell membranes by destabilising the interaction of lipids with other lipids and proteins. The cell membranes of most bacteria allow them to withstand stress, meaning that SDS can be tolerated up to a certain level with no obvious effects on the growth of the organism. Once the concentration increases beyond this "tolerated level" it leads to phenotypic defects and cell lysis, causing a decline in cell numbers.

A number of mutations are known to give rise to defects in bacterial cell membranes causing disruption to the lipid bilayer leading to SDS sensitivity, most notably deletion of components of the Tat protein transport system (Stanley *et al.*, 2001; Ize *et al.*, 2003; Joshi *et al.*, 2010). Deletion of *espA*, encoding a secreted component of the T7SS in *M. tuberculosis*, has also been shown to cause instability of the cell wall, as determined by increased sensitivity to SDS treatment (Garces *et al.*, 2010).

In order to determine the range of SDS concentrations that permit growth of *S. coelicolor* whilst still exerting stress, an initial experiment was carried out in which SFM and DNA media, selected as they are routinely used for culturing *S. coelicolor*, were supplemented with SDS at concentrations of 1 %, 0.1 %, 0.01 % and 0.001 %. Growth of *S. coelicolor* M145 on these media was observed over the course of one week allowing the tolerance of *S. coelicolor* to SDS to be established. Growth of *S. coelicolor* on DNA was reduced at 0.01 % SDS with inhibition observed at 0.1 % or above, whereas growth of *S. coelicolor* on SFM medium supplemented with SDS at a concentration of 0.1 % or above was inhibited (data not shown). Based on these

finding it was decided that DNA medium would be supplemented with SDS at concentrations ranging from 0.001 % to 0.01 % and that SFM medium would be supplemented with SDS at concentrations ranging from 0.01 % to 0.1 %. It should be noted that for the purposes of these experiments, SFM medium is routinely used to induce sporulation of *S. coelicolor* allowing membrane stability of T7SS mutant strains to be examined during progression through all stages in the lifecycle. DNA medium, on the other hand, promotes rapid vegetative growth but does not support aerial hyphae formation or sporulation allowing membrane stability of T7SS mutant strains to be examined during this mode of growth.

To determine whether deletion of genes encoding components of the putative T7SSs affect membrane stability of *S. coelicolor*, dilution series were prepared from pre-enumerated spore stocks of *S. coelicolor* strains M145, JKFSCO1, JKFSCO2 and JKFSCO3 so that 2 µl aliquots contained approximately $1x10^5$ spores down to $1x10^0$ in increments of 10 fold. These spore dilution series were then applied to the surface of DNA medium supplemented with SDS at concentrations of 0.001 %, 0.0025 %, 0.005 %, 0.0075 % and 0.01 % and SFM medium supplemented with SDS at concentrations of 0.01 %, 0.025 %, 0.05 %, 0.075 % and 0.1 %. Plates were incubated at 30°C for ten days, during which time growth of the strains exposed to different concentrations of SDS was assessed periodically. The results of this analysis can be seen in Figure 3.8.

It is clear that the ability of the *S. coelicolor* T7SS mutant strains to grow in the presence of SDS on both DNA (Figure 3.8A) and SFM (Figure 3.8B) media are not significantly affected when compared to the wild-type strain. However, the diffusible blue-pigmented antibiotic actinorhodin (note that actinorhodin can be blue or red depending on the pH of the medium) was detected visually at an earlier time-

point following growth of the wild-type strain on media supplemented with SDS than the T7SS mutant strains. This was particularly apparent after 65h of growth on DNA medium supplemented with 0.005 % and 0.0075 % SDS (Figure 3.8A) and SFM medium supplemented with 0.025 % 0.05 % SDS (Figure 3.8B). This was also observed at different time-points following inoculation for exposure to different concentrations of SDS (116h growth on DNA supplemented with 0.0025 % SDS; Figure 3.8A, or 88 hours growth on SFM supplemented with 0.0075 % SDS; Figure 3.8B). However, after ten days of growth, detectable levels of diffuse actinorhodin produced by the *S. coelicolor* T7SS mutant strains appeared similar to the wild-type strain following exposure to all concentrations of SDS (Figure 3.8).

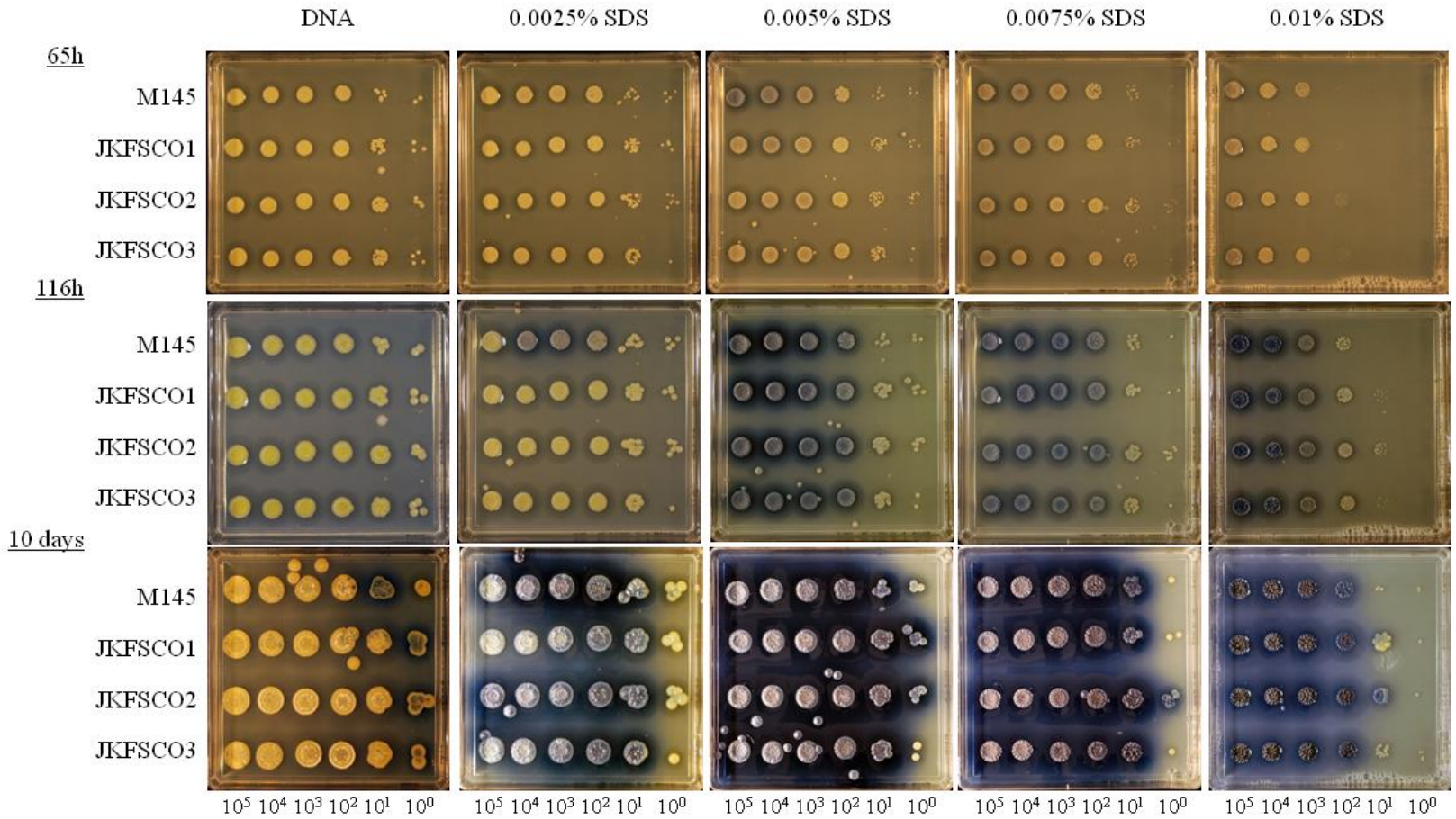

Figure 3.8A: Analysis of the effect of SDS on the growth of *S. coelicolor* M145, JKFSCO1, JKFSCO2 and JKFSCO3 strains. A dilution series was prepared from pre-enumerated spore stocks and 2 µl aliquots containing from approximately $1x10^5$ spores down to $1x10^0$ spores were applied to the surface of DNA medium supplemented with SDS at the indicated concentrations. Plates were incubated at 30°C for the indicated time periods prior to being photographed. The pictures shown represent three independent experiments.

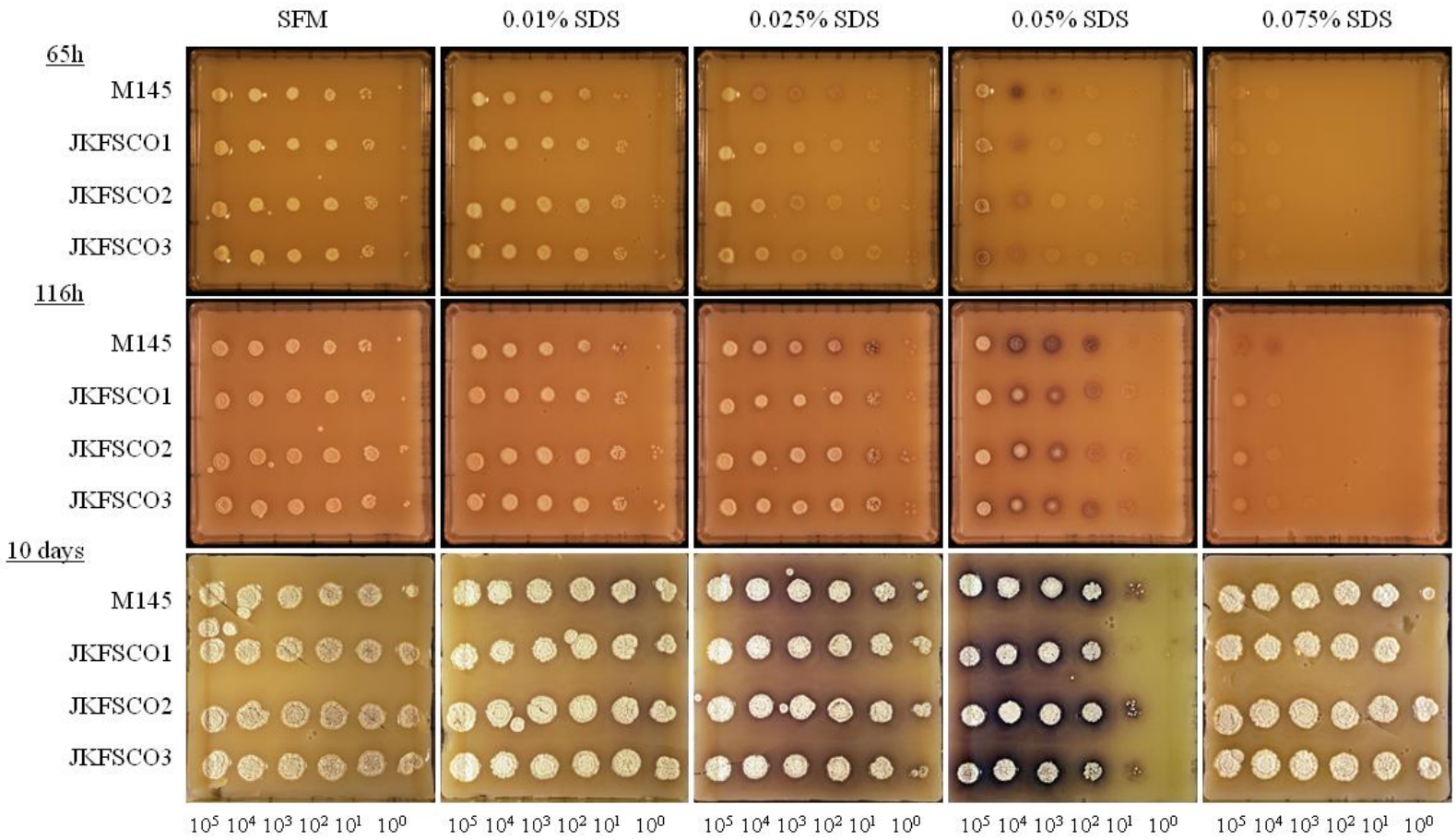

Figure 3.8B: Analysis of the effect of SDS on the growth of *S. coelicolor* M145, JKFSCO1, JKFSCO2 and JKFSCO3 strains. A dilution series was prepared from pre-enumerated spore stocks and 2 µl aliquots containing from approximately $1x10^5$ spores down to $1x10^0$ spores were applied to the surface of SFM medium supplemented with SDS at the indicated concentrations. Plates were incubated at 30°C for the indicated time period prior to being photographed. The pictures shown represent three independent experiments.

3.4.4 Analysis of the effect of heat on the viability of spores from *S. coelicolor* T7SS mutant strains

One of the identifying components of a T7SS is the presence of an ATPase of the FtsK/SpoIIIE family, which is encoded in the same genomic region as one or more WXG proteins, a multi-transmembrane spanning protein and a subtilisin-like serine protease. The FtsK/SpoIIIE class of ATPase is well known for playing roles in DNA translocation, chromosome segregation and sporulation in a range of bacteria. Interestingly, BldB (Sco5723) is a morphogenetic protein required for sporulation in the streptomycetes (Merrick, 1976; Champness, 1988; Pope *et al.*, 1998) and is encoded in the same genetic loci as T7SS components in *S. coelicolor* (Figure 3.3). It is therefore possible that BldB function is related to the T7SS and that formation or stability of mature spores may be affected by disruption of genes encoding components of the T7SS.

It has also been shown in *S. coelicolor* that combined deletion of the *smeA-sffA* genes leads to a defect in spore wall structure resulting in an increased sensitivity to heat (Ausmees *et al*., 2007). Although this effect has been mostly attributed to absence of SmeA, it is interesting to note that SffA also belongs to the family of FtsK/SpoIIIE ATPases. Furthermore, defects in other protein transport machineries have also been associated with temperature sensitivity, for example inactivation of the Tat protein export pathway in *S. scabies* resulted in increased sensitivity of spore germination to heat (Joshi *et al*., 2010).

I therefore sought to determine the effect of temperature on viability of spores by qualitatively analysing the growth of spores derived from the *S. coelicolor* strains M145, JKFSCO1, JKFSCO2 and JKFSCO3 following exposure to heat.

The effect of elevated temperatures on the viability of spores derived from the wild type and each of the mutant strains was assessed by harvesting spores of *S. coelicolor* strains M145, JKFSCO1, JKFSCO2 and JKFSCO3 and preparing spore stocks in 20 % glycerol as described in Section 2.1.1. Prior to treatment, a dilution series of each spore stock was prepared in sterile water and 100 µl aliquots from each of the 10^{-6} and 10^{-7} dilutions were spread onto the surface of LB agar plates. These were incubated at 30°C for 3 days after which time the colonies were counted, allowing calculation of the total viable count for the untreated spores.

To assess the effect of heat treatment on spore germination, dilution series were prepared in sterile water and the 10^{-4}, 10^{-5} and 10^{-6} dilutions for each strain were incubated at 60°C for either 15, 30 and 60 minutes. 100 µl aliquots from each dilution were then spread onto the surface of LB agar plates, incubated for three days at 30°C and the colonies counted to enumerate the total number of viable spores following treatment. This was then used to calculate the percentage of viable spores following treatment, by comparison to the untreated spores. The results of this analysis are shown in Figure 3.9.

Although there appears to be some differences between the viability of spores derived from each strain this was not consistently observed and as such has been attributed to natural variation in the data derived from this type of experiment. It can be concluded from this that deletion of the genes encoding ATPases of the putative T7SSs in *S. coelicolor* have no apparent influence on spore viability when exposed to high temperatures.

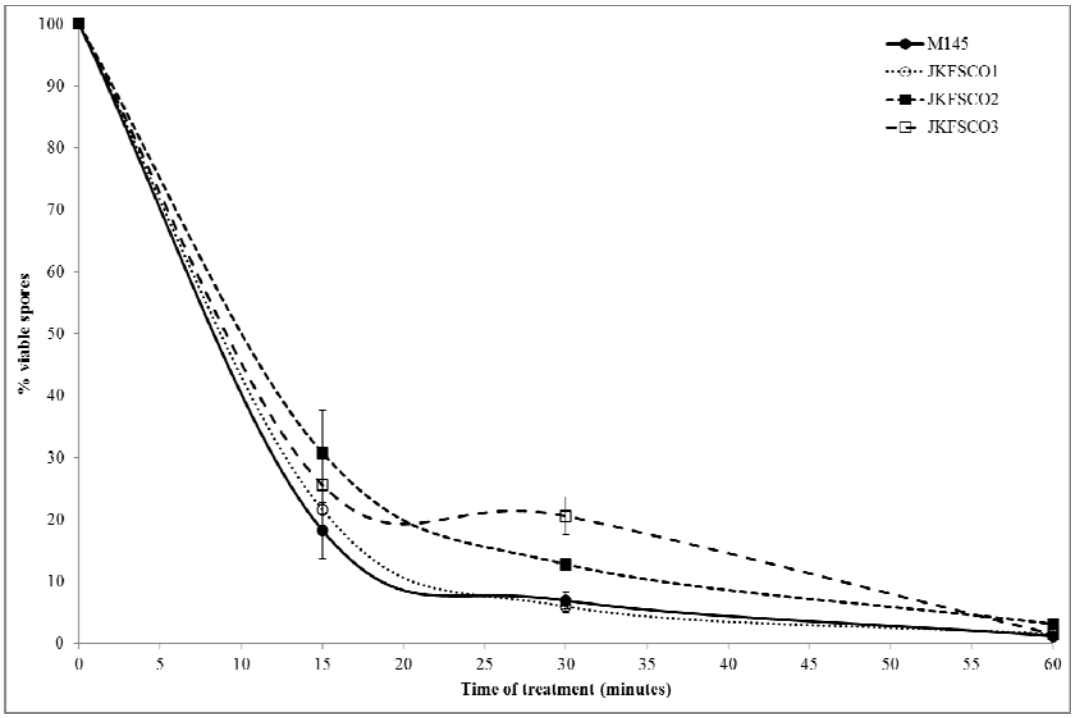

Figure 3.9: The effect of heat treatment on the viability of spores derived from *S. coelicolor* strains M145, JKFSCO1, JKFSCO2 and JKFSCO3. Freshly harvested spores were prepared in 20 % glycerol and used to prepare dilution series in water. Dilution series were then incubated at 60°C for 15, 30 and 60 minutes then 100 µl aliquots from the 10^{-4}, 10^{-5} and 10^{-6} dilutions were spread onto the surface of LB agar plates and colonies counted after three days incubation at 30°C. The number of viable spores following treatment was then expressed as a percentage of the number of viable spores from the freshly prepared spore suspension. Error bars represent the standard error of the mean, where n=3.

3.4.5 Analysis of the effect of osmotic stress on the growth of *S. coelicolor* T7SS mutant strains

Another form of stress that bacteria must tolerate in order to survive is perturbations in the osmotic balance of their environment. In order to do this they must maintain an intracellular osmotic pressure that is higher than that of their surroundings, usually mediated through regulation of gene expression as well as protein activity (reviewed by Krämer, 2010). In *S. coelicolor* a number of genes are known to be involved in the osmotic stress response, many of which have regulatory functions, for example the two-component regulatory system *osaAB* (Bishop *et al*., 2004), *rsbA* and *rsbB* (Lee *et al*., 2004) that are encoded in an operon with and act as an anti- and an anti-anti- sigma factor for σ^B (Cho *et al*., 2008; Lee *et al*., 2004; Lee *et al*., 2005), *osaC* (Fernandez-Martinez *et al.*, 2009) and σ^H (Sevcikova *et al*., 2010). It has been shown previously that strains of *S. coelicolor* and *S. scabies* that are defective in the Tat protein export system also both show an increased sensitivity to osmotic stress (Widdick *et al*., 2006; Joshi *et al*., 2010).

It is known that antibiotic production in *S. coelicolor* is affected under conditions of osmotic stress (Bishop *et al*., 2004) and salt stress (Sevcikova & Kormanec, 2004). As production of actinorhodin was induced by the addition of SDS to the growth medium at an earlier stage in growth of *S. coelicolor* M145 than the T7SS ATPase mutant strains it was deemed possible that this may also occur when the osmolarity of the growth medium was altered.

For these reasons I therefore sought to determine if mutants in the T7SSs of *S. coelicolor* behaved differently under conditions of osmotic stress than the parental strain, M145. In order to investigate this, two different osmolytes, sucrose and sodium chloride (NaCl), were chosen to ensure that any effects observed are due to

osmotic stress rather than other secondary effects such as salt-induced ionic stress. The two growth media, SFM and DNA, were again selected for these experiments, for the reasons outlined in Section 3.4.3.

3.4.5.1 Analysis of the effect of sodium chloride on the growth of *S. coelicolor* T7SS mutant strains

To determine the effect of sodium chloride (NaCl) on the growth of *S. coelicolor* T7SS mutant strains the same procedure was followed as described for testing the effect of SDS in Section 3.4.3, with the exception that the growth media was supplemented with NaCl to a final concentration of 5 % or 10 %.

Plates were incubated at 30°C for ten days, during which time growth of the strains was assessed periodically, and the results of this analysis can be seen in Figure 3.10. It is clear from this that the addition of 5 % NaCl to either DNA medium (Figure 3.10A) or SFM medium (Figure 3.10B) has no apparent effect on the growth and antibiotic production of the *S. coelicolor* T7SS mutant strains when compared to the parental strain M145.

It should be noted that very little growth was observed when any of the strains were grown on DNA or SFM media supplemented with 10 % NaCl, so these results were omitted from the final analysis.

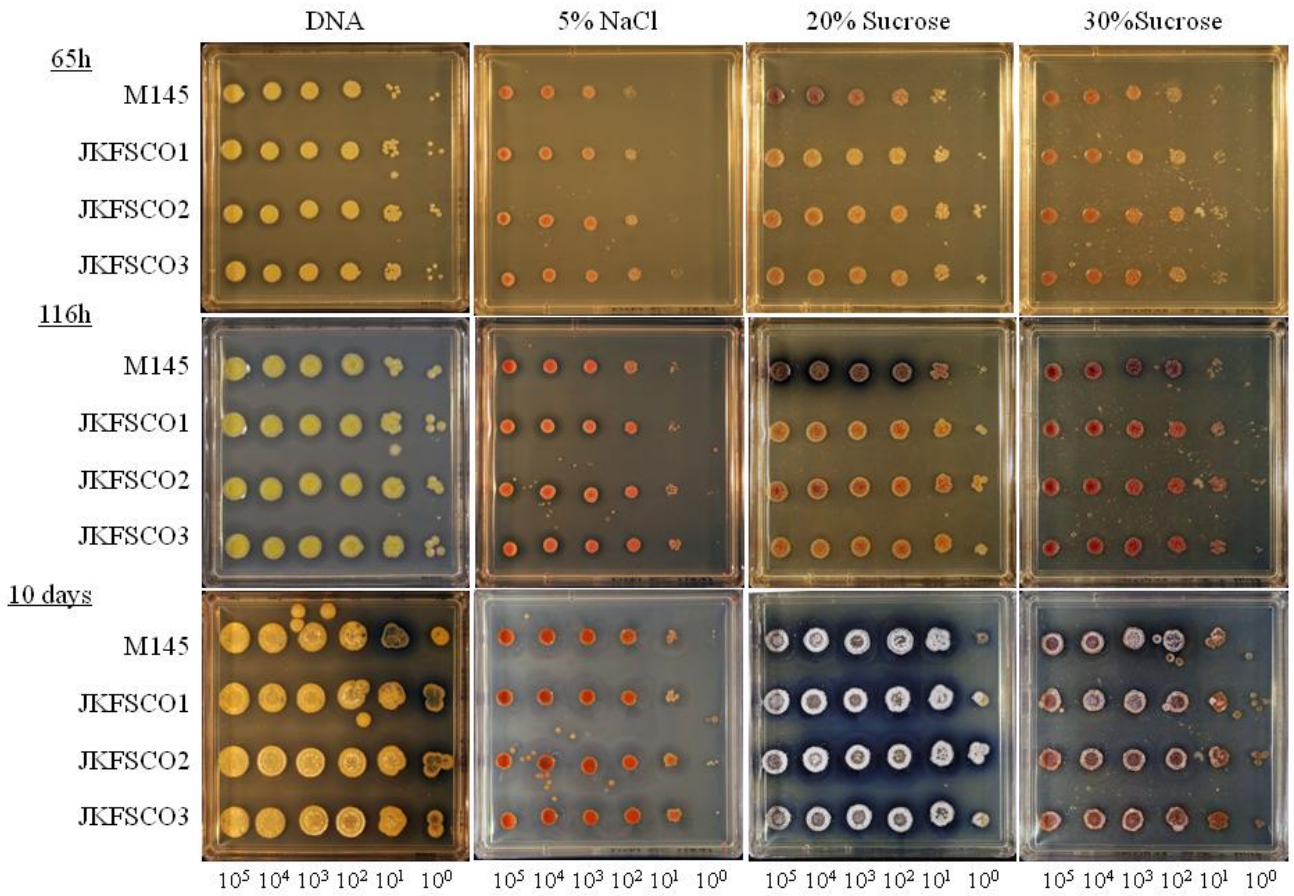

Figure 3.10A: The effect of osmotic stress on the growth of *S. coelicolor* strains M145, JKFSCO1, JKFSCO2 and JKFSCO3. A spore dilution series was prepared for each strain and 2 µl aliquots, containing from approximately $1x10^5$ spores down to $1x10^0$ spores were applied to the surface of DNA medium supplemented with either 5 % NaCl , 20 % sucrose or 30 % sucrose (as indicated above). Plates were incubated at 30°C for the indicated time periods prior to being photographed. The pictures shown represent three independent experiments.

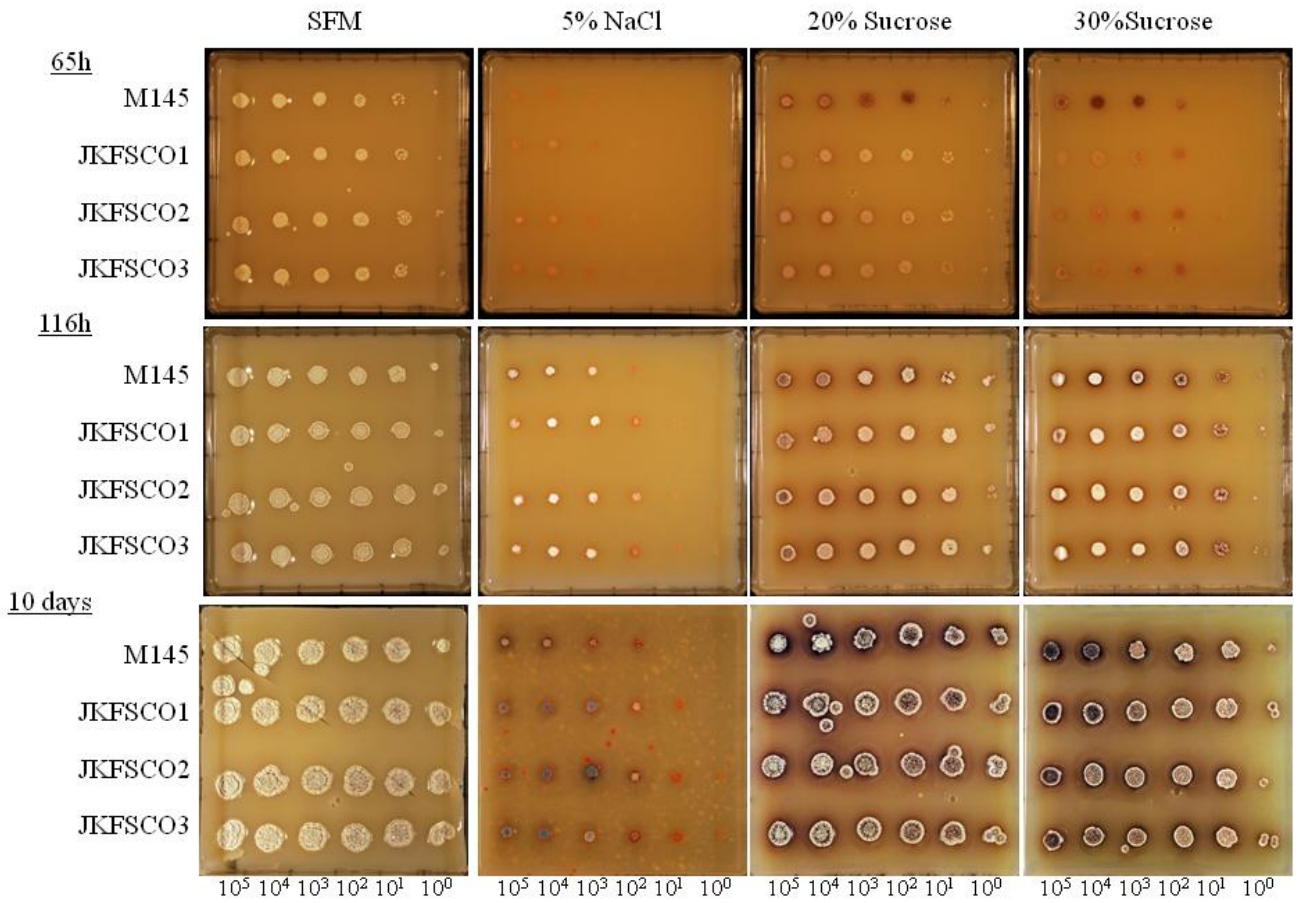

Figure 3.10B: The effect of osmotic stress on the growth of *S. coelicolor* strains M145, JKFSCO1, JKFSCO2 and JKFSCO3. A spore dilution series was prepared for each strain and 2 µl aliquots, containing from approximately $1x10^5$ spores down to $1x10^0$ spores were applied to the surface of SFM medium supplemented with either 5 % NaCl , 20 % sucrose or 30 % sucrose (as indicated above). Plates were incubated at 30°C for the indicated time periods prior to being photographed. The pictures shown represent three independent experiments.

3.4.5.2 Analysis of the effect of sucrose on the growth of *S. coelicolor* T7SS mutant strains

To determine the effect of sucrose on the growth of *S. coelicolor* T7SS mutant strains, the same procedure was followed as described for testing the effect of SDS in Section 3.4.3, with the exception that the growth media were supplemented with sucrose to a final concentration of 20 % or 30 %.

Plates were incubated at 30°C for ten days, during which time growth of the strains was assessed periodically and the results of this analysis can be seen in Figure 3.10. It is clear that the ability of the *S. coelicolor* T7SS mutant strains to grow in the presence of sucrose on both DNA (Figure 3.10A) and SFM (Figure 3.10B) media was similar to the wild-type strain. However, as was observed with the presence of SDS, the addition of sucrose to the media induced production of pigmented antibiotics which appeared delayed for the T7SS mutant strains. This became apparent after 65 hours of growth on DNA medium supplemented with 20 % sucrose or after 116 hours of growth on DNA medium supplemented with 30 % sucrose (Figure 3.10A) as well as after 65 hours of growth on SFM medium supplemented with 20 % or 30 % sucrose (Figure 3.10B).

However, after ten days of growth, production of the pigmented antibiotics by the *S. coelicolor* T7SS mutant strains appeared similar to the wild-type strain following exposure to both 20 % and 30 % sucrose.

3.5 Proteomic analysis of the secretomes of *S. coelicolor* wild type and T7SS mutant strains

As discussed above, most of the previous studies on the T7SS have focussed on *Mycobacteria* and *Staphylococcus* species. In the majority of cases studied the FSD protein is encoded in the same genetic loci as theWXG100 proteins, which are canonical substrates of the system. These proteins lack classical N-terminal signal sequences required for secretion by the Sec or Tat pathways, and little is known about how substrates of the T7SS are targeted for secretion. Two members of the WXG100 family of proteins from *M. tuberculosis*, ESAT-6 and CFP-10, are known to form a heterodimer (Renshaw *et al.*, 2005) and *in vitro* studies have shown that this heterodimer interacts with the secretion apparatus through several key residues present at the C-terminus of CFP-10 (Stanley *et al*., 2003; Champion *et al*., 2006).

However, the lack of a consistent signal sequence and very little homology between known substrates make it extremely difficult to predict proteins that are likely to be secreted by the T7SS using bioinformatic tools, such as those that are available for prediction of Sec or Tat substrate proteins (Nielsen *et al*., 1999; Rose *et al*., 2002; Bendtsen *et al*., 2005). Confounding the matter, substrate proteins, such as EspA and EspC (Fortune *et al.*, 2005; MacGurn *et al.*, 2005; Millington *et al.*, 2011), have been identified that are not encoded in the same genetic loci as the secretion apparatus. Furthermore, in some cases secretion of substrate proteins has been found to be mutually dependent on secretion of ESAT-6/CFP-10 (Fortune *et al*., 2005) whereas other cases have been reported where secretion of substrates is independent of ESAT-6/CFP-10 (McLaughlin *et al*., 2007). Therefore substrate proteins of the T7SS, outside of the WXG100 protein family, must be identified experimentally.

3.5.1 Western blot analysis to detect secretion of WXG100 proteins

Based on the studies of T7SS from other organisms, a strong prediction is that the secretion of the WXG proteins should be defective in at least some of the T7SS mutant strains constructed in this study. The WXG proteins Sco4509 and Sco5724/Sco5725 (which purified as a heterodimer) were isolated from *E. coli* strains overproducing these proteins, polyclonal antisera were raised in rabbits, and the antibodies kindly provided to me by Dr Martin Zoltner from the Palmer group. To analyse whether the secretion of any of Sco4509, Sco5724 and Sco5725 were affected in the T7SS mutant strains, protein samples were prepared from the cell wall washes of *S. coelicolor* strains M145, JKFSCO1, JKFSCO2 and JKFSCO3 which had been growing on SFM medium for 20 hours, as described in Section 2.5.4. Western blotting was subsequently carried out as described in Section 2.5.6 using the polyclonal antibodies at the concentration listed in Table 2.10 and representative results of five independent experiments are shown in Figure 3.11.

It should be noted that numerous attempts to increase the specific detection of the *S. coelicolor* WXG100 proteins using these antibodies were undertaken using a combination of different protein loads as well as a range of antibody concentrations. However, results obtained either lead to no or poor protein detection or were similar to those shown below.

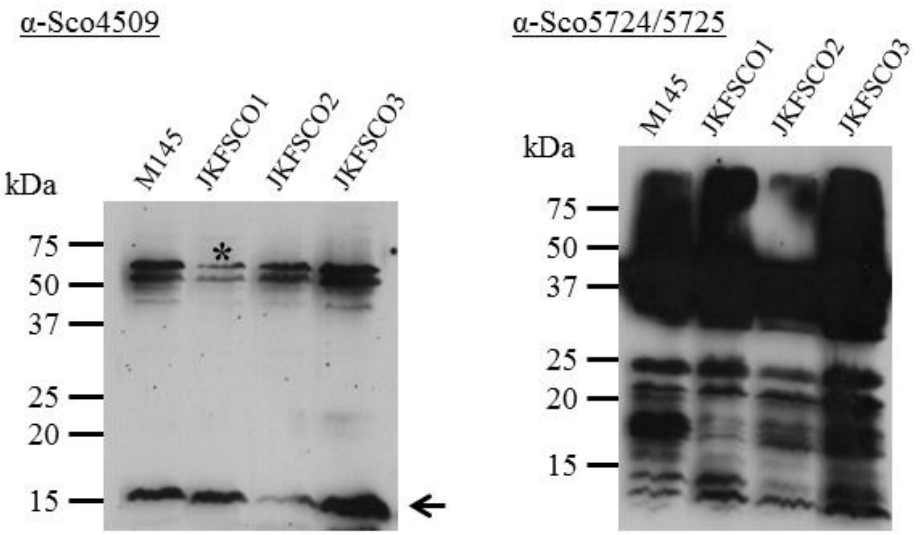

Figure 3.11: Western blot analysis of cell-wall associated protein samples prepared following 20 hours growth of *S. coelicolor* strains M145, JKFSCO1, JKFSCO2 and JKFSCO3. Proteins (20 µg) were separated on a 15 % SDS PAGE gel and transferred to nitrocellulose membrane prior to blotting. Primary antibodies (indicated at the top of each panel) were used at a the concentration given in Table 2.10 in blocking solution and secondary antibody (HRP-conjugated goat α-rabbit) at a concentration of 1:10,000. Detection was carried out using ECL reagent and films exposed for 1 minute. Protein samples were loaded in the order indicated above each panel.

The expected size of Sco4509 is 14 kDa. A band of approximately this size was observed in the cell-wall wash of each of the strains tested (indicated with an arrow in Figure 3.11, left panel). The band was present at similar intensity in the cell wall washes of the wild type strain and of JKFSCO1. This is rather surprising since JKFSCO1 is deleted for *sco4508*, the gene encoding the FSD protein that might be expected to be required for the export of Sco4509. Interestingly, there was rather less of the antigen present in the cell wall wash of strain JKFSCO2, which is deleted for the gene encoding the second putative FSD protein (*sco5734*). One possible interpretation for this observation is that Sco4509 is primarily secreted in a Sco5734-dependent manner. However it is clear that strain JKFSCO3, which lacks genes coding for both of the T7SS FSD-type ATPases, has an *even higher* level of antigen in the cell wall fraction than the wild type strain.

Several bands of approximately 50-70 kDa were also detected in the cell wall wash of each of the strains tested. It is notable that less of this antigen was present in the cell-wall wash of strain JKFSCO1, which is deleted for the gene encoding the first putative FSD protein (*sco4508*; indicated with an asterisk in Figure 3.11, left panel). One possibility is that Sco4509 forms a stable complex with other proteins that survives separation by SDS PAGE and that this is reduced in JKFSCO1 due to a reduced secretion in the absence of Sco4508. However, as this antigen was detected at wild-type levels in the cell-wall wash of JKFSCO3 that is deleted for the genes encoding both putative FSD proteins (*sco4508* and *sco5734*), this seems unlikely.

The significance of these observations is not clear. It is possible that the protein detected with this polyclonal antiserum is not Sco4509 (although the antiserum could cross-react with Sco4509 purified from *E. coli*; Martin Zoltner, personal communication). However, this would not explain why the levels of the

protein fluctuate so greatly between cell wall washes of the different strains. A second possibility is that the protein is Sco4509 and that there may be variable leakage of cytoplasmic proteins during sample preparation resulting in detection in the cell wall washes (perhaps due to subtle differences in the cell envelopes of the different strains). A third possibility is that Sco4509 is released from the cells following developmental cell lysis and that the protein is particularly stable or sticks to the cell walls of unlysed cells. However, what is clear is that from these results it cannot be concluded that secretion of Sco4509 is blocked in any of the putative T7SS mutant strains.

The expected sizes of Sco5724 and Sco5725 are 13.8 and 11.5 kDa, respectively. A number of bands both above and below 15 kDa were observed in the cell-wall fraction prepared from *S. coelicolor* M145, JKFSCO1, JKFSCO2 and JKFSCO3 (Figure 3.11, right panel). However, all of the bands observed were detected in each of the samples. This strongly indicates non-specific binding of the antibody to other proteins present in the samples. As was the case for Sco4509, it cannot be concluded that secretion of Sco5724 or Sco5725 is blocked in any of the mutant strains.

3.5.2 2D SDS PAGE analysis

Studies on bacterial protein secretion systems have utilised a number of approaches aimed at identification of substrates. In general, comparative studies are undertaken whereby the secreted protein fraction (secretome) of a deletion mutant in a gene encoding an essential component of a protein secretion pathway is compared to that derived from the wild-type parental strain.

One method of comparing secretome samples is 2-dimensional (2D) gel electrophoresis that separates proteins firstly by their isoelectric point followed by their molecular mass on an SDS PAGE gel, providing a "snapshot" of the proteins present in each sample. Comparison of the stained proteins in each gel allows identification of protein spots present in the wild-type sample but absent from mutant sample. These protein spots are then excised, analysed by mass spectrometry (MS) and the resulting data processed by MASCOT, which identifies proteins by comparison of peptide sequences identified by MS to a primary sequence database (Perkins *et al.*, 1999).

In both Gram-positive and Gram-negative bacteria a number of Tat-secreted proteins have been identified using 2D gel electrophoresis (Jongbloed *et al.*, 2002; Jongbloed *et al.*, 2004; Posey *et al.*, 2006; Widdick *et al*., 2006; De Buck *et al*., 2008; Joshi *et al.*, 2010). Most notable, the Palmer laboratory has been instrumental in the identification of Tat-secreted proteins using 2D gel electrophoresis from *S. coelicolor* (Widdick *et al*., 2006) and *S. scabies* (Joshi *et al.*, 2010). This technique has also been used to identify a number of proteins secreted by the T7SS in *Mycobacteria* (Mattow *et al.*, 2003; MacGurn *et al.*, 2005; Abdallah *et al.*, 2009), so is applicable to the study carried out here.

In all bacteria studied to date, deletion of the gene coding for the FtsK/SpoIIIE ATPase of the T7SS has been shown to block secretion of the WXG100 proteins, as well as other substrates of the system. Although cross-talk between different T7SS gene clusters is not a commonly reported phenomenon, cases have been reported where deletion of genes from one T7SS locus results in increased secretion of proteins substrates of a disparate T7SS. Therefore, to rule out the possibility of intracellular complementation, a double knockout mutant of *sco4508* and *sco5734* was also analysed alongside strains harbouring single mutations in each of these genes.

Widdick and colleagues (2006) observed that when secretome samples were prepared from *S. coelicolor* grown in liquid culture, significant lysis of bacteria occurs, leading to severe contamination of the sample with cytoplasmic proteins. This led to development of a technique for isolating extracellular (cell-wall associated) proteins from *Streptomyces* grown on cellophane disc on the surface of an agar plate, as described in Section 2.5.4. Since phenotypic analysis of the mutant strains grown on different growth media failed to reveal conditions under which the *S. coelicolor* strains JKFSCO1, JKFSCO2 and JKFSCO3 showed pleiotropy when compared to the wild-type strain, M145 (described in Section 3.4.1) there was no indication as to the conditions that might favour expression of the T7SSs. SFM was therefore used, as this is the standard medium used in laboratories for growth of *S. coelicolor* supports "normal" progression through all stages in the lifecycle of this organism.

S. coelicolor strains M145, JKFSCO1, JKFSCO2 and JKFSCO3 were cultured on the surface of cellophane discs on SFM agar plates for 48 hours then cellwall wash samples prepared as described in Section 2.5.4. Proteins in each sample were then resolved by 2D SDS PAGE, as described in Sections 2.5.7. The Coomassie stained gel images obtained can be seen in Figure 3.12. Each image represents one of three gels run for each sample prepared.

Gel images were inserted into a Microsoft Office PowerPoint® document allowing alternation between images for visual comparison between the gels prepared from the wild-type and each of the mutant samples. This comparison was also carried out by physically overlaying the gel for comparison on top of a transilluminator (the pre-cast gels are attached to a sturdy plastic backing that allows easy handling).

Although some differences were observed between the protein pattern of the wild-type strain and mutant strains in individual gels these could not be observed

consistently in each of the three gels prepared for each sample. No proteins were therefore identified from this experiment, possible reasons for which are discussed below and in Section 3.7.

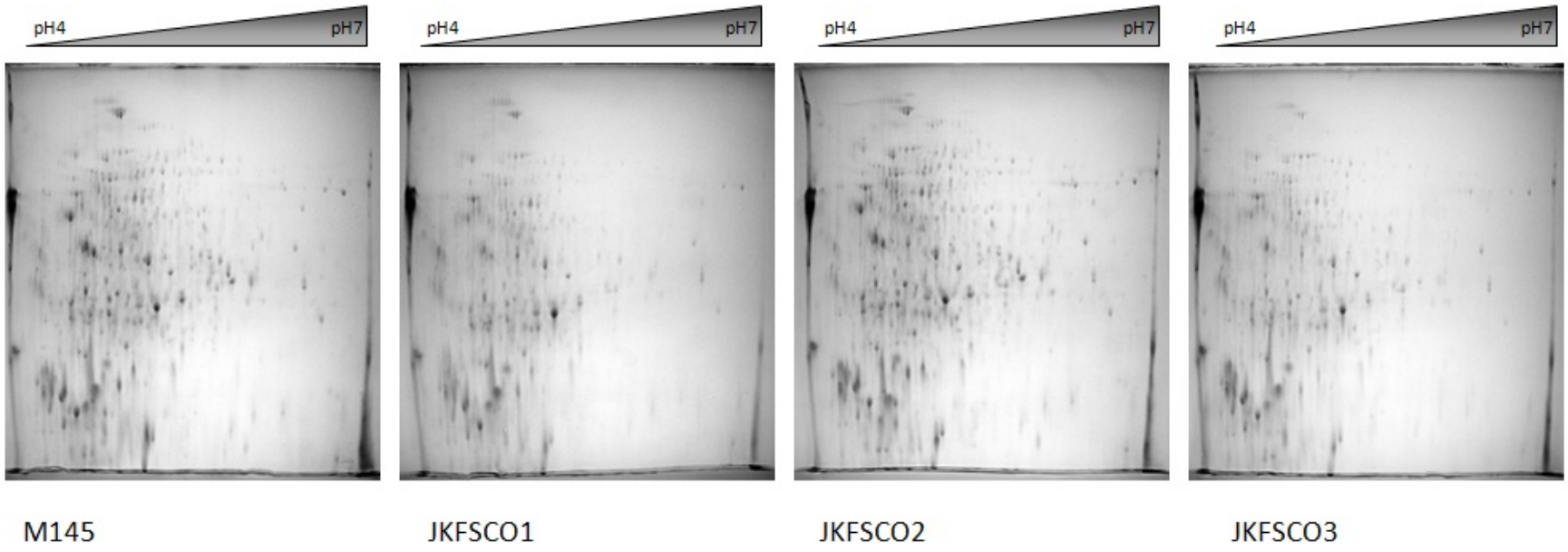

Figure 3.12: Two-dimensional SDS PAGE separation of proteins present in cell wall washes of *S. coelicolor* strains M145, JKFSCO1, JKFSCO2 and JKFSCO3. Strains were cultured on SFM and incubated at 30°C for 48 hours. Cell wall protein samples were then prepared as described in Section 2.5.4. Protein pellets were resuspended in 2D sample buffer and the concentration estimated using a 2D Quant Kit (GE Healthcare). 900 µg of protein was separated by isoelectric focusing (pH gradient 4–7) followed by SDS PAGE. Proteins were stained with Coomassie blue prior to imaging. Images were analysed visually.

During the course of this study a paper was published by Akpe san Roman et al. (2010) indicating that secretion of the WXG100 proteins, Sco5724 (EsxB) and Sco5725 (EsxA) takes place at an early stage of growth i.e. up to 24 hours following inoculation, after which time these proteins were no longer detectable in the culture supernatant. Transcriptional expression data was also provided to me indicating that both *sco4508* and *sco5734* are most highly expressed during vegetative growth (i.e. within the first 24 hours; Dr Andrew Hesketh, personal communication). Taken together these data suggest that at least one of the T7SSs of *S. coelicolor* functions only during vegetative growth phase. For this reason cellwall wash samples were also prepared from each of the strains cultured on the surface of cellophane discs on SFM agar plates for 20 hours and resolved by 2D SDS PAGE. The Coomassie stained gel images obtained can be seen in Figure 3.13 - each image represents one of three similar gels run for each sample prepared. The remaining images can be found in the appendix at the end of this thesis (Figure A1).

Unfortunately, a malfunction in the rubber seals of the electrophoresis unit resulted in buffer leakage from the upper cathodic chamber to the lower anodic chamber. This prevented the current reaching its normal level and led to a greatly increased time for the gel run (>30h as opposed to approximately 6-8 hours). The bromophenol blue dye front in each gel did not resolve to the same position. Once the proteins in each gel were stained the resolution of the proteins in each gel was seen to vary between gels especially at the lower size range where a solid line of unresolved protein was observed. This hindered the comparison process and no proteins were identified as being unique to any one sample.

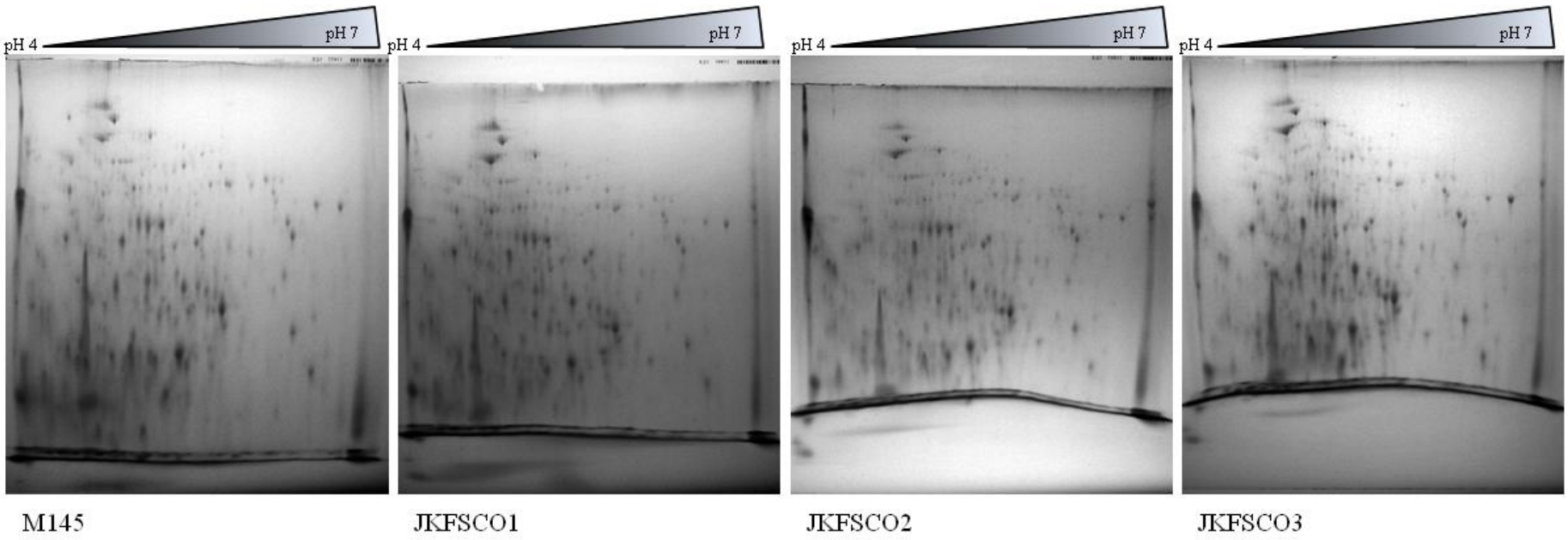

Figure 3.13: Two-dimensional SDS PAGE separation of proteins present in cell wall washes of *S. coelicolor* strains M145, JKFSCO1, JKFSCO2 and JKFSCO3. Strains were cultured on SFM and incubated at 30°C for 20 hours. Cell wall protein samples were then prepared as described in Section 2.5.4. Protein pellets were resuspended in 2D sample buffer and the concentration estimated using a 2D Quant Kit (GE Healthcare). 900 µg of protein was separated by isoelectric focusing (pH gradient 4–7) followed by SDS PAGE. Proteins were stained with Coomassie blue prior to imaging. Images were analysed visually.

3.6 Analysis of the ability of bacteriophage to infect *S. coelicolor* T7SS mutant strains

It has been reported that components of the putative T7SS from *Bacillus subtilis* are encoded in an operon with *yueB* that codes for a membrane protein which is essential for irreversible binding of the bacteriophage SPP1 to the bacterial surface and ejection of phage DNA into the host cell (São-José *et al.*, 2004, 2006). Although YukE (a WXG100 family protein) and YukA (an FtsK/SpoIIIE ATPase) are not essential for phage infection, strains with mutations in the genes encoding these proteins give smaller plaques upon SPP1 infection (São-José *et al.*, 2004). The authors have speculated that the membrane components of the putative T7SS of *B. subtilis* may play a role in transporting the double–stranded phage DNA into the host cell.

To determine whether the T7SS of *S. coelicolor* plays a role in phage infection, plaque assays were carried out as described in Section 2.7 using three different bacteriophages, and the *S. coelicolor* strains M145, JKFSCO1, JKFSCO2 and JKFSCO3 as hosts. The results of this analysis can be seen in Figure 3.14.

It can clearly be seen that two of the phages, ΦHau3AI and ΦR4, that are known to carry out lytic infection of wild-type *S. coelicolor*, are also able to infect the T7SS mutant strains in a similar fashion as the wild-type strain, as ascertained by number and size of plaque formation (Figure 3.14). A third phage, ΦC31, that does not normally form plaques on wild-type *S. coelicolor*, was shown to form plaques on strains JKFSCO2 and JKFSCO3 but not JKFSCO1. This implies that Sco5734, the gene for which is deleted in JKFSCO2 and JKFSCO3, may play a role in resistance to this phage. However, it should be noted that the phage growth limitation (PGL) system, an unusual mechanism that confers resistance of *S. coelicolor* to ΦC31

infection (Chinenova *et al.*, 1982) is prone to spontaneous mutation at a high frequency (Laity, 1993). Given that JKFSCO3 is derived from JKFSCO2 the possibility that both of these strains also harbour an additional mutation in a gene encoding a component of the PGL system cannot be ruled out and would require further experimental testing (for example attempting to complement the ΦC31 lytic phenotype by providing *sco5734* in *trans*).

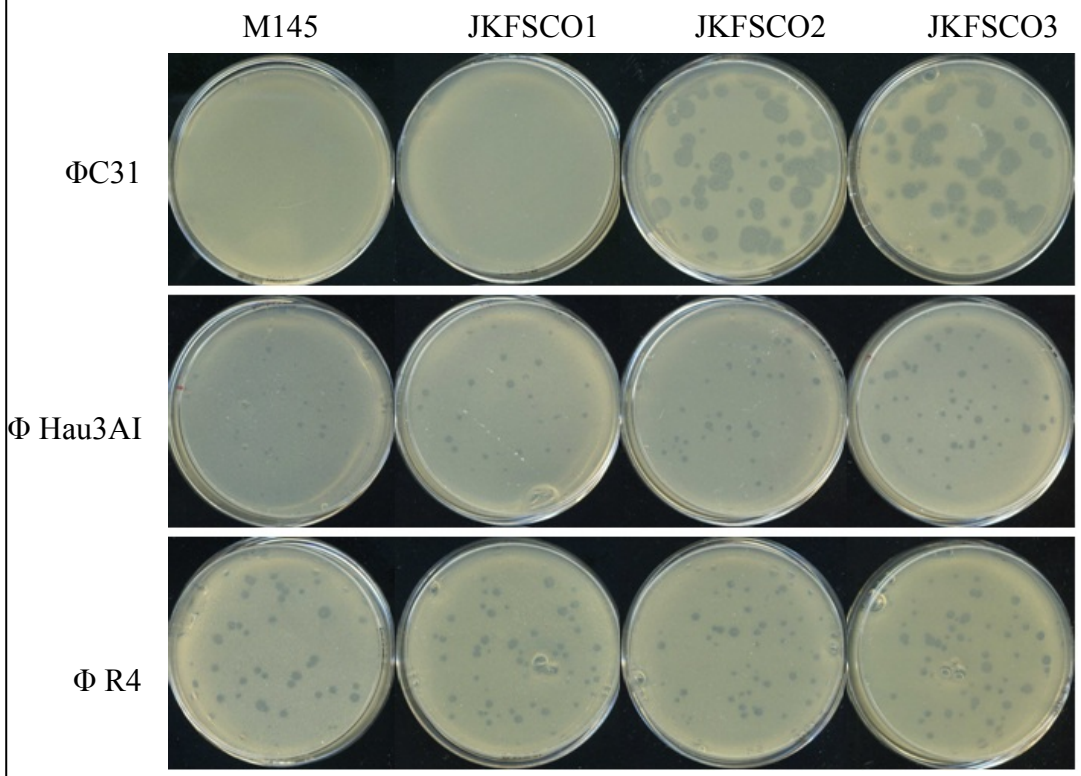

Figure 3.14: Analysis of the ability of bacteriophage to infect *S. coelicolor* strains M145, JKFSCO1, JKFSCO2 and JKFSCO3. The surface of DNA medium was co-inoculated with a phage suspension and approximately 10^7-10^8 spores derived from the strain to be examined. Plates were incubated at 30°C for 18 hours to allow plaque formation prior to being photographed.

3.7 Discussion

3.7.1 The genome of *S. coelicolor* encodes two putative T7SSs that are not essential for viability

In this study bioinformatics analysis was undertaken to identify putative T7SSs encoded in the genome of the model streptomycete *S. coelicolor*. It was shown that, in addition to the putative T7SS identified by Gey van Pittius *et al.* (2001) a second system also appeared to be present. To analyse potential roles of each of these T7SS, single and combined mutations were constructed in genes coding for the FSD proteins of each T7SS cluster. Mutant strains in each of the FtsK/SpoIIIE ATPase genes were obtained easily, giving the first indication that this system is not essential for growth and viability of the organism.

3.7.2 A role for the T7SSs of *S. coelicolor* in developmental regulation?

When various phenotypic analyses of the strains harbouring mutations in the FtsK/SpoIIIE ATPase genes were undertaken, using a range of growth media, the strains displayed similar growth behaviour to the wild-type. However, it was noted that when stress was applied to the strains through addition of SDS or sucrose to the growth media each mutant showed a delay in the production of the pigmented antibiotic actinorhodin.

As discussed briefly in Chapter 1, antibiotic production in *S. coelicolor* is controlled by multiple regulatory pathways and is also intimately linked to the developmental cycle, with antibiotics being produced at the transition phase from vegetative to aerial growth. Although development of the mutant strains constructed in this study appeared indistinguishable from wild-type, one theory as to a possible role for these systems may be formed by examination of the genetic neighbourhood of the

T7SS-encoding genes. It was observed that *bldB*, which is required for sporulation in *S. coelicolor*, is found in the same genetic locus as *sco5724* and *sco5725* (coding for two potential WXG100 proteins). Interestingly Sco4541, the WXG100 protein identified in this study, and a probable component of the second T7SS is encoded next to the *sco4542* and *sco4543*, genes that encode a BldB-family protein and WhiJ, respectively (Eccleston e*t al.*, 2006; Aínsa *et al.*, 2010). As *bld* and *whi* genes are regulators of aerial hyphae formation and sporulation in *S. coelicolor*, this genetic association suggests the Type T7SS may contribute to developmental regulation. Perhaps the system is only necessary under conditions that the organism may encounter at times in its natural environment, rather than in the artificial laboratory situation.

As discussed previously, during the course of the experiments described in this chapter a paper was published by Akpe San Roman *et al.* (2010) describing the characterisation of one of the two *S. coelicolor* T7SS gene clusters described here. The findings of Akpe San Roman *et al.* (2010) support the association of the T7SS with development. The authors isolated transposon insertion mutants in multiple genes in the *sco5734* region and found that insertional inactivation of *sco5724*, which was shown to to have a polar effect on *sco5725* (both of these encoding predicted WXG100 proteins) resulted in a sporulation defect. Spores were produced later in these strains and they were aberrantly sized and appeared to contain multiple nucleoids. The authors also found that transcription of these two genes was elevated in a *bldB* mutant during early sporulation. Given the genetic association of *bldB*-family genes to those encoding components of the T7SS, it is tempting to speculate that the delayed production of actinorhodin production under conditions of stress observed in this thesis is due to an effect on *bldB* expression. This would, however, warrant further investigation.

Consistent with the data presented in this chapter, Akpe San Roman *et al.* (2010) observed no growth defect for their strain which carried a transposon insertion in

sco5734, the gene encoding the predicted FtsK/SpoIIIE ATPase from the same genetic locus. This is rather odd given the striking phenotype reported for the *sco5724* mutant strain and given that these genes are both expected to encode components of the same T7SS. The authors rationalised this observation by suggesting that the Sco5724/Sco5725 WXG100 proteins have an intracellular function, which would explain why no growth defect was observed for the FtsK/SpoIIIE ATPase mutant strain. It was further suggested that the heterodimer formed by the WXG100 proteins acts to regulate nucleoid condensation during segregation and that it is may do this by sequestering regulatory protein(s) during vegetative growth (Akpe San Roman *et al.*, 2010). Given that BldB has been shown to be required for developmental regulation (Pope *et al*., 1998), its genetic association with the T7SS in this, and many other streptomycetes, as well as the fact that BldB is thought to interact with additional proteins (Eccleston *et al.*, 2006) make it an excellent candidate for this role.

This proposal can be likened to the situation observed for *M. tuberculosis*, where EspR regulates expression of Esx-1 but is also secreted by the system (Raghavan *et al.*, 2008). Akpe San Roman *et al.* (2010) have also suggested an alternative protein to BldB, Sco6675, as a potential intracellular protein regulated by the T7SS since this is an orthologue of EspR. However, it should also be noted that WhiJ belongs to the SinR family of transcriptional regulators (Aínsa *et al.*, 2010) which is the same family as EspR (Raghavan *et al.*, 2008). Given the findings presented here on the association of genes encoding WhiJ, BldB-family and WXG100 proteins and that WhiJ is also thought to have an unidentified interacting partner (Aínsa *et al.*, 2010) WhiJ may also be a good candidate for a possible T7SS-regulated protein. As deletion of *whiJ* has been shown to suppress the developmental effects associated with deletion of genes encoding BldB-family proteins (Aínsa *et al.*, 2010) another possibility is that WhiJ, BldB-family

proteins and WXG100 proteins all interact. This could be ascertained by a bacterial two-hybrid study.

In contrast with the inconsistent results presented in this chapter, Akpe San Roman *et al.* (2010) were able to demonstrate that FtsK/SpoIIIE ATPase (Sco5734) was absolutely required for the secretion of Sco5724 and Sco5725 to the growth medium. This was achieved following introduction of genes coding for C-terminal His-tagged copies of each protein at a heterologous location into the mutant backgrounds. Given that the signal sequences for secretion of WXG family proteins appear to reside at their extreme C-termini (Champion *et al*., 2006; DiGiuseppe Champion *et al*., 2009) it is surprising that addition of a C-terminal his-tag did not abolish secretion. Indeed I considered constructing His-tagged variants of Sco5724 and Sco5725 since the polyclonal antiserum used in this study did not seem to reproducibly recognise these proteins. However only N-terminally His-tagged variants were constructed and these proteins were not secreted (data not shown). Interestingly, Akpe San Roman *et al.* (2010) were able to show that secretion of Sco5724 and Sco5725 were also dependent upon the predicted membrane associated protein (Sco5721) that is an orthologue of Rv3869. However, secretion of the WXG100 proteins was shown to occur in the absence of the predicted serine protease (Sco5722) or the putative pore protein (Sco5735). Given that the homologue of Sco5735 is essential for secretion of EsxA and EsxB in *M. tuberculosis* (Stanley *et al*., 2003), this finding is rather surprising, and it may be that there is cross-functionality between some of the T7SS protein orthologues in *S. coelicolor*.

3.7.3 Are there non-WXG100 substrate proteins of the putative T7SS in *S. coelicolor*?

The discovery that non-WXG100 substrate proteins are exported by T7SSs in other organisms (Fortune *et al.*, 2005; McLaughlin *et al.*, 2007; Raghavan e*t al.*, 2008) suggested that there may be additional substrates of the T7SS in *S. coelicolor*. In this thesis identification of putative substrate proteins was attempted using 2-dimensional PAGE of cell wall washes from the different mutant strains. The initial analysis was carried out using proteins from cell-wall wash samples prepared following 48 hours grown on SFM, in keeping with the methods previously employed in the Palmer group (Widdick *et al.*, 2006; Joshi *et al.*, 2010). However, no consistent differences in protein spots were seen when the gels were compared proteins were observed. Subsequently Akpe San Roman *et al.* (2010) demonstrated that the WXG100 proteins Sco5724 and Sco5725 were only detectable prior to 24 hours growth, therefore cell wall washes were also prepared and analysed following 20 hours of growth. However, this also failed to reveal any protein spots unique to any one sample.

It is possible that substrates of the T7SS are not expressed under the conditions tested here, and for example samples could be prepared following growth on a range of different media. This approach has been shown to be effective for identification of proteins secreted by the Tat system of *S. coelicolor* (Widdick e*t al.*, 2006). A further possibility is that even though the proteins are differentially missing from some of the sample they evade detection following 2D-PAGE as the staining method is not sensitive enough to detect proteins of low abundance. More sensitive stains could be employed, or liquid based proteomics methods could be used, where proteins present in samples are detected using mass spectrometry methods. Some of these methods are extremely sensitive and can detect proteins at very low abundance. Other possible reasons for the failure to identify protein substrates may be that their isoelectric points fall outside the

range tested here (a pH gradient of 4-7 was used as the majority of secreted proteins are expected to fall within this range, as demonstrated by Widdick *et al.* (2006).

An interesting alternative is that protein substrates of the T7SSs were not detected because the T7SSs in *Streptomyces* function primarily in DNA transport, analogous to the T4SS discussed in Section 1.3.14 of this thesis. This hypothesis is supported by findings in *M. smegmatis* where the *esx*-1 locus is required for conjugal DNA transfer (Flint *et al.*, 2004; Coros *et al.*, 2008). It may be possible to investigate this by comparing levels of extracellular DNA (eDNA) present in the extracellular fractions from the mutant and wild-type strains. One way to do this could involve preparing eDNA from liquid cultures by filter sterilising the culture supernatant, or from surface grown cultures by washing the biomass with a high ionic strength buffer to release any DNA. Samples could then analysed by quantitative PCR (qPCR) using random primers in a method similar to that described by Lou *et al.* (2011). Alternatively, eDNA could be visualised using fluorescence microscopy following staining with 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one) (DDAO) as described by Schooling *et al.* (2009). Another possibility would be to analyse a culture by flow cytometry following staining of eDNA with a fluorescent dye, which has been shown to be an effective way of detecting DNA attached to the cell surface of *E. coli* (Castan *et al.*, 2002).

Along the same line of reasoning it may also be possible that the streptomycete T7SS is required for DNA uptake rather than DNA or protein secretion, as ESX-1 mutants of *M. smegmatis* recipient strains are hypo-conjugative (Coros *et al.*, 2008). This would be analogous to the 'Com' system found in *Helicobacter pylori* (Hofreuter *et al.*, 2001) and *Bacillus subtilis* (Briley *et al.*, 2011) that is related to the T4SS and is required for natural competence. It should be noted, however, that natural competence of *S. coelicolor* has not been reported.

3.7.4 A role for the T7SS of *S. coelicolor* in phage infection?

The results shown in the last part of this chapter suggest that Sco5734 may be required for resistance to lysis by the bacteriophage ΦC31 as strains JKFSCO2 and JKFSCO3 (which both lack *sco5734*) displayed susceptibility whereas M145 and JKFSCO1 were resistant to lysis by this phage (Figure 3.14). However, it is known that the phage growth limitation (PGL) system is required for resistance to ΦC31 and that it is prone to a high rate of spontaneous mutation resulting in a susceptible phenotype (Chinenova *et al.*, 1982; Bedford *et al.*, 1995). As JKFSCO3 is derived from JKFSCO2 it is possible that each of these strains harbour an identical mutation in a *pgl* gene. Complementation of these strains should therefore be carried out in order to determine if the phage susceptible phenotype observed can be attributed directly to the absence of Sco5734.

4. CHARACTERISATION OF THE PUTATIVE T7SS FROM STREPTOMYCES SCABIES

4.1.1 Introduction

S. scabies was first described by Thaxter in 1892, where it was referred to as *Oospora scabies*, the organism inciting common scab symptoms on potato. Symptoms of infection in a susceptible host, such as *Solanum tuberosum*, are typically lesions on the surface of potato tubers. These lesions can vary in severity depending on a number of factors including inherent resistance of the host, the virulence of the infecting strain as well as the environmental conditions during the period of infection (Bouchek-Mechiche *et al*., 2000).

It should be noted that the host range of *S. scabies* is not only restricted to potato. Disease has also been commonly observed in other root crops such as radish, carrot and beet (Loria *et al.*, 2006). It has even been seen to damage the pericarp of peanuts, where it is known as peanut wart (Kritzman *et al*., 1996). Hooker demonstrated in 1949 that both monocot and dicots are susceptible to infection, and subsequent studies carried out on model plants such as *Arabidopsis thaliana* and *Nicotiana tabacum*, indicated that the host range may encompass all higher plants (Hooker,1949; Leiner *et al.*, 1996). In these other plant hosts, infection has been demonstrated to cause damage to the root system and meristems (Hooker, 1949).

As *S. scabies* is able to infect a wide plant host range this indicates that the plant cell targets are highly conserved features present in most, if not all, higher plants. It has been observed that scab lesions develop on potatoes throughout the growing season but do not develop after the tuber has stopped expanding (Loria *et al*., 2006). When taken together with observations that in other plant hosts root infections are not established on mature or fibrous roots, but that the organism does prevent further growth of new roots

(Loria *et al.*, 1996), it is apparent that for infection to occur there is the requirement for the active expansion of plant tissue (Loria *et al*., 2006).

The primary virulence determinant of scab-causing *Streptomyces* spp. is thaxtomin A that has been reported to act as an inhibitor of cellulose synthase (Scheible *et al.*, 2003). This explains why active expansion of plant tissue is required for the establishment of infection. Several other virulence factors are also known to be produced by *S. scabies*, most notably a protein that causes tissue necrosis called Nec1 (Bukhalid & Loria, 1997; Joshi *et al.*, 2007a). Although Nec1 is a secreted protein its plant cell target is unknown, however it is thought to suppress the plant defence response (Bukhalid & Loria, 1997; Joshi *et al.*, 2007a). These virulence factors have been discussed in Section 1.2.2.

Protein secretion is a common theme in studies of bacterial pathogenesis and is routinely discussed in analyses of bacterial genome sequences (Bell *et al*., 2004; Bentley *et al*., 2002; Cole *et al*., 1998). Protein secretion systems are essential for the surface localisation of proteins involved in interactions with hosts, as well as delivery of virulence factors to the extracellular environment or directly into the host cell. The role of protein secretion has been most intensely studied in Gram negative bacterial pathogens, as they possess complex secretion machineries in order to export proteins across the double membrane as well as, in some cases, also across the membrane of the host cell (reviewed by Saier, 2006). As discussed in Chapter 3, Gram positive bacteria probably possess fewer secretion pathways through which proteins may be transported. The general secretory (Sec) pathway is the most intensely studied (reviewed by Driessen & Nouwen, 2008) but more recently alternative routes of protein secretion have been described, such as the Tat pathway (Berks *et al*., 1996) and the T7SS (Stanley *et al*., 2003).

Protein secretion systems may contribute to the normal physiology of many non-pathogenic bacteria but are additionally utilised for the secretion of virulence factors in closely related pathogenic species. One example which is highly relevant to this study is the streptomycete Tat pathway; deletion of *tatC*, encoding an essential component of the Tat translocation machinery, from the genome of *S. coelicolor* led to severe pleiotropic defects and blocked secretion of numerous proteins of varying function (Widdick *et al.*, 2006). Similar pleiotropic defects were also observed when *tatC* was deleted from the genome of *S. scabies*, indicating that the pathway performs similar physiological roles, but it has also been shown that a number of individual proteins secreted by the Tat pathway in *S. scabies* contribute directly to virulence (Joshi *et al.*, 2010).

The most recently discovered protein secretion system in Gram-positive bacteria, the T7SS, is encoded in the genomes of many non-pathogens but has gained attention for the contribution it makes to the virulence of *M. tuberculosis* (Stanley *et al*., 2003) and *S. aureus* (Burts *et al.*, 2005). The discovery of genes encoding a putative T7SS in the plant pathogen *S. scabies*, described below in Section 4.2.1, led us to not only to attempt characterisation of the system along similar lines as for *S. coelicolor* in Chapter 3, but also to investigate a potential role for the T7SS in the virulence of *S. scabies*.

4.1.2 Aims of this chapter

The aim of the work in this chapter was to characterise Type VII protein secretion in *S. scabies*, specifically to determine whether the system was functional, its role in *S. scabies* physiology and to identify substrate proteins of the machinery. Furthermore, as *S. scabies* is a plant pathogen, I also sought to determine if the T7SS

contributes to the virulence of this organism by conducting a number of plant virulence bioassays using T7SS mutant strains.

RESULTS

4.2 Analysis of the T7SS-encoding genes of *S. scabies*

4.2.1 Identification of genes encoding a putative T7SS in *S. scabies*

Sequencing of the genome of *S. scabies* was performed by the Sanger Centre in collaboration with Rosemary Loria (Cornell University, USA). This was completed in 2006 and can be found at http://www.sanger.ac.uk/Projects/S_scabies/. The annotation of the genome has also recently been completed and made available in the public NCBI database.

Since the genome of *S. coelicolor* was shown to encode components of a T7SS (Section 3.2; Pallen *et al.*, 2002) I hypothesised that this was probably also true for *S. scabies*. To determine whether this was the case, BLASTP analysis was initially performed using the amino acid sequences of components of the putative T7SS from *S. coelicolor* (the encoding genes can be seen in Figure 3.3). From this analysis, an FtsK/SpoIIIE domain (FSD) protein, Ssc58621, was identified as being highly homologous to Sco5734 (40 % identity and 55 % similarity over 99% of the protein). Ssc58621 was also the top hit when Sco4508 was used as a query, but showed lower homology with 33 % identity and 51 % similarity over 35% of the protein.

Further evidence that this region encodes a putative T7SS in *S. scabies* was provided when Scab58651 was found to be homologous to Sco5735, with 39 % identity and 47 % similarity over 19% of the protein. The *S. scabies* genome does not encode any proteins with significant homology to Sco5721, which has since been shown to be a component of the T7SS in *S. coelicolor* (Akpe San Roman *et al.*, 2010). Analysis of the genetic region surrounding *ssc58621* also failed to uncover any homologues to the two

predicted serine proteases encoded in the genetic region of the cognate *S. coelicolor* T7SS, Sco5722 and Sco5731.

As WXG100 proteins are one of the signatures for the presence of a T7SS, BLASTP analysis was carried out using the *S. coelicolor* WXG100 protein sequences, Sco5724, Sco5725 and Sco4509, to query the *S. scabies* database. However, this approach failed to identify any WXG100 proteins encoded by the *S. scabies* genome. Visual inspection of DNA around the region of *ssc58621* for the presence of genes encoding proteins of approximately 100-150 amino acid residues provided several candidate WXG100 protein sequences including *ssc58661, ssc58671* and *ssc58681*. However, none of the proteins encoded by these genes contained a canonical W-X-G motif, and when used as a query for BLASTP analysis no WXG100 domain was detected.

At this stage it was decided to include more WXG100 protein sequences to carry out this analysis. I reasoned that *Streptomyces* protein homologues would be much more closely related to each other than to homologues from other species, so identification of further WXG100 proteins encoded in the genomes of other sequenced *Streptomyces* species was sought. To this end BLASTP analysis was performed using the WXG100 protein sequences of the *S. coelicolor* proteins against all available streptomycete protein sequences. Sco5725 was found to be homologous to Sav2533 from *S. avermitilis*, which was in turn found to share weak homology with Ssc58681 from *S. scabies*, a protein encoded in the same region as the putative T7SS FSD protein. As this protein had already been analysed by BLASTP, the translated protein sequences of this and two similarly sized neighbouring genes were analysed using PHYRE (Kelley & Sternberg, 2009), which is a secondary structure prediction program. Both Ssc58671 and Ssc58681 were predicted to be members of the EsxAB dimer-like superfamily of proteins with estimated precision of 95 % (E-value 0.02) and 85 % (E-value 0.34)

respectively. The criteria for assigning proteins to this family have been described previously in Section 3.2. For each of the *S. scabies* WXG100 proteins identified here the WXG motif is replaced with FXXXG, where X is a non-conserved amino acid.

All of the protein-coding ORFs of *S. scabies* were also analysed by BLASTP for the presence of a BldB-family protein and WhiJ as these are encoded next to a WXG100 domain containing proteins in the genome of *S. coelicolor* (Figure 3.3). Two BldB-family proteins were found to be encoded by *ssc58751* and *ssc58761* and the closest WhiJ orthologue encoded by *ssc58741*. The arrangement of this putative T7SS gene cluster on the chromosome of *S. scabies* can be seen in Figure 4.1.

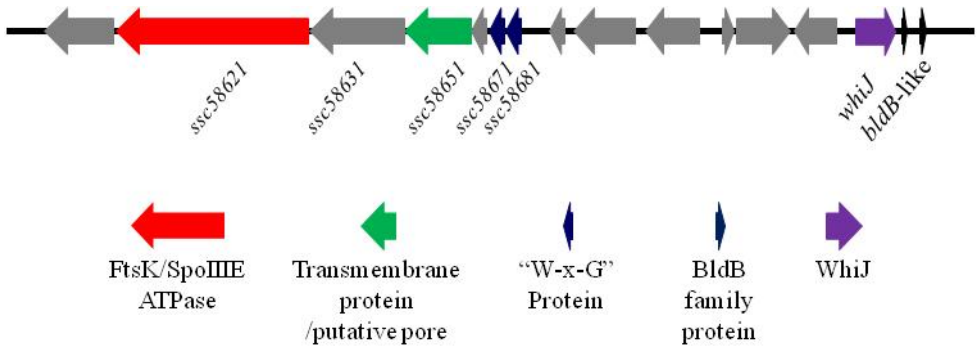

Figure 4.1: Arrangement of T7SS-encoding genes on the chromosome of *S. scabies*.

4.2.2 Expression analysis of the *S. scabies* T7SS genes

To aid subsequent study of the T7SS of *S. scabies* I sought to determine whether the genes in the *S. scabies* T7SS region of the chromosome, identified in Section 4.2.1, are co-expressed and whether they are transcribed under standard laboratory growth conditions or only in response to the presence of plant-derived material in the growth medium.

To determine this, messenger RNA was isolated, as described in Section 2.4.1, from mycelia of *S. scabies* strain 87.22 which had been growing for 20 hours in either

TSB (Table 2.2), a standard artificial medium, or TDM medium (Table 2.2), which contains cellobiose and is known to induce expression of virulence genes (Johnson *et al.*, 2007; Joshi *et al.*, 2007b; Bignell *et al.*, 2010). This mRNA was used as a template to prepare cDNA, as described in Section 2.4.2, for use as a template in PCR reactions, as described in Section 2.2.5. Primer pairs were used for amplification of an internal region of a single gene or the junction region between neighbouring genes. The primers used to amplify each region and the expected size of the products can be found in Table 4.1 and are depicted with double-headed arrows in Figure 4.2A.

Region amplified	**Forward primer**	**Reverse primer**	**Size of product (bp)**
ssc58681- ssc58671	Ssc58681 inside_fwd	Ssc586271 inside_rev	580
ssc58671-ssc58661	ssc58671_RTPCR_fwd	ssc58661_RTPCR_rev	486
ssc58661-ssc58651	ssc58661_RTPCR_fwd	ssc58651_RTPCR_rev	532
ssc58651-ssc58631	ssc58651_RTPCR_fwd	ssc58631_RTPCR_rev	801
ssc58631-ssc58621	ssc58631_RTPCR_fwd	ssc58621_RTPCR_rev	739
ssc58621-ssc58611	ssc58621_RTPCR_fwd	ssc58611_RTPCR_rev	750
ssc58621	Ssc58621 inside_fwd	Ssc58621 inside_rev	477
ssc58651	Ssc58651 inside_fwd	Ssc58651 inside_rev	1033
ssc58671	Ssc58671 inside_fwd	Ssc586271 inside_rev	244
ssc58681	Ssc58681 inside_fwd	Ssc58681 inside_rev	271
murX	DRB21	DRB22	144

Table 4.1: Primers used in RT-PCR analysis to detect expression of individual or neighbouring genes from the *S. scabies* T7SS chromosomal region. The expected size of each PCR product is shown.

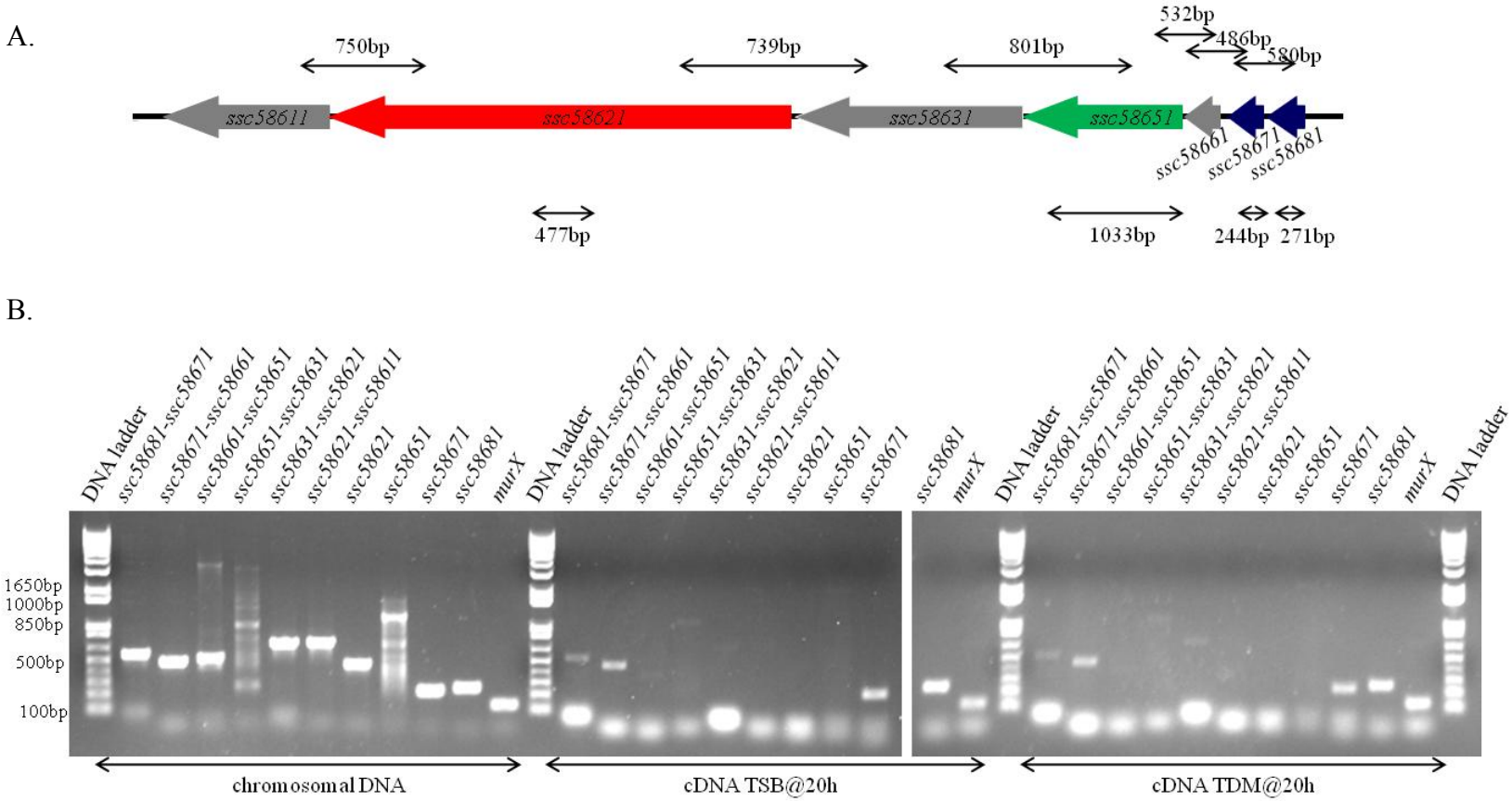

Figure 4.2: RT-PCR analysis to detect expression of individual or neighbouring genes from the *S. scabies* T7SS-encoding chromosomal region. Schematic representation the *S. scabies* T7SS chromosomal region with double headed arrows indicating the expected size of PCR products obtained following co-expression of genes (top, A) or individual genes (bottom, A). Agarose gel showing products obtained following PCR analysis of *S. scabies* 87.22 chromosomal DNA (left, B), cDNA from a 20 hours TSB culture of *S. scabies* 87.22 (middle, B) or cDNA from a 20h TDM culture of *S. scabies* 87.22 (right, B).

PCR analysis was carried out on each cDNA preparation using the primer pair listed in Table 4.1 (for more details of these also see Table 2.6). As a positive control for each PCR reaction, chromosomal DNA, prepared as described in Section 2.2.3, was used as a template. The results of this analysis can be seen in Figure 4.2B. To ensure the RNA preparations were not contaminated with chromosomal DNA during the extraction procedure, PCR reactions were also carried out using the RNA preparations as a template, yielding no products.

When PCR reactions were carried out using chromosomal DNA as a positive control, products of the expected size were obtained for each region (Figure 4.2B). Although it can be seen that multiple bands were obtained for the *ssc58651* or *ssc58651-ssc58631* regions, the brightest band resolved to the expected position following agarose gel electrophoresis.

Identical products were obtained following RT-PCR analysis of RNA samples prepared following growth on both media (Figure 4.2B, middle and right). From this it can be concluded that the T7SS of *S. scabies* is not specifically expressed as a response to the presence of plant material.

Strong bands can clearly be seen following RT-PCR with primers that amplify the *ssc58681-ssc58671* and *ssc58671-ssc58661* regions, which indicates that the genes *ssc58681, ssc58671* and *ssc58661* are co-transcribed. A faint band was obtained following RT-PCR with primers that amplify the *ssc58661-ssc58651* region, however, this band resolved to a lower position than expected following agarose gel electrophoresis so no conclusion can be drawn from this. Faint bands were also obtained following RT-PCR with primers that amplify the *ssc58651-ssc58631* and *ssc58631-ssc58621* regions, this time of the expected size, indicating that *ssc58651, ssc58631* and *ssc58621* are co-transcribed. However, no band was obtained following the PCR reaction to detect the *ssc58621-ssc58611* region indicating that these genes are most likely not co-transcribed.

The presence of a strong band following RT-PCR with primers that amplify an internal region of *ssc58681* and *ssc58671* indicated that these genes are expressed during growth in both TSB and TDM medium. No bands of the correct size were obtained for RT-PCR analysis with primers that amplify an internal region of *ssc58621* or *ssc58651*. This implies that these genes are not being expressed under these conditions. However, this conflicts with the previously mentioned evidence showing that bands were obtained following RT-PCR with primers that amplify the *ssc58631-ssc58621* and *ssc58651-ssc58631*. Taken together these data indicate that genes *ssc58681* to *ssc58621* form a transcriptional unit. Analysis of the chromosomal region surrounding these genes using BPROM, a web-based program that predicts promoter regions in bacterial genome sequences, showed a possible promoter region approximately 100 bp upstream of *ssc58681*. The proposed operon structure of the T7SS-encoding genes from *S. scabies* is shown in Figure 4.3.

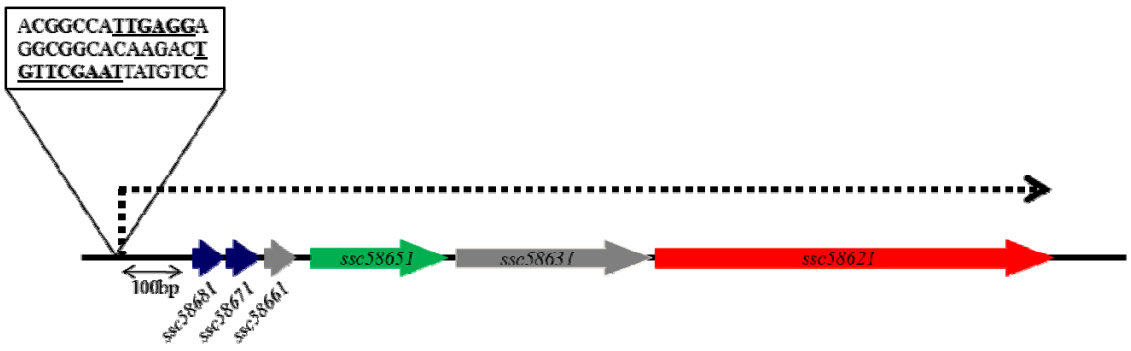

Figure 4.3: Proposed operon structure of genes from the *S. scabies* T7SS-encoding chromosomal region. The transcriptional unit is indicated with the dashed arrow above the genes. A possible promoter sequence approximately 100 bp upstream of *ssc58681* is shown in bold and underlined (predicted using BPROM tool sited at Softberry).

4.3 Genetic inactivation of the putative T7SS of *S. scabies*

4.3.1 Chromosomal deletion of *ssc58621*, *ssc58651*, *ssc58671* and *ssc58681*

The genes *ssc58621* (encoding the putative FSD protein), *ssc58651* (encoding the putative pore protein), *ssc58671* and *ssc58681* (encoding the probable WXG100 proteins) were selected for disruption, and the individual deletions were each marked with an apramycin resistance cassette. To this end, PCR amplification of the disruption cassette, FRT-*oriT*-*aac*(3)*IV*- FRT, (using pIJ773 as a template) was carried out using primer pairs "Ssc58621 KO Fwd" and "Ssc58621 KO Rev", "Ssc58651 KO Fwd" and "Ssc58651 KO Rev", "Ssc58671 KO Fwd" and "Ssc58671 KO Rev" or "Ssc58681 KO Fwd" and "Ssc58681 KO Rev" (Table 2.6) and the PCR products were purified away from the template DNA by agarose gel electrophoresis as described in Section 2.3.1.1.

The *S. scabies* chromosomal region encoding *ssc58621* to *ssc58681* is present on cosmid clone 212, and each selected gene was subjected to PCR-targeted gene replacement as described in Section 2.3.1.2. The mutated cosmids were then transferred from *E. coli* to *S. scabies* 87.22 by conjugation, as described in Section 2.3.1.3, after which they integrated into the chromosome at the *ssc58621*, *ssc58651*, *ssc58671* or *ssc58681* genomic regions by homologous recombination. Double-crossover mutants were identified by replica plating, selecting for apramycin resistance (specified by the disruption cassette) and kanamycin sensitivity (from the cosmid backbone) as described in Section 2.3.1.4. The resultant strains were designated JKFSCAB1 (Δ*ssc58621*), JKFSCAB2 (Δ*ssc58651*), JKFSCAB3 (Δ*ssc58671*) and JKFSCAB4 (Δ*ssc58681*).

4.3.2 **Verification of *ssc58621, ssc58651, ssc58671* and *ssc58681* mutant strains by Southern blot analysis**

To verify chromosomal deletion of *ssc58621*, *ssc58651, ssc58671* and *ssc58681* chromosomal DNA was prepared from each of the strains, along with the parental strain, as described in Section 2.2.3 and analysed by Southern blot.

Chromosomal DNA (5 µg) from each strain was then subjected to overnight restriction digest with either *Sal*I (*S. scabies* 87.22, JKFSCAB1, JKFSCAB2 and JKFSCAB3) or *Mlu*I (*S. scabies* 87.22 and JKFSCAB4). The resultant DNA fragments were resolved by agarose gel electrophoresis and transferred to a nylon membrane as described in Section 2.2.11.

Probes for each gene to be tested were prepared by PCR using primer pairs "ssc58621 South Fwd" and "ssc58621 South Rev", ssc58651 South Fwd" and "ssc58651 South Rev", ssc58671 South Fwd" and "ssc58671 South Rev" or "ssc58681 South Fwd" and "ssc58681 South Rev" (Table 2.6), as described in Section 2.2.11.3. Each probe was designed to hybridise to the chromosomal region immediately upstream of the gene targeted for deletion, so that binding to a DNA fragment would be detected for both the wild-type and mutant strain. Due to an alteration in restriction pattern resulting from replacement of the targeted gene with the FRT-*oriT*-*aac*(3)*IV*- FRT cassette, a size difference of this DNA fragment should be observed if the disruption cassette recombined at the correct position on the chromosome. The expected size of DNA fragments that the probe will hybridise to for wild-type and mutant strains can be found in Table 4.2, and a schematic diagram outlining the detection procedure, using *ssc58621* as an example, can be seen in Figure 4.4.

Gene targeted for deletion	Chromosomal DNA digested with	Size if wild-type gene detected (bp)	Size if gene deletion detected (bp)
ssc58621	*Sal*I	2036	741
ssc58651	*Sal*I	2736	430
ssc58671	*Sal*I	643	362
ssc58681	*Mlu*I	1076	1894

Table 4.2: Expected size of DNA fragments for probe hybridisation in wild-type and mutant strains during Southern blot analysis

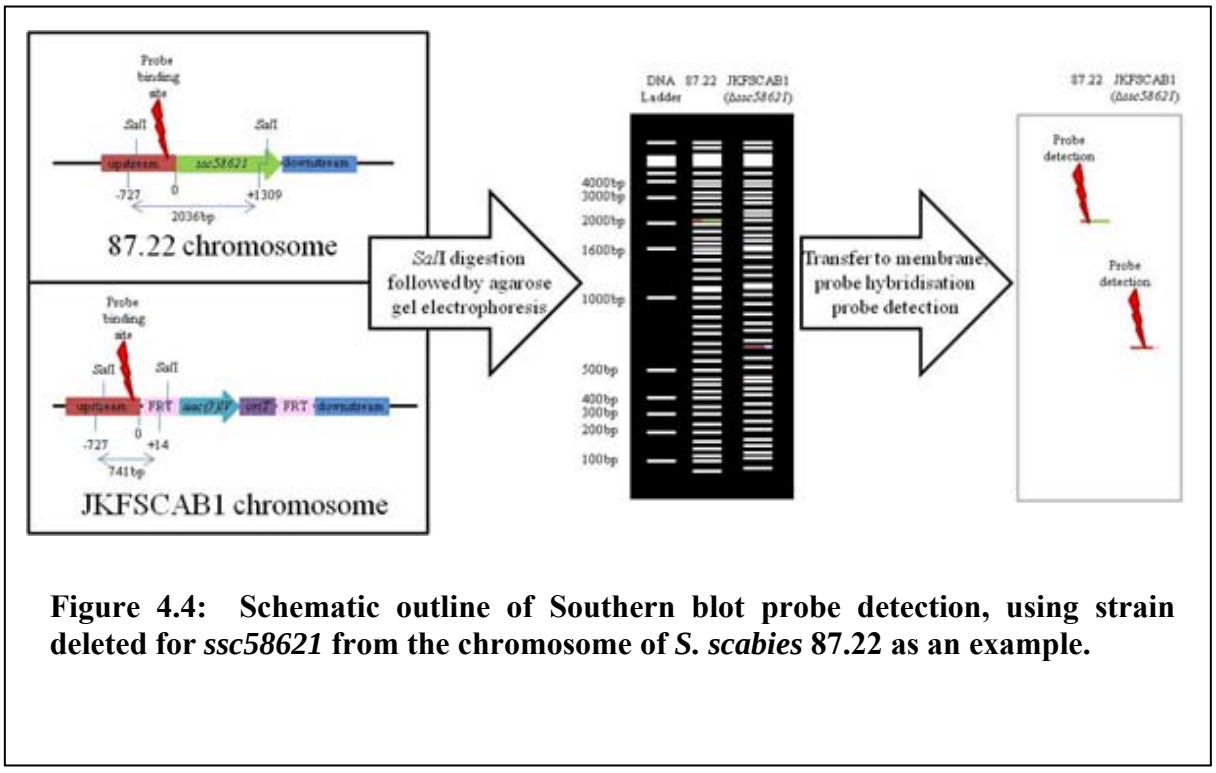

Figure 4.4: Schematic outline of Southern blot probe detection, using strain deleted for *ssc58621* from the chromosome of *S. scabies* 87.22 as an example.

The nylon membrane was cut into sections so that each contained DNA fragments derived from *S. scabies* 87.22 alongside the DNA fragments derived from one each of the four mutant strains. Each section was then incubated with the appropriate probe corresponding to the gene targeted for deletion. Hybridisation of this to a membrane-bound DNA fragment was then visualised, as described in Section 2.2.11.4. The results of this can be seen in Figure 4.5.

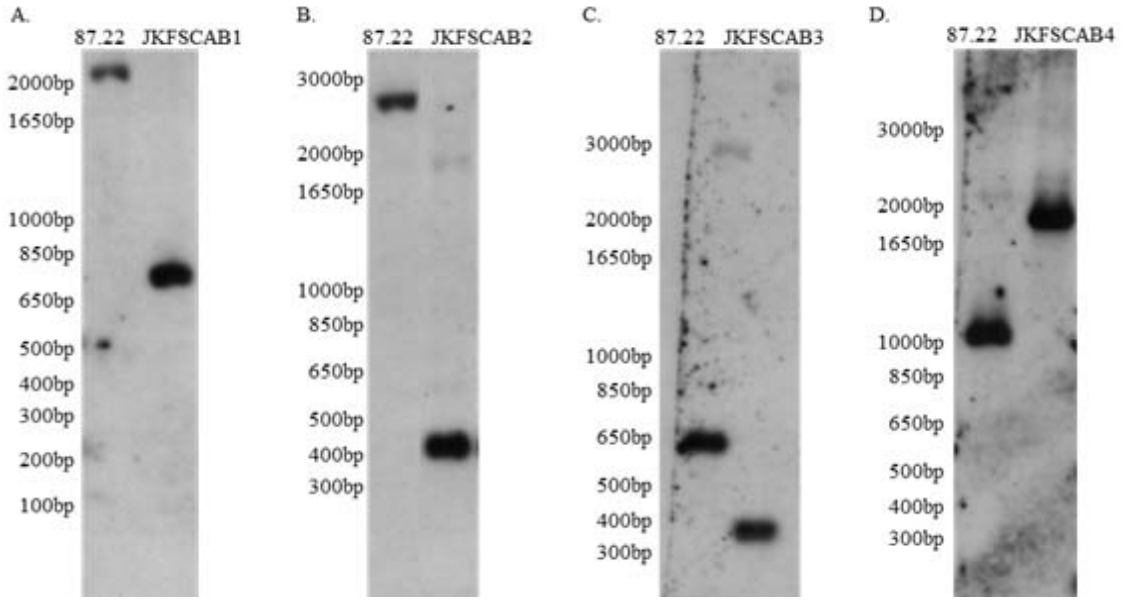

Figure 4.5: Southern blot to confirm deletion of T7SS genes from the chromosome of *S. scabies* 87.22. *Sal*I-digested DNA from *S. scabies* 87.22 and JKFSCAB1 was probed with a DNA probe that hybridises upstream of *ssc58621* (A), *Sal*I-digested DNA from *S. scabies* 87.22 and JKFSCAB2 was probed with a DNA probe that hybridises upstream of *ssc58651* (B), *Sal*I-digested DNA from *S. scabies* 87.22 and JKFSCAB3 was probed with a DNA probe that hybridises upstream of *ssc58671* (C) and *Mlu*I-digested DNA from *S. scabies* 87.22 and JKFSCAB4 was probed with a DNA probe that hybridises upstream of *ssc58681* (D)

It is clear that the *ssc58621* probe bound to a *Sal*I restriction fragment of chromosomal DNA derived from the parental strain 87.22 of approximately 2000 bp (Figure 4.5A, lane 1) whereas it bound to a *Sal*I restriction fragment of chromosomal DNA derived from JKFSCAB1 of approximately 750 bp (Figure 4.5A, lane 2). For *ssc58651*, the probe bound to a *Sal*I restriction fragment of chromosomal DNA derived from the parental strain 87.22 of just below 3000 bp (Figure 4.5B, lane 1) whereas it bound to a *Sal*I restriction fragment of chromosomal DNA derived from JKFSCAB2 of approximately 400 bp (Figure 4.5B, lane 2). The *ssc58671* probe bound to a *Sal*I restriction fragment of chromosomal DNA derived from the parental strain 87.22 of approximately 650 bp (Figure 4.5C, lane 1) whereas it bound to a *Sal*I restriction fragment of chromosomal DNA derived from

JKFSCAB3 of approximately 350 bp (Figure 4.5C, lane 2). Finally, the *ssc58681* probe bound to a *Mlu*I restriction fragment of chromosomal DNA derived from the parental strain 87.22 of approximately 1000 bp (Figure 4.5D, lane 1) whereas it bound to a *Mlu*I restriction fragment of chromosomal DNA derived from JKFSCAB4 of just below 2000 bp (Figure 4.5D, lane 2).

In each case the expected size shift of DNA between the wild-type and mutant strains that the probe bound to was observed (Table 4.2), confirming replacement of the wild-type copy of each gene on the chromosome with the FRT-*oriT*-*aac*(3)*IV*- FRT disruption cassette.

4.4 Phenotypic analysis of *S. scabies* T7SS mutant strains

4.4.1 Growth analysis of *S. scabies* T7SS mutant strains on solid media

To analyse any phenotypic effects associated with loss of components encoding the T7SS, *S. scabies* T7SS mutant strains were cultured alongside the parental strain 87.22 on nine different types of solid media and inspected periodically over the course of one week to visually assess their growth rate, morphology and sporulation pattern. The media utilised in this analysis are routinely used in laboratories working with *Streptomyces* and the properties of each have been described previously in Section 3.4.1.

An equal number of spores derived from each strain were applied to the surface of the growth media and streaked out with an inoculation loop to cover each section of the plate. The plates were then incubated at 30°C and observed daily over the course of a week to look for phenotypic differences between the wild-type and mutant strains cultured on each media. Pictures were then taken of growth on the surface of the agar following five days incubation, the results of which are shown in Figure 4.6.

Some differences in strain behaviour were clearly revealed by these growth tests on some of the growth media, whereas on other media no apparent differences were seen. Thus,

for example, after five days of growth on MM+1 % mannitol (Figure 4.6F) and DNA medium (Figure 4.6H) each of the mutants displayed a similar growth and sporulation pattern to the wild type strain. Moreover, growth and sporulation of JKFSCAB2 (which is deleted for the gene that encodes the predicted pore protein) appeared similar to the wild type strain on all media tested.

However, clear differences between wild type and T7SS mutant strains JKFSCAB1, JKFSCAB3 and JKFSCAB4 were observed in aerial hyphae formation and sporulation pattern following growth on several media. Although growth of JKFSCAB2, JKFSCAB3 and JKFSCAB4 on R2YE (Figure 4.6D) and R2 (Figure 4.6E) was similar to the wild type strain, JKFSCAB1 (which is deleted for *scab58621* encoding the FtsK/SpoIIIE ATPase) appeared to form aerial hyphae more readily than any of these strains, observed as a white appearance to the surface of the biomass. Similarly, when grown on SFM, (Figure 4.6A) JKFSCAB1 also showed increased sporulation in comparison to the wild type strain, which is observed as darker grey pigmentation.

By contrast, a decrease in aerial hyphae formation and therefore sporulation was observed in comparison to the wild type strain when JKFSCAB3 and JKFSCAB4 (which are deleted for *scab58671* and *scab58681* encoding the WXG100 proteins) were grown on SFM (Figure 4.6A). This effect was also observed when these strains were grown on OBM (Figure 4.6B), IPM (Figure 4.6C), MM+1 % glucose (Figure 4.6G) and, most prominently, on YME (Figure 4.6I). On YME JKFSCAB3 and, to a greater extent, JKFSCAB4 appeared severely deficient in aerial hyphae formation (Figure 4.6I).

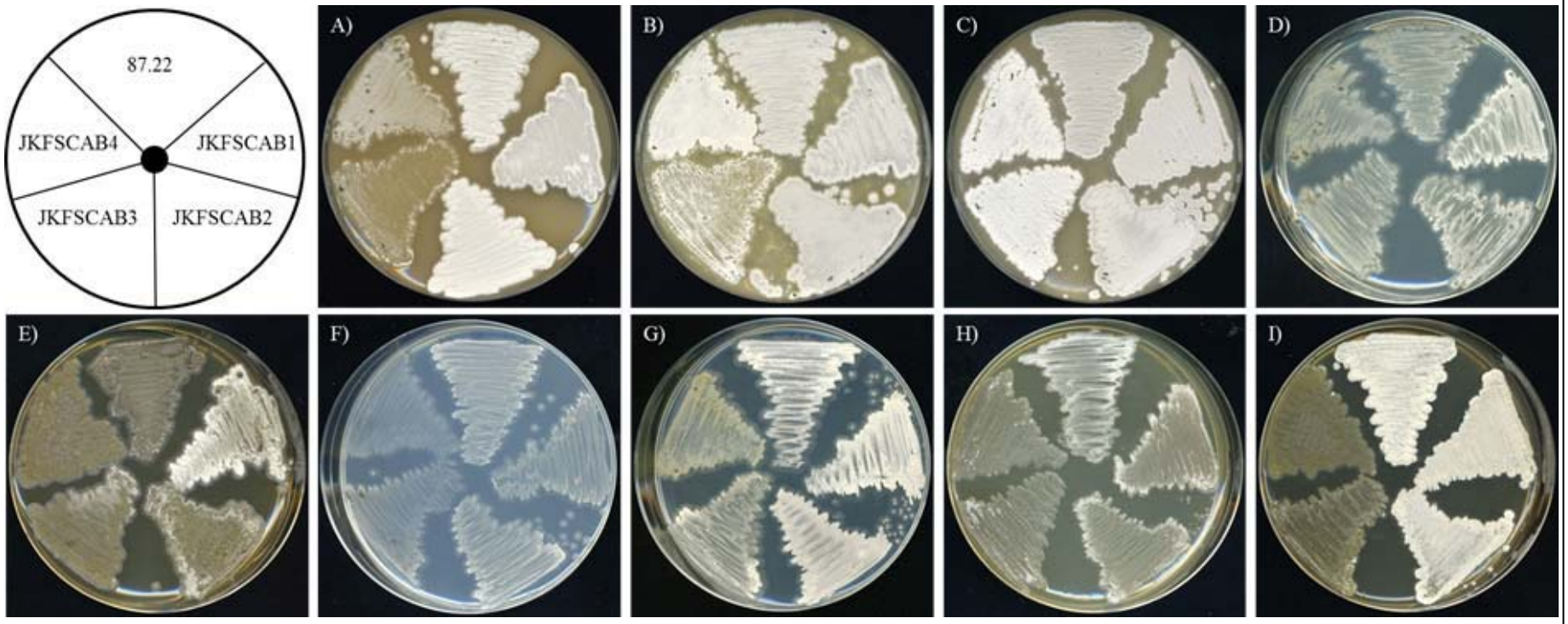

Figure 4.6: Growth analysis of *S. scabies* 87.22, JKFSCAB1, JKFSCAB2, JKFSCAB3 and JKFSCAB4 strains cultured on solid media and incubated at 30°C for five days. Equal numbers of spores were streaked from glycerol stock and grown on the different types of solid media SFM (A), OBM (B), IPM (C), R2 (D), R2YE (E), MM+1 % mannitol (F), MM+1 % glucose (G), DNA (H) and YME (I). The pictures shown represent five independent experiments.

From this it can be concluded that there are pleiotropic effects on the development of *S. scabies* associated with deletion of the genes encoding WXG100 proteins and the FSD protein but not for the gene encoding the putative pore protein.

It is not uncommon in *Streptomyces* for secondary mutations to arise during deletion of a gene, especially if the targeted gene makes an important contribution to the physiology of the organism. These are known as suppressor mutations as they suppress the effects of the primary mutation, for example, the lethal effects of *chpE* (encoding one of the eight chaplin proteins) deletion from the chromosome of *S. coelicolor* could be suppressed by loss of the other chaplins, loss of the rodlets or inactivation of the Tat pathway (Di Berado *et al.*, 2008). Similarly, it has also been observed that complementation of genes in involved in lipoprotein biogenesis in *S. coelicolor* and *S. scabies* failed to restore a wild-type growth pattern (Thompson *et al.*, 2010; Widdick *et al.*, 2011).

Suppressor mutants generally arise at a very low frequency and after a prolonged incubation period if the gene targeted for deletion performs an essential function. Although deletion of the genes encoding the WXG100 proteins occurred as readily as deletion of the other T7SS genes targeted here, it is still formally possible that the observed effects on sporulation in the *S. scabies* strains deleted for genes encoding the WXG100 proteins are the result of secondary mutations. In order to investigate this, an attempt to complement the sporulation defect was undertaken by constructing a plasmid, pScab_WxG_operon, containing the *S. scabies* chromosomal DNA sequence spanning the *ssc58681-ssc58671* region, including 225 nt upstream and 93 nt downstream (to allow for inclusion of the natural upstream promoter region and any possible downstream elements). This plasmid was then transferred from *E. coli* to *S. scabies* strains 87.22, JKFSCAB3 and JKFSCAB4 by conjugation, as described in Section 2.3.1.3, after which it integrated into the chromosome at the φC31 attachment site. Introduction of the plasmid encoding the *S. scabies* WXG100

genes into the chromosome of the wild-type strain 87.22 was intended to act as a control, with the expectation that introduction of this plasmid would have no deleterious effect on the phenotype of the organism.

The strains harbouring pScab_WxG_operon were then applied to the surface of YME medium alongside the parental strains, incubated at 30°C and the aerial hyphae formation pattern of each strain observed daily over the course of ten days. The result of this can be seen in Figure 4.7.

Following two days growth on YME medium the entire surface of the biomass formed by strain 87.22 showed aerial hyphae development (observed as a white appearance to the surface of the biomass). However, aerial hyphae development was only evident at the edges of the biomass formed by strains 87.22+pScab_WxG_operon, JKFSCAB3+pScab_WxG_operon and JKFSCAB4+pScab_WxG_operon. As expected, following two days growth no aerial hyphae formation was evident on the surface of the biomass formed by strains JKFSCAB3 and JKFSCAB4.

Following six days growth on YME medium the entire surface of the biomass formed by strain 87.22 developed spores (observed as a grey appearance to the surface of the biomass). This was also observed for strain 87.22+pScab_WxG_operon, however the spores appeared darker than those of 87.22 indicating that more spores were produced following introduction of a second copy of the WXG100 protein-encoding genes into the *S. scabies* chromosome. Aerial hyphae development was observed just at the edges of the biomass formed by strains JKFSCAB3 and JKFSCAB4. This defect in aerial hyphae formation was partially alleviated by introduction of pScab_WxG_operon into the chromosome of *S. scabies* strains JKFSCAB3 and, to a lesser extent, JKFSCAB4 following six days growth on YME medium. Thus partial complementation of the mutant strains was afforded by supplying additional copies of the WxG100 protein-encoding genes *in trans*.

The observation that introduction of a second copy of the WXG genes into the chromosome of strain 87.22 initially inhibited aerial hyphae formation then later resulted in an apparent increase in sporulation was unexpected. It is most likely that the introduction of additional copies of the WXG100 protein-encoding genes leading to an increased level of these proteins is responsible for the effects observed.

As partial complementation of the WXG100 mutant strains, JKFSCAB3 and JKFSCAB4 was observed following introduction of pScab_WxG_operon, this indicates that the defect in aerial hyphae formation can be attributed to the absence of the WXG100 proteins. However, as aerial hyphae formation was not restored to wild-type levels this indicates that the level of the WXG100 proteins is important or that additional factors may also be involved and this will discussed in more detail in Section 4.8.

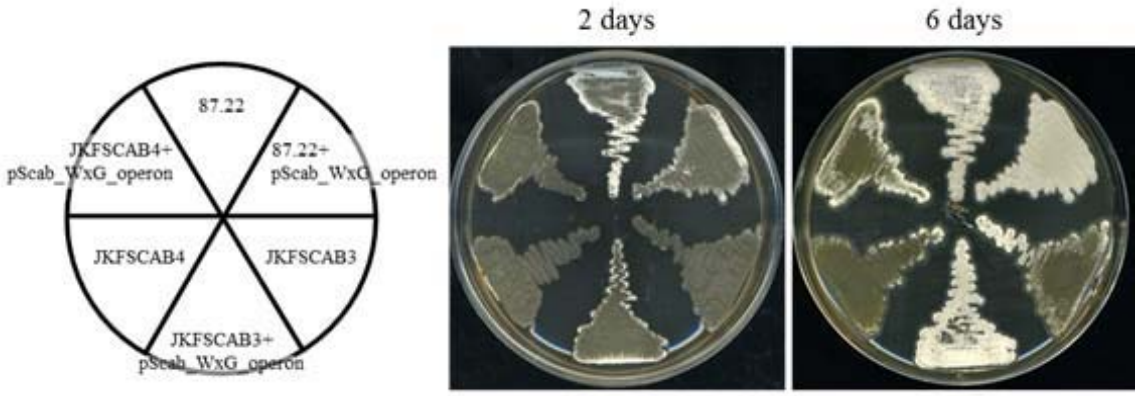

Figure 4.7: Complementation analysis of *S. scabies* 87.22, 87.22+pScab_WxG_operon, JKFSCAB3, JKFSCAB3+pScab_WxG_operon, JKFSCAB4 and JKFSCAB4+pScab_WxG_operon. Strains were cultured on YME medium and incubated at 30°C for the indicated time periods.

4.4.2 Growth analysis of *S. scabies* T7SS mutant strains in liquid culture

For reasons outlined in Section 3.4.2, measurement of total cytosolic protein was used to assess the growth rate of *S. scabies* strains grown in liquid culture. TSB medium was

inoculated with a spore stock and incubated and sampled as described in Section 2.1.4. At regular time intervals, samples were withdrawn, total cytosolic protein was prepared and its concentration assessed, as described in Section 2.5.1. Total cytosolic protein concentration values correlate with growth of *S. scabies* allowing production of growth curves from the data collected over the time-course. The results of growth rate analysis of the *S. scabies* strains 87.22, JKFSCAB1, JKFSCAB2, JKFSCAB3 and JKFSCAB4 are presented in Figure 4.8.

Both JKFSCAB1 and JKFSCAB2 displayed a similar growth pattern to the wild-type strain 87.22, entering logarithmic growth phase between 21-24 hours post inoculation and reaching a peak by 33 hours post inoculation. On the other hand, strains JKFSCAB3 and JKFSCAB4 grew more vigorously than the wild type strain following the initial lag phase, showing earlier entry into logarithmic growth phase at 18 hours post inoculation. However, the rate of growth remained similar to that of the wild type, JKFSCAB1 and JKFSCAB2 strains for the duration of the logarithmic growth period as each slope had a similar gradient (Figure 4.8). JKFSCAB4 entered stationary phase at 27 hours post inoculation, whereas all other strains examined, including the wild type, entered stationary phase at 33 hours post inoculation. However, the final levels of total cytosolic protein were similar for all of the strains, indicating that similar biomasses were achieved at the end of the growth cycle.

Although *S. scabies* does not sporulate in liquid culture, the difference in growth of JKFSCAB3 and JKFSCAB4 in liquid culture compared to strain 87.22 reflects the observations made following growth of these strains on solid media (strains JKFSCAB3 and JKFSCAB4 failed to develop normally with JKFSCAB4 being most severely affected, Figure 4.6).

It can be concluded from this that the presence of the FSD protein homologue, Ssc58621 and the putative pore protein Ssc58651 of the T7SS are not essential for normal

laboratory growth of *S. scabies*. However, as strains harbouring deletions of the genes encoding Ssc58671 and Ssc58681 grew more vigorously during the early growth phase in liquid medium this would indicate that the WXG100 proteins are important during early development.

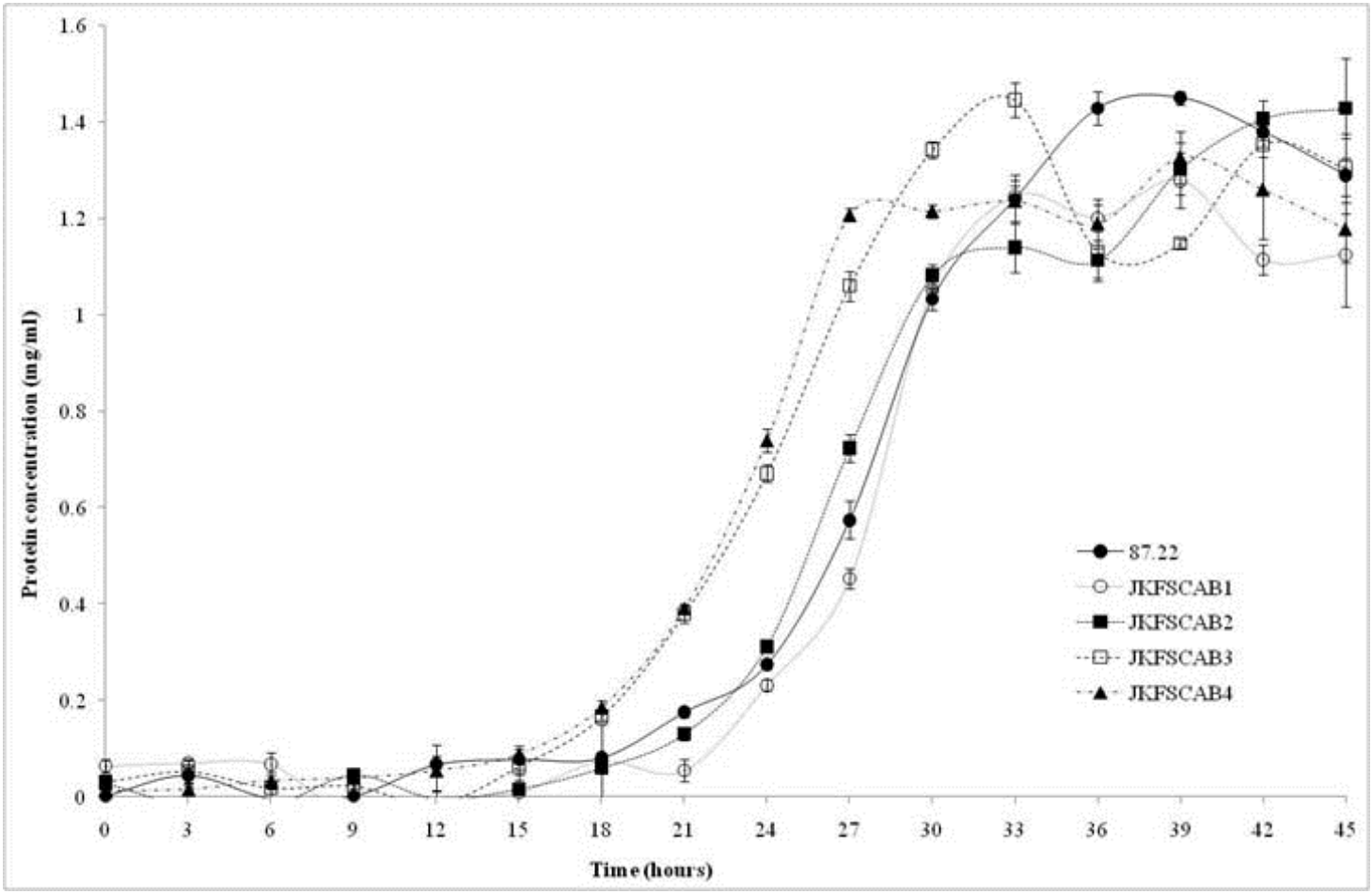

Figure 4.8: Growth rate analysis of *S. scabies* strains 87.22, JKFSCAB1, JKFSCAB2, JKFSCAB3 and JKFSCAB4 in liquid TSB medium. 100 ml aliquots of TSB medium was inoculated with spores of the strain of interest, to a density of $1x10^6$ per ml of culture medium, and incubated at 30°C with shaking. 1 ml samples were withdrawn from the cultures every three hours and total cytosolic protein prepared as described in Section 2.5.1. Protein concentration was assessed using the BioRad DC protein assay. Error bars represent the standard error of the mean, where n=3.

4.4.3 Analysis of the effect of SDS on the growth of *S. scabies* T7SS mutant strains

As discussed in Section 3.4.3, treatment of cells with the ionic detergent sodium dodecyl sulphate (SDS) is an effective way of disrupting cell membranes by destabilising the interaction of lipids with other lipids and proteins, and mutations in protein secretion systems may lead to defects in cell envelope stability which manifest as increased SDS sensitivity.

In order to determine the range of SDS concentrations that permit growth of *S. scabies* whilst still exerting stress, an initial experiment was carried out in which IPM and YME media were supplemented with SDS at concentrations of 1 %, 0.1 %, 0.01 % and 0.001 %. These media were selected as they are routinely used for culturing *S. scabies* and, in the case of YME, a defect in aerial hyphae formation and sporulation has been observed for two of the four T7SS mutants growing on this medium (Figure 4.6) so it is possible that additional deleterious effects brought about by mutations in the putative T7SS of *S. scabies* may be revealed when grown on this media under conditions of stress. Growth of *S. scabies* 87.22 on these media was observed over the course of one week allowing the tolerance of *S. scabies* to SDS to be established. Growth of *S. scabies* on YME was inhibited at concentrations of 0.01 % SDS whereas growth of *S. scabies* on IPM medium supplemented with SDS at a concentration of 0.1 % was reduced with inhibition observed at 1 % (data not shown). Based on these finding it was decided that YME medium would be supplemented with SDS at concentrations ranging from 0.001 % to 0.01 % and that IPM medium would be supplemented with SDS at concentrations ranging from 0.01 % to 0.1 %.

To determine whether deletion of genes encoding components of the putative T7SS affects membrane stability of *S. scabies*, a dilution series was prepared from pre-enumerated spore stocks of strains *S. scabies* 87.22, JKFSCAB1, JKFSCAB2, JKFSCAB3 and JKFSCAB4 so that 2 µl aliquots contained approximately $1x10^5$ spores down to $1x10^0$ in increments of 10 fold. These spore dilution series were then applied to the surface of YME

medium supplemented with SDS at concentrations of 0.001 %, 0.0025 %, 0.005 %, 0.0075 % and 0.01 % and IPM medium supplemented with SDS at concentrations of 0.01 %, 0.025 %, 0.05 %, 0.075 % and 0.1 %. Plates were incubated at 30°C for nine days, during which time growth of the strains exposed to different concentrations of SDS was assessed periodically. The results of this analysis can be seen in Figure 4.9 (note that very little growth was observed on YME medium at 0.0075 % SDS or above so these plates were omitted from the final analysis). Close inspection of the results indicate that the ability of the *S. scabies* T7SS mutant strains to grow in the presence of SDS on both YME and IPM media is not affected when compared to the wild-type strain.

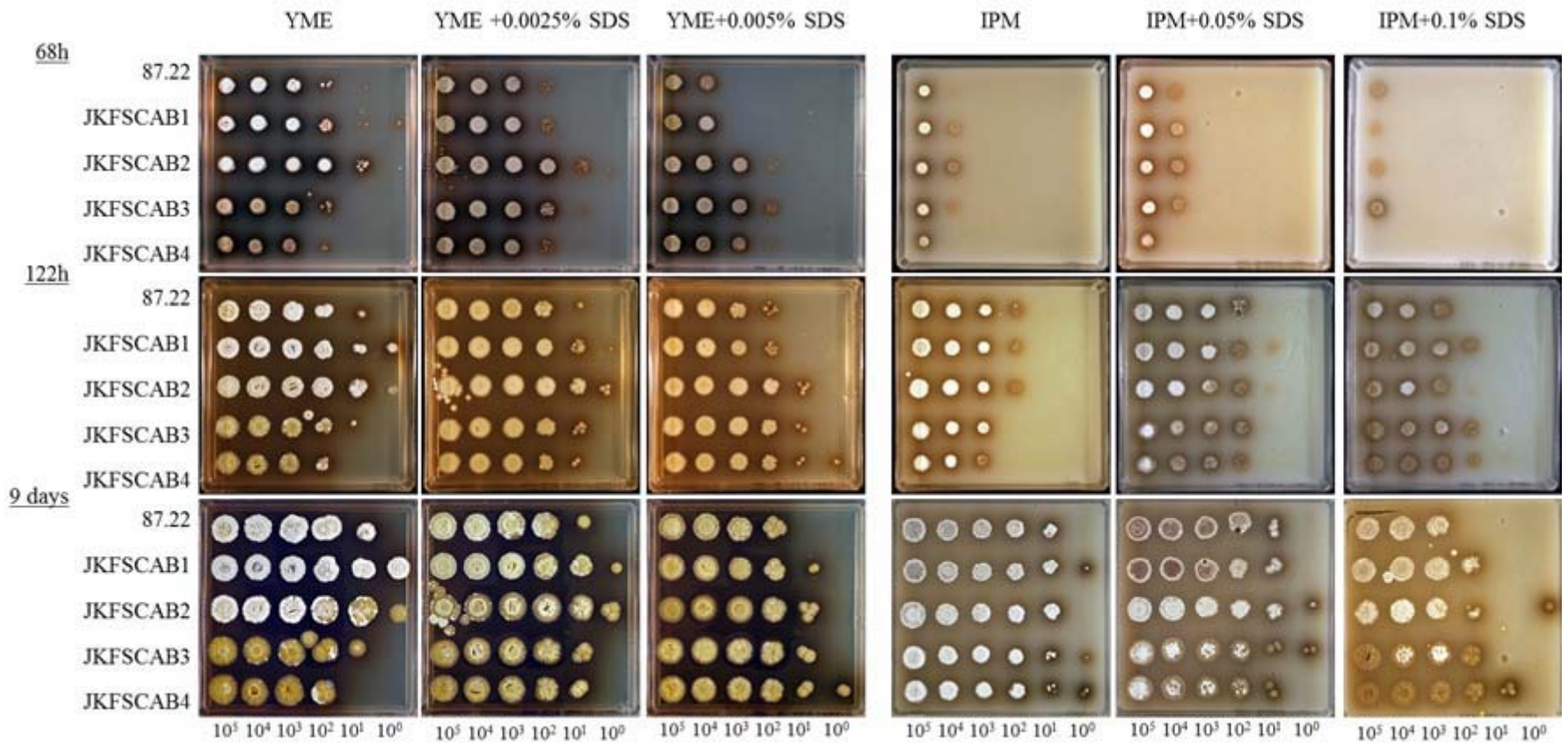

Figure 4.9: Analysis of the effect of SDS on the growth of *S. scabies* 87.22, JKFSCAB1, JKFSCAB2, JKFSCAB3 and JKFSCAB4 strains. A dilution series was prepared from pre-enumerated spore stocks and 2 µl aliquots containing from approximately $1x10^5$ spores down to $1x10^0$ spores were applied to the surface of YME and IPM medium supplemented with SDS at the indicated concentrations. Plates were incubated at 30°C for the indicated time periods prior to being photographed. The pictures shown represent three independent experiments

4.4.4 Analysis of the effect of osmotic stress on the growth of *S. scabies* T7SS mutant strains

As discussed in Section 3.4.5, treatment of *S. coelicolor* strains carrying mutations in genes coding for the FSD proteins of the two T7SS resulted in premature production of the pigmented antibiotic actinorhodin when the cells were stressed osmotically (Figure 3.10). Although it should be noted that *S. scabies* does not produce either of the pigmented antibiotics actinorhodin or undedycprodigiosin, it was hypothesised that exposure of the *S. scabies* T7SS mutants to osmotic stress may also reveal physiological defects.

In order to investigate this, the osmolyte sucrose was used to supplement the two growth media, IPM and YME, that were again selected for these experiments for the reasons outlined in Section 4.4.3.

4.4.4.1 Analysis of the effect of sucrose on the growth of *S. scabies* T7SS mutant strains

To determine the effect of sucrose on the growth of *S. scabies* strains T7SS mutant strains the same procedure was followed as described for testing the effect of SDS in Section 4.4.3 with the exception that the growth media was supplemented with sucrose to a final concentration of 20 % or 30 %. Plates were incubated at 30°C for nine days, during which time growth of the strains was assessed periodically and the results of this analysis can be seen in Figure 4.10.

Although some variation was observed in growth between different strains cultured on individual plates, this was not a consistent observation and can be attributed to fluctuations in the spore number applied with each aliquot. It is therefore clear that the ability of the *S. scabies* T7SS mutant strains to grow in the presence of sucrose on both YME and IPM media is similar to the wild-type strain (Figure 4.10).

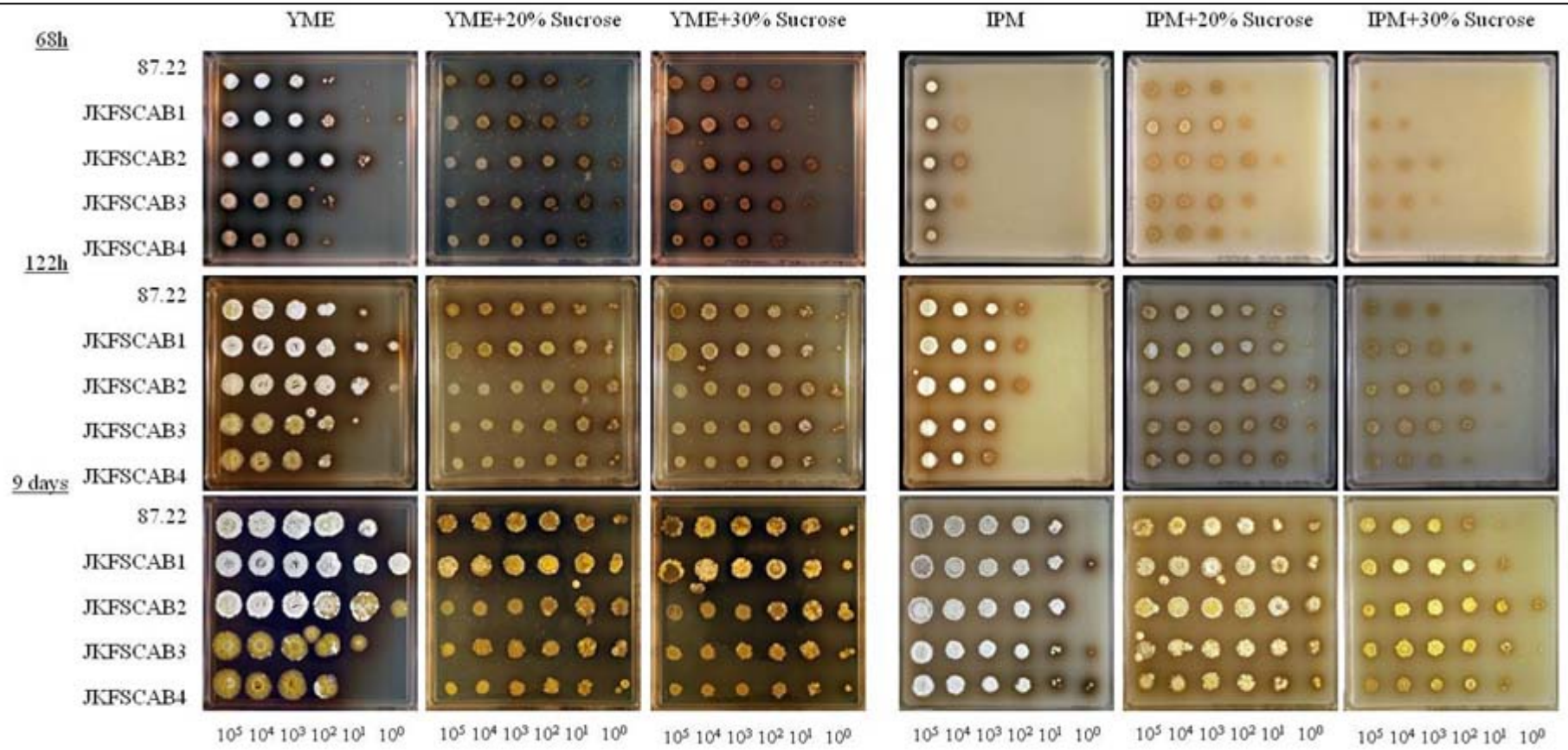

Figure 4.10: The effect of osmotic stress on the growth of *S. scabies* strains 87.22, JKFSCAB1, JKFSCAB2, JKFSCAB3 and JKFSCAB4. A spore dilution series was prepared for each strain and 2 µl aliquots, containing from approximately $1x10^5$ spores down to $1x10^0$ spores were applied to the surface of YME and IPM medium supplemented with either 20 % sucrose or 30 % sucrose (as indicated above). Plates were incubated at 30°C for the indicated time periods prior to being photographed. The pictures shown represent three independent experiments

4.4.5 Analysis of the effect of heat on the viability of spores derived from *S. scabies* T7SS mutant strains

A further physiological effect caused by absence of the Tat protein transport machinery in *S. scabies* is a reduced ability of spores to germinate after a heat shock (Joshi *et al.*, 2010). The effect of temperature on viability of spores of *S. scabies* that lacked components of the T7SS was therefore also assessed, by qualitative analysis of the growth of spores derived from the *S. scabies* strains 87.22 JKFSCAB1, JKFSCAB2, JKFSCAB3 and JKFSCAB4 following exposure to heat. To this end, spore stocks of *S. scabies* strains 87.22, JKFSCAB1, JKFSCAB2, JKFSCAB3 and JKFSCAB4 were prepared in 20 % glycerol as described in Section 2.1.1. Prior to treatment, a dilution series of each spore stock was prepared in sterile water and 100 µl aliquots from each of the 10^{-6} and 10^{-7} dilutions were spread onto the surface of LB agar plates. These were incubated at 30°C for three days after which time the colonies were counted, allowing calculation of the total viable count for the untreated spores.

To assess the effect of heat treatment on spore germination, dilution series were prepared in sterile water and the 10^{-4}, 10^{-5} and 10^{-6} dilutions for each strain were incubated at 50°C for either 15, 30 and 60 minutes. 100 µl aliquots from each dilution were then spread onto the surface of LB agar plates, incubated for three days at 30°C and the colonies counted to enumerate the total number of viable spores following treatment. This was then used to calculate the percentage of viable spores following treatment, by comparison to the untreated spores. The results of this analysis are shown in Figure 4.11.

Although some differences were observed between the viability of spores derived from each strain this could not be attributed to a specific strain when this experiment was replicated. As such this has been attributed to natural variation in the data. It can be

concluded from this that deletion of the genes encoding components of the putative T7SS in *S. scabies* does not appear to influence spore viability when exposed to high temperatures.

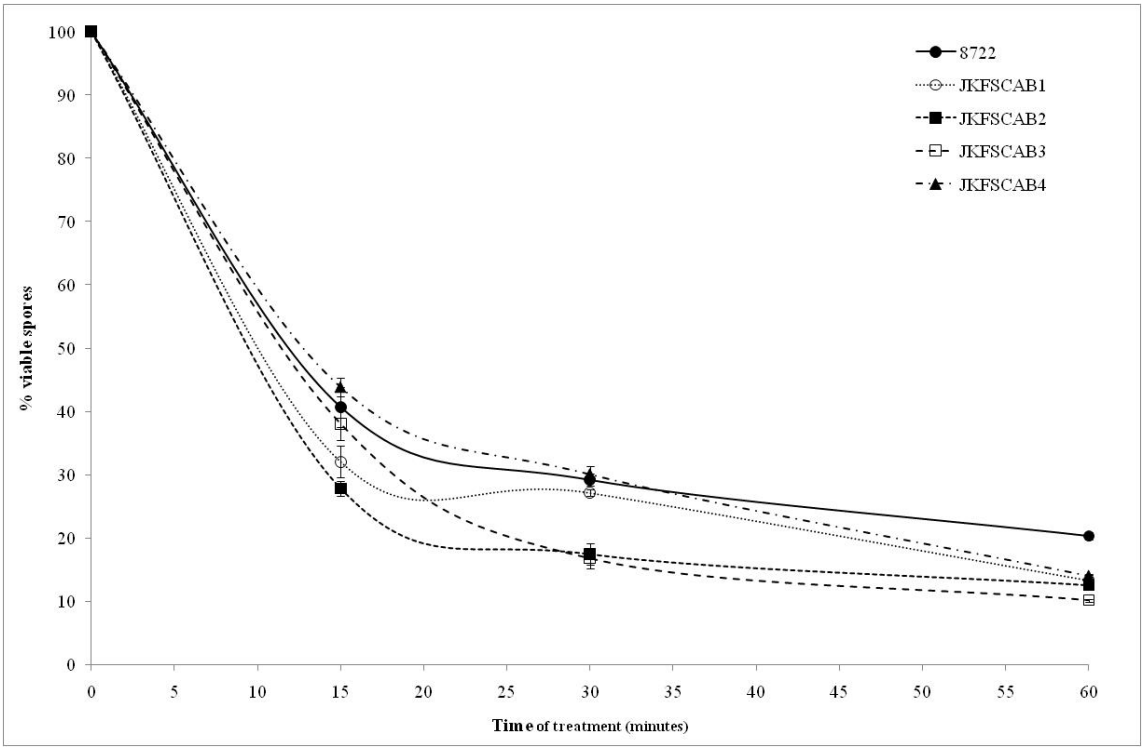

Figure 4.11: The effect of heat treatment on the viability of spores derived from *S. scabies* strains 87.22, JKFSCAB1, JKFSCAB2, JKFSCAB3 and JKFSCAB4. Freshly harvested spores were prepared in 20 % glycerol and used to prepare dilution series in water. Dilution series were incubated at 50°C for 15, 30 and 60 minutes then 100 µl aliquots from the 10^{-4}, 10^{-5} and 10^{-6} dilutions were spread onto the surface of LB agar plates and colonies counted after three days incubation at 30°C. The number of viable spores following treatment was then expressed as a percentage of the number of viable spores from the freshly prepared spore suspension. Error bars represent the standard error of the mean, where n=3.

4.4.6 Analysis of spores by scanning electron microscopy

S. scabies, along with a number of other streptomycetes, produce spore chains that coil to give a spiral arrangement (Lambert & Loria, 1989a). The observation that some of the *S. scabies* T7SS mutant strains, in particular JKFSCAB3 and JKFSCAB4, displayed a media-dependent defect in aerial hyphae formation and sporulation raised the possibility that spore chains may have altered morphology in comparison to the wild-type strain.

To investigate this, *S. scabies* strains 87.22, JKFSCAB1, JKFSCAB2, JKFSCAB3 and JKFSCAB4 were cultured on IPM medium and samples of each strain showing spore development were prepared following ten days growth as described in Section 2.6, prior to analysis by SEM. Sample preparation and assistance with operation of the microscope was provided by Martin Kierans**,** Centre for High Resolution Imaging and Processing (CHIPS), School of Life. Sciences, University of Dundee. The results of this can be seen in Figure 4.12.

It is clear that whereas the strains, 87.22, JKFSCAB1 and JKFSCAB2 formed masses of spiral shaped spore chains (Figure 4.12, panel A, B and C) the WXG100 mutant strains JKFSCAB3 and JKFSCAB4 showed very little evidence of spiral spore chain formation (Figure 4.12, panel D and E). When examined under higher magnification it can also be seen that the spore chains derived from strains 87.22, JKFSCAB1 and JKFSCAB2 are uniform in size (Figure 4.12, panel F, G and H), whereas those derived from strains JKFSCAB3 and JKFSCAB4 form chains of differently sized spores (Figure 4.12, panel I and J). This indicates that whereas the FSD and putative pore proteins of the T7SS are dispensable for spore formation under the conditions tested the WXG100 proteins are required for the correct formation of spore chains in *S. scabies*.

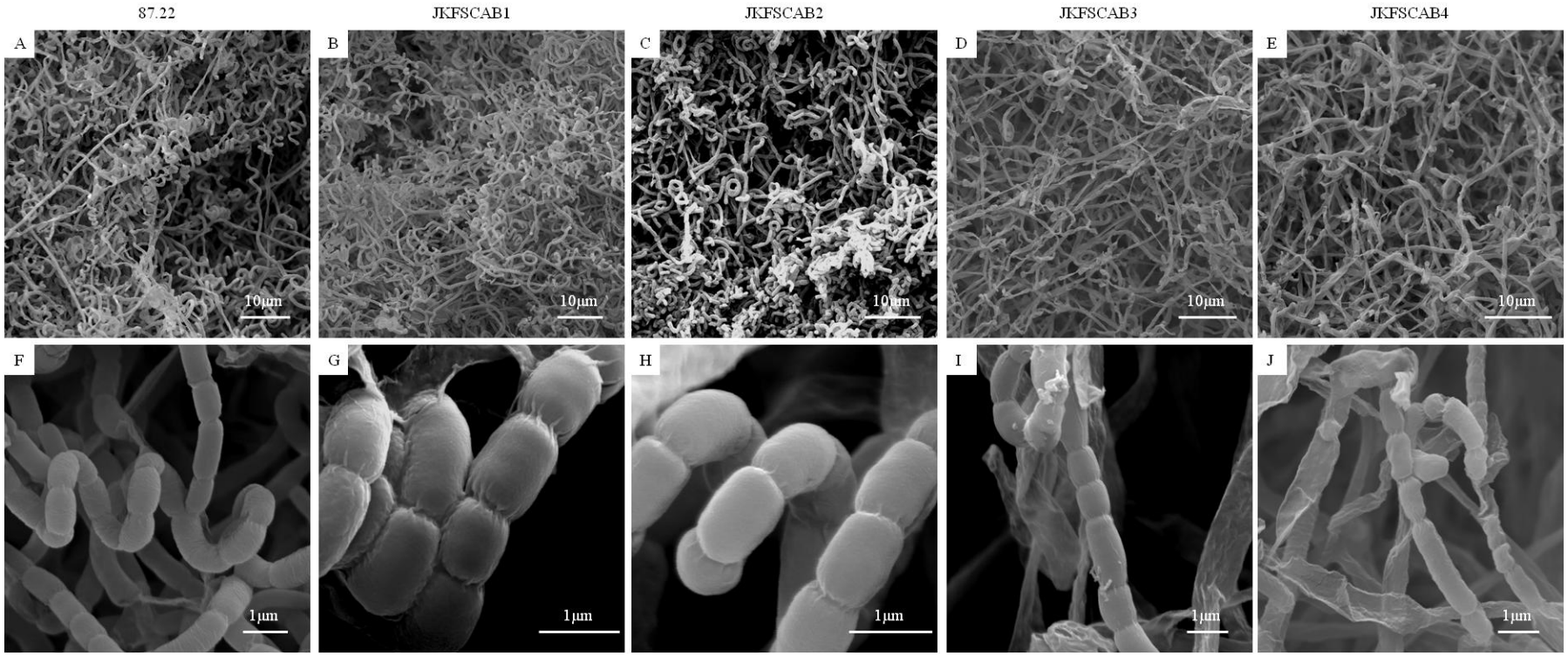

Figure 4.12: Scanning electron microscopy images of spores derived from *S. scabies* strains 87.22 (A & F), JKFSCAB1 (B & G), JKFSCAB2 (C & H), JKFSCAB3 (D & I) and JKFSCAB4 (E & J).

4.5 Proteomic analysis of the secretomes of *S. scabies* wild type and T7SS mutant strains

As discussed in Section 3.5 the WXG100 proteins, encoded in the same genetic loci as the FSD protein, are canonical substrates of the T7SS and therefore serve as useful indicators of functionality of the T7SS. However, other substrate proteins of T7SSs are often encoded in different genetic loci from the secretion apparatus (Fortune *et al.*, 2005; MacGurn *et al.*, 2005). Together with the fact that they appear to lack a consistent signal sequence and share very little homology with other known substrates these must be identified experimentally.

4.5.1 Western blot analysis to detect T7SS-dependent secretion of WXG100 proteins

Based on the studies of T7SS from other organisms, a strong prediction is that the secretion of the WXG proteins should be defective in the mutant strain encoding the FSD protein of the T7SS, JKFSCAB1, constructed in this study.

To analyse whether the secretion of Ssc58671 and Ssc58681 was affected in JKFSCAB1, pSsc58671_6H and pSsc58681_6H, that express each of the WXG100 proteins containing a C-terminal his-tag from the strong constitutive promoter *ermE* from *S. erythraea*, were introduced into *S. scabies* strains 87.22 and JKFSCAB1. Protein samples were then prepared from whole cells and the culture supernatant of *S. scabies* strains 87.22 and JKFSCAB1 harbouring these constructs which had been growing in TSB medium for 20 hours, as described in Section 2.5.2 and 2.5.3. Cell-wall wash samples were also prepared following 20 hours growth on YME, as described in Section 2.5.4. Proteins were separated by SDS PAGE as described in Section 2.5.5 and western blotting was subsequently carried out as described in Section 2.5.6 using the monoclonal α-His_4 antibody at the concentration listed in Table 2.10. However, even when the film was exposed for a period of several hours no protein could be detected in any sample (data not shown). The cross-reactivity of the

antibody and functioning of the ECL detection system were confirmed as a signal was detected for the His-tagged protein standard used. It is possible that the addition of a C-terminal his-tag interferes with the correct function of the WXG100 proteins causing them to be rapidly degraded, however, it should be noted that earlier attempts using an N-terminal His-tag also failed to lead to detection of the proteins by Western blot (data not shown). Alternatively, there may be a problem with expression of the proteins for unknown reasons.

4.5.2 2D SDS PAGE analysis of cell-wall associated proteins from *S. scabies* 87.22, JKFSCAB1, JKFSCAB3 and JKFSCAB4 strains

As discussed in Section 3.5.2, a number of comparative proteomic studies have been carried out in both Gram-positive and Gram-negative bacteria leading to identification of substrates of the Tat system (Jongbloed *et al.*, 2002; Jongbloed *et al.*, 2004; Posey *et al.*, 2006; Widdick *et al.*, 2006; De Buck *et al.*, 2008; Joshi *et al.*, 2010). Two of these studies were conducted by the Palmer laboratory and focussed on the identification of Tat-substrate proteins from *S. coelicolor* (Widdick *et al.*, 2006) and its pathogenic relative *S. scabies* (Joshi *et al.*, 2010) using 2D gel electrophoresis. As this technique has also been used to identify a number of proteins secreted by the T7SS in *Mycobacteria* (Mattow *et al.*, 2003; MacGurn *et al.*, 2005; Abdallah *et al.*, 2009) this approach was deemed applicable to the study carried out here.

In all bacteria studied to date, loss of the FSD protein of the T7SS has been shown to block secretion of the WXG100 proteins as well as other substrates of the system. We therefore utilised JKFSCAB1, harbouring a marked deletion in the gene encoding the FSD protein, to study the secretome of *S. scabies* by 2D-gel electrophoresis aimed at identification of substrates of the T7SS. As some substrate proteins of the T7SS in *Mycobacteria* have been shown to be mutually dependent on the WXG100 proteins for secretion we

hypothesised that the same may also be true for *S. scabies*. Therefore, we also utilised strains JKFSCAB3 and JKFSCAB4, harbouring mutations in each of the genes encoding a WXG100 protein, for analysis by 2D-gel electrophoresis.

The strain JKFSCAB2, harbouring mutation in *ssc58651* (hypothesised to encode the pore protein of the T7SS), was not utilised for analysis by 2D-gel electrophoresis as deletion of *sco5735*, encoding the orthologous protein from *S. coelicolor*, has been shown not to be essential for secretion of the WXG100 proteins (Akpe San Roman *et al.*, 2010).

Phenotypic analysis of the mutant strains grown on different media revealed that when *S. scabies* strains JKFSCAB3 and JKFSCAB4 were grown on YME medium they showed pleiotropy compared to the wild-type strain, 87.22 (Figure 4.6). This indicated that the T7SS may be active during growth in this medium. Therefore the *S. scabies* strains 87.22, JKFSCAB1, JKFSCAB3 and JKFSCAB4 were cultured on the surface of cellophane discs on YME agar plates for 48 hours then cell-wall wash samples prepared as described in Section 2.5.4. Proteins in each sample were then resolved by 2D SDS PAGE, as described in Sections 2.5.7. The Coomassie stained gel images obtained can be seen in Figure 4.13.

Gel images were inserted into a Microsoft Office PowerPoint® document allowing alternation between images for visual comparison between the gels prepared from the wild-type samples and each of the mutant samples. This comparison was also carried out by physically overlaying the gel for comparison on top of a trans-illuminator (the pre-cast gels are attached to a sturdy plastic backing that allows easy handling).

Although some differences were observed between the protein pattern of the wild-type strain and mutant strains in individual gels these could not be observed consistently in each of the three gels prepared for each sample. No proteins were therefore identified as altered between samples in this experiment.

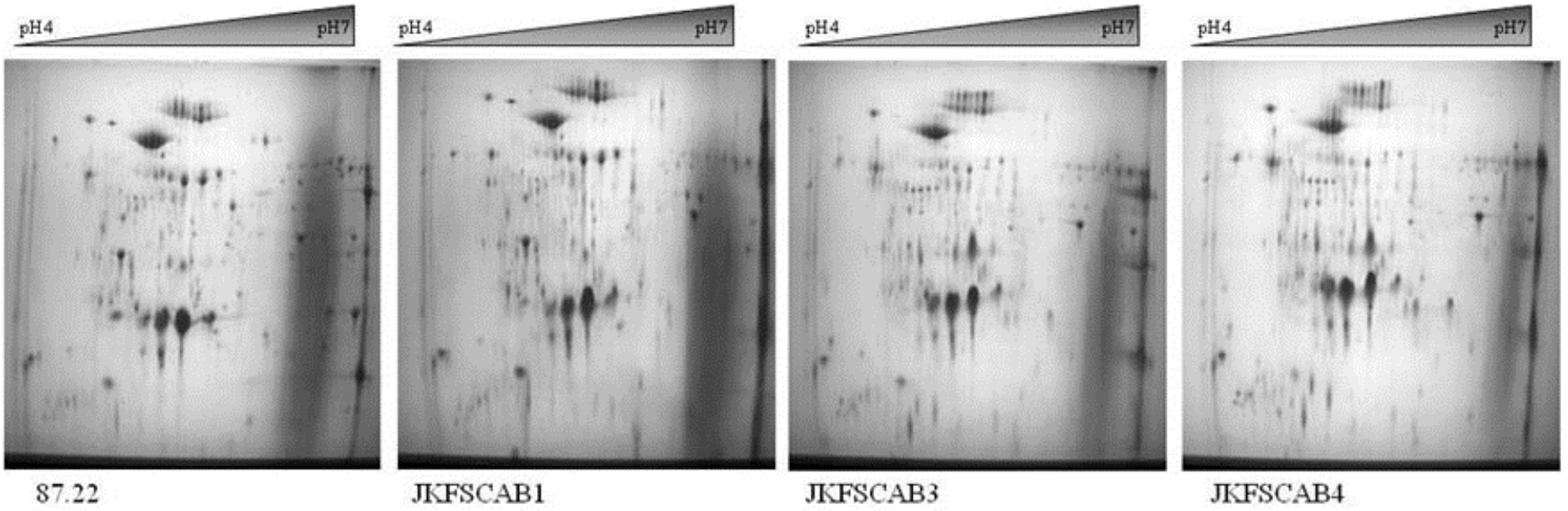

Figure 4.13: Two-dimensional SDS PAGE separation of proteins present in the cell wall wash of *S. scabies* strains 87.22, JKFSCAB1, JKFSCAB3 and JKFSCAB4. Strains were cultured on YME and incubated at 30°C for 48 hours. Cell wall protein samples were then prepared as described in Section 2.5.5. Protein pellets were resuspended in 2D sample buffer and the concentration estimated using a 2D Quant Kit (GE Healthcare). 900 µg of protein was separated by isoelectric focusing (pH gradient 4–7) followed by SDS PAGE. Proteins were stained with Coomassie blue prior to imaging. Images were analysed visually.

As discussed in Section 3.5.2 a paper published by Akpe san Roman and colleagues (2010) indicates that secretion of the WXG100 proteins in *S. coelicolor* occurs prior to 24 hours following inoculation, so the same may also be true of the T7SS of *S. scabies*. For this reason cell-wall wash samples were also prepared from each of the strains cultured on the surface of cellophane discs on YME agar plates for 20 hours and resolved by 2D SDS PAGE and compared as described above. The Coomassie stained gel images obtained can be seen in Figure 4.14 - each image represents one of three similar gels run for each sample prepared. The remaining images can be found in the appendix at the end of this thesis (Figure A2).

However, no proteins were identified as being unique to any one sample.

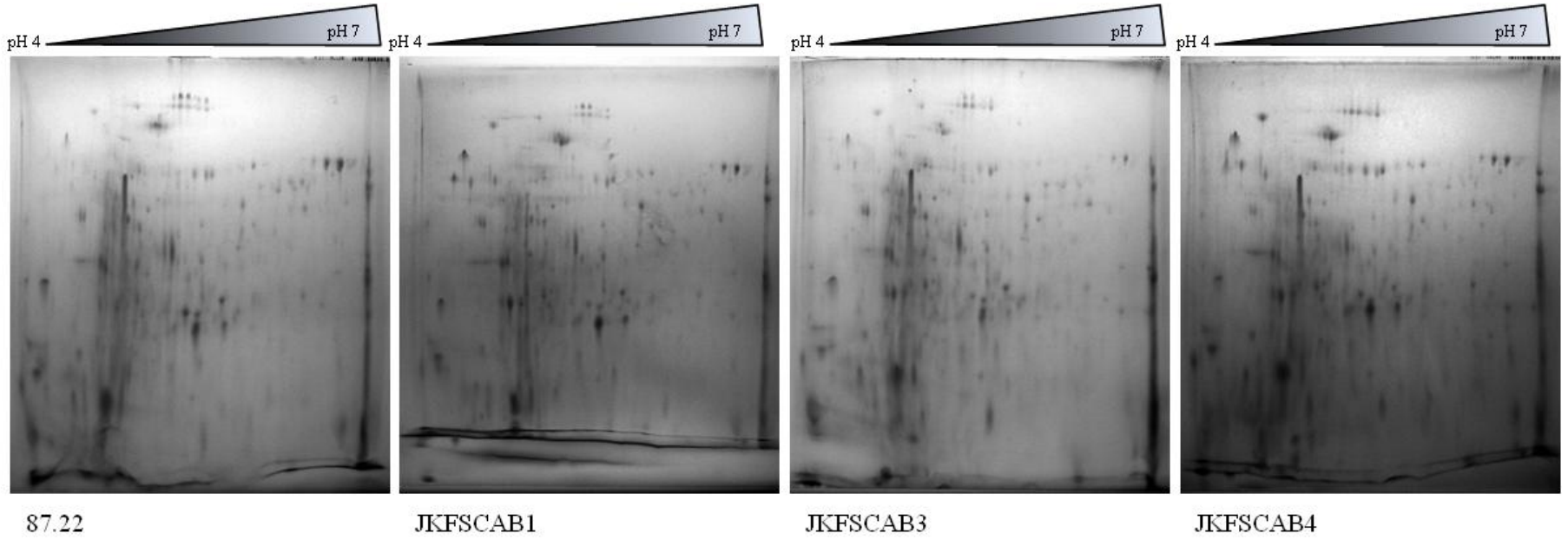

Figure 4.14: Two-dimensional SDS PAGE separation of proteins present in the cell wall wash of *S. scabies* strains 87.22, JKFSCAB1, JKFSCAB3 and JKFSCAB4. Strains were cultured on YME and incubated at 30°C for 20hours. Cell wall protein samples were then prepared as described in Section 2.5.5. Protein pellets were resuspended in 2D sample buffer and the concentration estimated using a 2D Quant Kit (GE Healthcare). 900 µg of protein was separated by isoelectric focusing (pH gradient 4–7) followed by SDS PAGE. Proteins were stained with Coomassie blue prior to imaging. Images were analysed visually.

4.6 Analysis of the ability of bacteriophage to infect *S. scabies* T7SS mutant strains

To determine whether the T7SS of *S. scabies* plays a role in phage infection, plaque assays were carried out as described in Section 2.7 using three different bacteriophages and the *S. scabies* strains 87.22, JKFSCAB1, JKFSCAB2, JKFSCAB3 and JKFSCAB4 as hosts. The results of this analysis can be seen in Figure 4.15.

It can be seen that two of the phage, Φ Hau3AI and Φ R4, that infect wild-type *S. scabies* are also able to infect the T7SS mutant strains in a similar fashion as the wild-type strain, as ascertained by number and size of plaque formation (Figure 4.15). A third phage, ΦC31, that infects wild-type *S. scabies* also infects strains JKFSCAB1 and JKFSCAB2 but strains JKFSCAB3 and JKFSCAB4 appear resistant, as ascertained by the absence of plaque formation. This implies that the WXG100 proteins Ssc58671 and Ssc58681, the genes for which are deleted in JKFSCAB3 and JKFSCAB4, may be required for lytic infection by this phage. However, it should be noted that introduction of the plasmid pScab_WxG_Operon into JKFSCAB3 and JKFSCAB4 (see Section 4.4.1) failed to render these strains sensitive to infection with ΦC31 (data not shown).

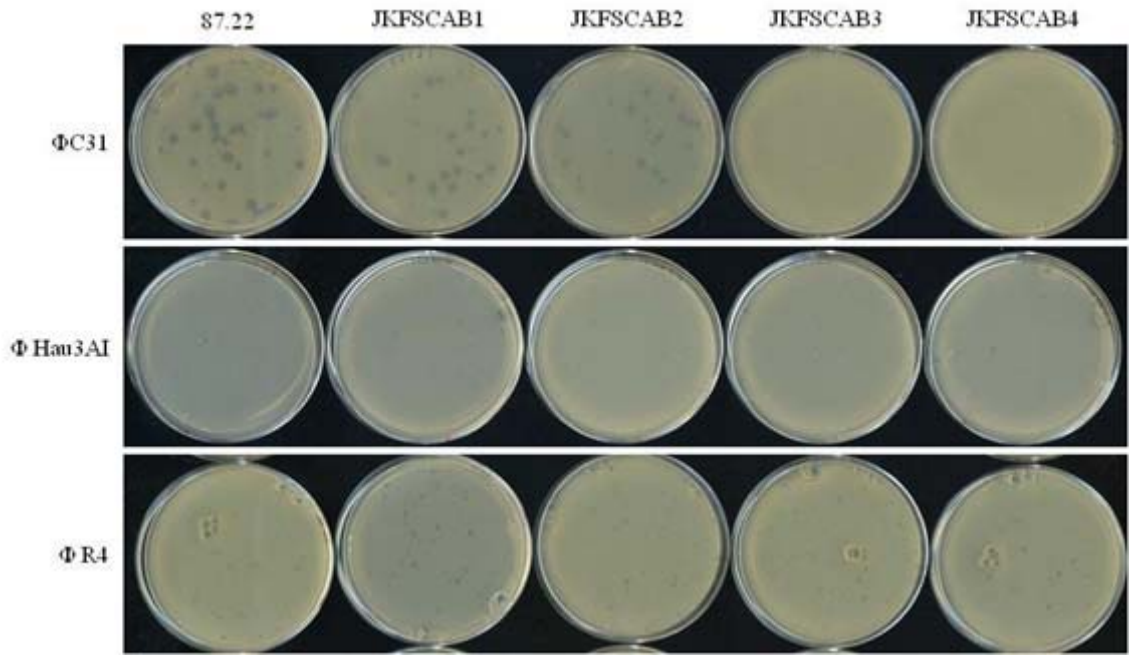

Figure 4.15: Analysis of the ability of bacteriophage to infect *S. scabies* strains 87.22, JKFSCAB1, JKFSCAB2, JKFSCAB3 and JKFSCAB4. The surface of DNA medium was co-inoculated with a phage suspension and approximately 10^7-10^8 spores derived from each strain examined. Plates were incubated at 30°C for 18 hours to allow plaque formation prior to being photographed.

4.7 Plant virulence bioassays

In order to investigate any potential role for the T7SS in the virulence of *S. scabies*, a number of virulence bioassays were used. These assays were developed in the laboratory of Prof. Rosemary Loria at Cornell University to allow the virulence of scab-causing *Streptomyces* species to be assessed with relative ease and speed. These experiments were carried out during a four month visit to the Loria lab, between September 2010 and January 2011. The assays involve inoculation of plants or plant tissue with spores derived from the strain of interest, followed by monitoring of disease progression. Each assay utilised in this study is described below.

4.7.1 Radish seedling virulence assay

Ideally, virulence assays involving pathogens of crop plants should be carried out in the economically important host(s) under standard growing conditions. In the case of *S. scabies* this would require tests to be carried out primarily on potato plants over an entire growing season. This is not only time-consuming, as the development of fully grown potato tubers can take many months, but also labour intensive and requires the availability of a large area in which to grow the plants. Immature mini-tubers can be produced that are suitable for virulence testing (Loria & Kempter, 1986) requiring less space, but the production of these is still time-consuming.

It is therefore advantageous from the viewpoint of carrying out virulence testing in the laboratory that *S. scabies* has a wide host range (Hooker, 1949; Leiner *et al.*, 1996). One of the quickest virulence assays developed for use with *S. scabies* involves the use of *Raphanus sativus* (radish) seedlings (Leiner *et al.*, 1996). This bioassay has been reported to mainly detect thaxtomin A production and results can be obtained in under a week (Bignell *et al.*, 2010). As radish is also an economically important host it was decided that this assay would be used in the first instance to assess any effect that deletion of genes encoding components of the T7SS has on the ability of *S. scabies* to cause disease.

This assay was carried out as described in Section 2.8.1, with 36-40 h old seedlings that showed a similar level of germination being selected for inoculation with either sterile water (serving as a negative control) or *S. scabies* strains 87.22, JKFSCAB1, JKFSCAB2, JKFSCAB3 and JKFSCAB4. Fresh spores of each inoculation strain were diluted to give an OD_{600} of 0.5 and 200 µl of this was used to inoculate the seedlings. Following six days incubation at 21(+/-2) °C with a 16 hour photoperiod the roots were carefully cut from the agar using a scalpel, assessed for disease symptoms and photographed. The results of this assay are shown in Figure 4.16.

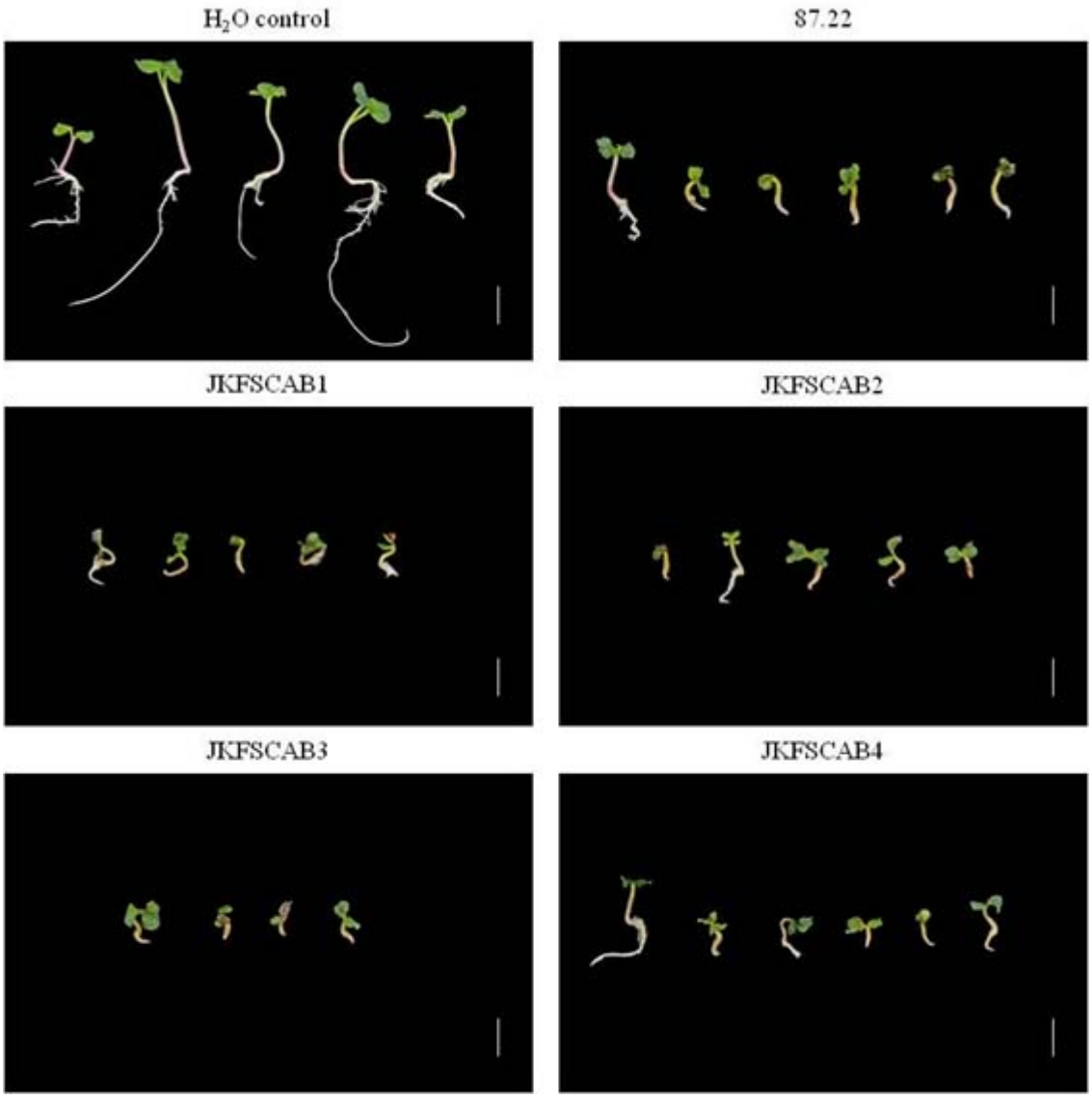

Figure 4.16: Virulence testing of *S. scabies* T7SS mutant strains using the radish seedling virulence assay. Radish seeds were surface sterilised for 5 minutes in 70 % ethanol followed by 10 minutes in 15 % bleach then rinsed ten times in 50 ml of sterile H_2O. Seeds were then allowed to germinate in the dark for 36-40 hours in a petri dish lined with filter paper that was pre-wet with 2 ml of sterile H_2O. Seedlings of equal size were transferred to each of six wells in 1.5 % water agar in a deep petri dish then inoculated with 200 µl of a freshly prepared spore suspension derived from the strain of interest (as indicated above each panel) adjusted to an OD_{600} of 0.5. Petri dishes were sealed with parafilm and incubated at 21(+/-2) °C with a 16 hour photoperiod for six days prior to excision of roots from the agar. Seedlings were assessed for disease symptoms and photographed. Scale bars = 1 cm.

It is obvious that the root growth of seedlings inoculated with the wild-type strain, 87.22 (Figure 4.16, top right panel) is severely stunted when compared to water inoculated seedlings (Figure 4.16, top left panel). Closer inspection of the roots inoculated with strain 87.22 reveals that there is browning at both the root tip and at regions where the formation of secondary roots would have occurred in non-inoculated plants. This phenotype is a common symptom of *S. scabies* root infection (Loria *et al.*, 2006). Similar effects were observed when radish seedlings were inoculated with each of the *S. scabies* T7SS mutant strains (Figure 4.16, middle and bottom panels).

Individual seedlings inoculated with strains 87.22 (Figure 4.16, top right panel), JKFSCAB2 (Figure 4.16, middle right panel) and JKFSCAB4 (Figure 4.16, bottom right panel) were observed to have slightly more developed roots than other seedlings, however, this is reflective of the natural variability observed between non-inoculated seedling (Figure 4.16, top left panel). This experiment was replicated five times with observations consistent with the results presented here. From this it can be concluded that the T7SS does not measurably contribute to the virulence phenotype of *S. scabies* on radish seedlings.

Pictures showing replicates of this experiment can be found in the appendix of this thesis in Figures A3, A4 and A5.

4.7.2 *Arabidopsis* virulence assay

The most commonly used organism for carrying out laboratory-based studies into plant physiology and genetics is *Arabidopsis thaliana* (reviewed by Sommerville & Koornneef, 2002). It was the first plant to have its genome sequenced, revealing that its five chromosomes are likely to encode nearly 26,000 proteins (Lin *et al.*, 1999; Mayer *et al.*, 1999; Theologis *et al.*, 2000; Salanoubat *et al.,* 2000; Tabata *et al.*, 2000). Besides the availability of a genome sequence, *Arabidopsis* possesses several qualities making it a

popular plant for cultivation in the laboratory. The plants are small and as such can be cultivated where space is limited and are also adaptable to aseptic growth on laboratory medium (Langridge, 1994). They are extremely easy to grow, being commonly referred to as a "weed" (Meyerowitz, 1989; Langridge, 1994) and have a short lifecycle of approximately 6-8 weeks from germination to mature seed. This allows studies to be conducted in a short time period. Each plant produces many thousands of seeds making it not only inexpensive to source but also allowing experiments to be performed on a large number of subjects providing suitable numbers for accurate statistical analysis.

For the purposes of the study carried out here it should be noted that *Arabidopsis* is also a member of the *Brassicaceae* family which includes economically important crops such as radish and cabbage. As *Arabidopsis* is slower growing than radish, used in the previous assay (Section 4.7.1), it is expected that the symptoms would develop over a longer time period. This may be of advantage in helping to reduce the strong effects exerted by the virulence determinants thaxtomin and Nec1 on the virulence phenotype. If this is the case it may allow more subtle differences in *S. scabies* virulence to be observed.

This assay has been used in previous studies to assess the virulence of scab-causing *Streptomyces* species harbouring mutations in genes thought to be involved in virulence (Joshi & Loria, 2007; Joshi *et al.*, 2010). In these studies the *A. thaliana* cultivar Columbia (Col-0) was used. However, a comparative study carried out in which virulence of *S. scabies* was assessed on different cultivars of *A. thaliana* revealed that Landsberg erecta (Ler-0) is more susceptible to infection than Col-0 (Dr MV Joshi & Prof R Loria, personal communication). Indeed, one study in which the phytotoxic effect of thaxtomin A was examined utilised the *A. thaliana* cultivar Ler-0 (Scheible *et al.*, 2003; Lerat *et al.*, 2009). It was therefore decided that virulence assays would be conducted in both Col-0 and Ler-0.

However, due to contamination issues only the results of the *Arabidopsis* virulence assays with cultivar Ler-0 were suitable for presentation in this thesis.

This assay was carried out as described in Section 2.8.2, with four day old *A. thaliana* Ler-0 seedlings being inoculated with either sterile water (serving as a negative control) or *S. scabies* strains 87.22, JKFSCAB1, JKFSCAB2, JKFSCAB3 and JKFSCAB4. Fresh spores of each inoculation strain were diluted to give an OD_{600} of 0.5 and 10 µl of this was used to inoculate the root of each seedling. Following four weeks incubation at 21(+/-2) °C with a 16 hour photoperiod the plants were assessed for disease symptoms and photographed. The results of this assay are shown in Figure 4.17.

Although it can be seen that many of the *Arabidopsis* plants inoculated with the *S. scabies* wild-type strain 87.22 have stunted roots and less growth in the aerial regions in comparison to the water inoculated control plants, a significant proportion of the plants inoculated with strain 87.22 still appeared relatively healthy, showing development of roots, stems and leaves. This indicated that there was substantial and unexpected variability in the assay. Similar effects were observed when *Arabidopsis* seedlings were inoculated with each of the *S. scabies* T7SS mutant strains (Figure 4.17). Although the results show that these strains can infect *Arabidopsis* seedings, given the inconsistencies observed between plants inoculated with the same strain, a comparison could not be conducted between wild-type inoculated plants and those inoculated with T7SS mutant strains of *S. scabies*. Therefore no definitive conclusions could be drawn from this assay.

The partial *A. thaliana* Col-0 data obtained (due to the previously mentioned contamination issues) can be found in Figures A6 and A7 in the appendix of this thesis.

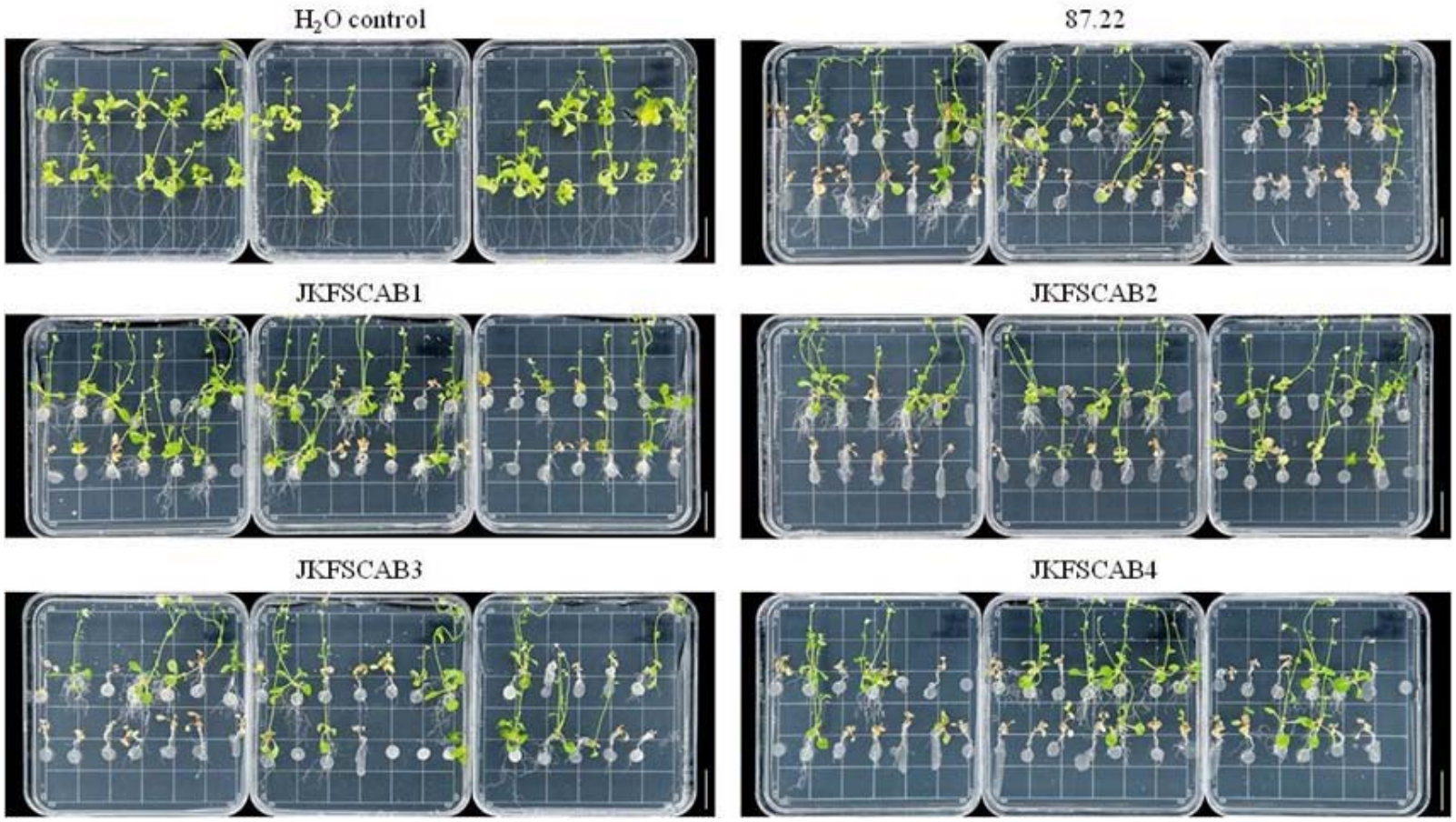

Figure 4.17: Virulence testing of *S. scabies* T7SS mutant strains using the *Arabidopsis* virulence assay. *Arabidopsis* seeds were surface sterilised for 3 hours by exposure to chlorine gas generated following the addition of 2 ml of 37 % HCl to 48 ml bleach. Seeds were stored in 1 ml of 0.1 % phytagar (Sigma) in the dark at 4°C in the dark for 2-5 days prior to being transferred onto MS/1 %sucrose/0.8 % agar in square petri dishes by pipetting. Plates were sealed with parafilm and incubated in an upright position at 21(+/-2) °C with a 16 hour photoperiod for four days. Root tips were then inoculated with 10 µl of a freshly prepared spore suspension derived from the strain of interest (as indicated above each panel) adjusted to an OD_{600} of 0.5. Petri dishes were sealed with parafilm and incubated in an upright position at 21(+/-2) °C with a 16 hour photoperiod for 4 weeks prior to being photographed. Scale bars = 1 cm.

4.7.3 *Nicotiana* virulence assay

Another organism that is commonly used in laboratories to study plant physiology and genetics is *Nicotiana tabacum*, commonly referred to as tobacco. Tobacco also possesses a number of traits, like *Arabidopsis,* that make it ideal for study in the laboratory - the plants are relatively small, they can be grown aseptically on laboratory medium and seeds are readily available. Tobacco in itself is widely sought after for medicinal and recreational use however for the purposes of this study it should be noted that it also belongs to the *Solanaceae* family, which includes a number of economically important crops such as tomato and potato, the latter being the most economically important host for *S. scabies*. It should also be noted that *Nicotiana* has been used previously to assess the virulence of scab-causing *Streptomyces* species harbouring mutations in genes thought to be involved in virulence (Joshi *et al.*, 2007a; Joshi & Loria, 2007; Lerat *et al.*, 2009; Bignell *et al.*, 2010; Joshi *et al.*, 2010).

This assay was carried out as described in Section 2.8.3, with *Nicotiana tabacum* seedlings being inoculated with either sterile water (serving as a negative control) or *S. scabies* strains 87.22, JKFSCAB1, JKFSCAB2, JKFSCAB3 and JKFSCAB4. Pre-enumerated spore stocks of each inoculation strain were diluted to give approximately $5x10^7$ spores per ml of sterile H_2O. One ml of this solution was used to inoculate the surface of the agar in each box containing seven day old seedlings. Following nine weeks incubation at 21(+/-2)°C with a 16 hour photoperiod the plants were assessed for disease symptoms and photographed. The results of this assay are shown in Figure 4.18.

It is obvious that the growth of seedlings inoculated with the wild-type strain *S. scabies* 87.22 (Figure 4.18, second from top) is severely reduced when compared to the water inoculated seedling (Figure 4.18, top). The plant height is greatly reduced (Figure 4.18, A) and inspection of the roots reveals that they are underdeveloped, possessing much less root

mass (estimated visually) and very little evidence of lateral roots (Figure 4.18, C). Similar effects were observed when *Nicotiana* seedlings were inoculated with each of the *S. scabies* T7SS mutant strains (Figure 4.18, bottom four panels). A close-up of the roots can also be found in Figure A8 in the appendix of this thesis.

Although all *S. scabies* inoculated plants are much shorter than the water inoculated control plants (Figure 4.18, A), the overall condition of the plants appear healthy, with little etiolation of the stems and leaves. In fact, when viewed from above it is difficult to distinguish water inoculated plants from *S. scabies* infected plants (Figure 4.18, B). This is consistent with reports that foliage of potato plants infected with *S. scabies* usually shows no evidence of infection.

Individual magenta boxes containing plants inoculated with *S. scabies* strains were observed to have more bacterial growth roots than others (seen as a large yellow area covering the surface of the agar, especially prominent for strain JKFSCAB4 (Figure 4.18, bottom, B). The reason for this is unclear, however, this was not a specific characteristic of any one strain as it was observed for each strain tested and appeared randomly when this work was replicated, giving results that were consistent with those presented here. From this it can be concluded that the T7SS does not contribute significantly to the virulence phenotype of *S. scabies* in *N. tabacum* seedlings.

A similar assay performed using the related *Nicotiana benthomiana*, showing more severe disease symptoms due to innoculation with a greater number of spores, can be found in Figures A9 and A10 in the appendix of this thesis.

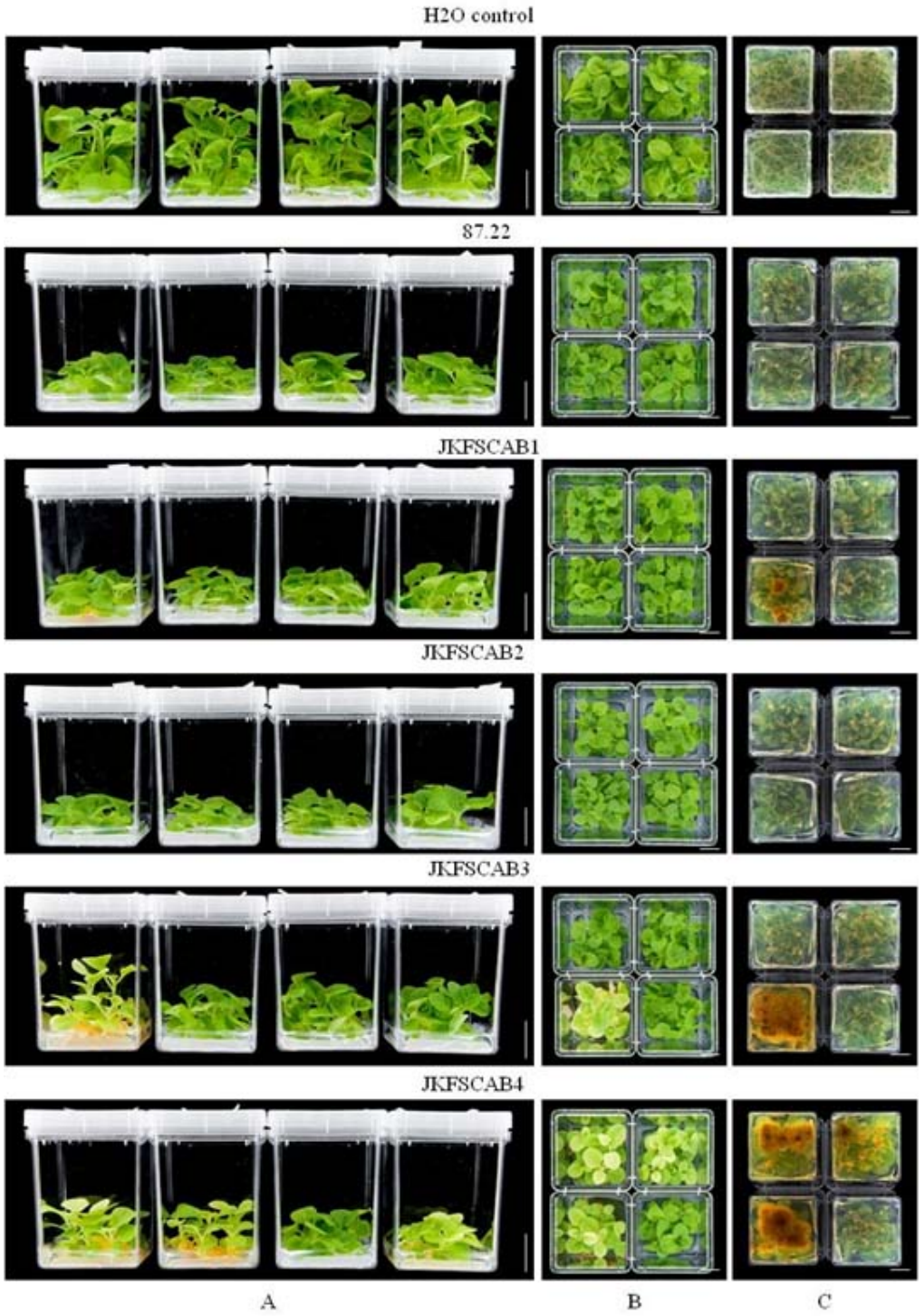

Figure 4.18: Virulence testing of *S. scabies* T7SS mutant strains using the *N. tabaccum* virulence assay. Tobacco seeds were surface sterilised for 3 hours by exposure to chlorine gas generated following the addition of 2 ml of 37 % HCl to 48 ml bleach. Seeds were stored in 1 ml of 0.1 % phytagar (Sigma) in the dark at 4°C in the dark for 2-5 days then transferred onto MS/1 %sucrose/0.8 % agar in sterile magenta boxes. These were incubated in at 21(+/-2) °C with a 16 hour photoperiod for seven days prior to the surface of the agar in each box being flooded with 1 ml of sterile water containing approximately $5x10^7$ spores from a frozen spore stock derived from the strain of interest (as indicated above each group of plants). Plants were incubated at 21(+/-2) °C with a 16 hour photoperiod for 9 weeks prior to being photographed from the side (A), above (B) and below (C). Scale bars = 2 cm.

4.7.4 Potato plant virulence assay

As discussed above in Section 4.7.1, it is preferable to carry out virulence testing of a plant pathogen in the economically important host. Unfortunately the space and time required for normal growth and maturation of potato plants make it difficult to carry out virulence testing of *S. scabies.* However, it is common practice for labs working on potato pathogens to maintain potato plants in sterile micro-propagation systems where cuttings are taken at the site of axilliary bud formation and planted into agar-based laboratory medium.

I therefore worked in conjunction with Dr MV Joshi and Dr IM Francis during two separate visits to the laboratory of Prof. R Loria at Cornell University, to develop an assay whereby micro-propagated potato plants could be used to assess virulence of *S. scabies* strains harbouring mutations in components of the putative T7SS. This assay was carried out as described in Section 2.8.4, with cuttings of the scab-susceptible potato variety Chippewa being inoculated with either sterile water (serving as a negative control) or *S. scabies* strains 87.22, JKFSCAB1, JKFSCAB2, JKFSCAB3 and JKFSCAB4. Axillary bud cuttings were dipped in a freshly prepared spore suspension of each inoculation strain that had been diluted to an OD600 of 0.5. One cutting was planted per tube of potato sub-culture medium and incubated at 21(+/-2) °C with a 16 hour photoperiod for 21 days after which time the plants were assessed for disease symptoms and photographed. The results of this assay are shown in Figure 4.19.

It is obvious that root development of cuttings inoculated with the wild-type strain *S. scabies* 87.22 (Figure 4.19, second from left plant) has been severely inhibited when compared to the water inoculated cutting (Figure 4.19, extreme left plant). Similar effects were observed for cuttings inoculated with each of the *S. scabies* T7SS mutant strains (Figure 4.19, last four plants). The stem length and leaf size of all *S. scabies* infected potato plants were also reduced when compared to the water inoculated control plant. Although a

difference in height was observed between plants infected with different *S. scabies* strains this can be attributed to natural variation in plant height as this is observed between replicates of non-inoculated plants as well as those inoculated with the same strain. From this it can be concluded that the T7SS does not appear to contribute to the virulence phenotype of *S. scabies* in potato plants cultivated in this *in vitro* growth system.

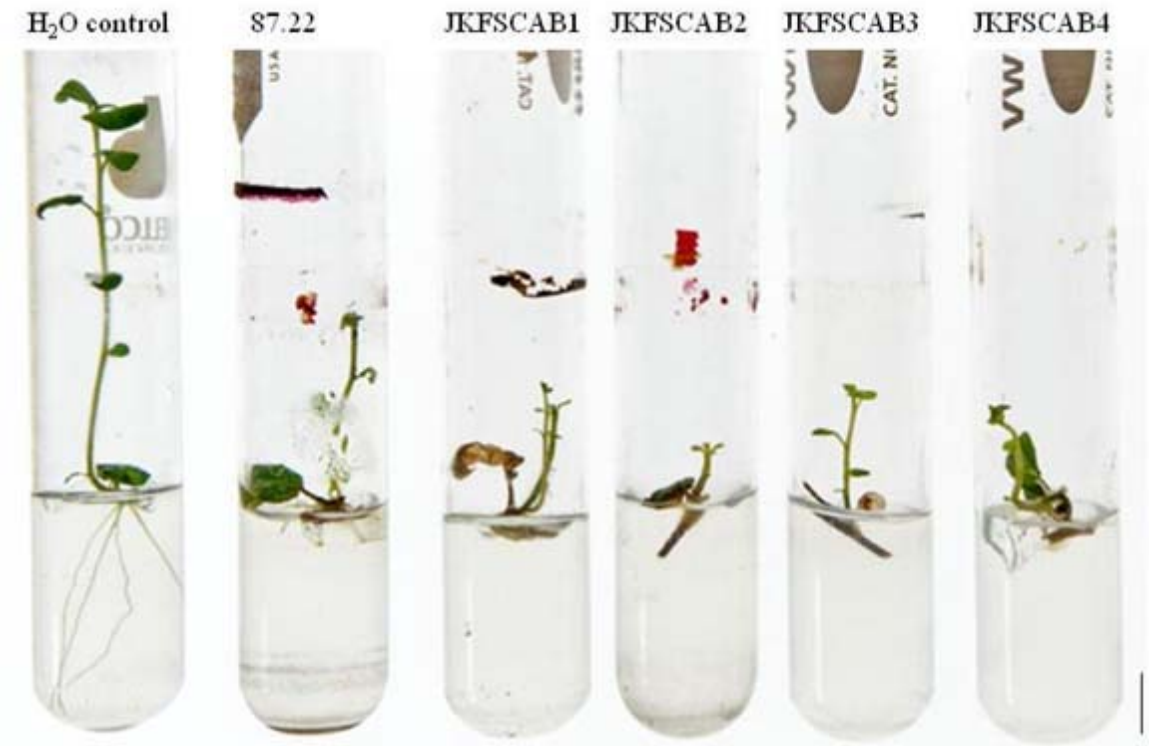

Figure 4.19: Virulence testing of *S. scabies* T7SS mutant strains using the potato plant virulence assay. Cuttings were taken at the site of axillary bud formation of potato plants of the Chippewa variety. The cutting was dipped in 5 ml of a freshly prepared spore suspension derived from the strain of interest (as indicated above each plant) that had been adjusted to an OD_{600} of 0.5. One cutting was planted per tube, in triplicate, and incubated at 21(+/-2) °C with a 16 hour photoperiod for 21 days prior to being photographed. Scale bars = 1 cm.

4.7.5 Potato tuber slice disc assay

One of the assays used to test the effect of virulence factors produced by scab-causing *Streptomyces* species is to apply the purified virulence factor or secreted fraction of strains producing the given virulence factor to discs of potato tuber tissue (Loria *et al.*, 1995; Joshi *et*

al., 2007a; Bignell *et al.*, 2010). This assay has also been used to assess the virulence of scab-causing *Streptomyces* species harbouring mutations in putative virulence genes (Seipke & Loria, 2008; Mann, 2009).

This assay was carried out as described in Section 2.8.5, using commercially available potatoes. Tuber slices were inoculated with agar plugs excised from plates of the strains 87.22, JKFSCAB1, JKFSCAB2, JKFSCAB3 and JKFSCAB4 that were showing confluent sporulation. The results of this assay are shown in Figure 4.20.

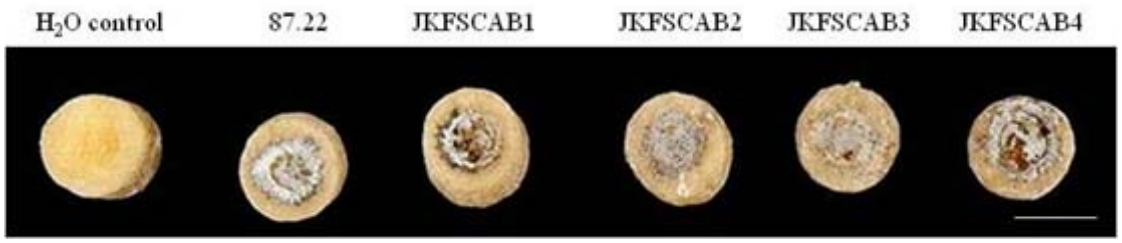

Figure 4.20: Virulence testing of *S. scabies* T7SS mutant strains using the potato tuber slice disc assay. Potato tubers were surface sterilised for 10 minutes in a 15 % bleach solution and washed twice in 1L of sterile H_2O for 1 minute. Cores of approximately 1 cm in diameter were aseptically excised from the tuber then cut into discs of 5 mm thickness. These were transferred to a petri dish lined with sterile filter paper pre-wet with 2 ml of sterile H_2O. Each potato tuber slice was then inoculated spore-side down with an agar plugs from plates showing confluent sporulation. The inoculation strain is indicated above each tuber slice. The petri dishes was sealed with parafilm and incubated in the dark at room temperature for seven days prior to being photographed. Scale bars = 1 cm.

As expected, no effect was observed in the non-inoculated control disc. However, lesions were observed on the potato tuber slice inoculated with *S. scabies* strain 87.22. Lesions were also observed on potato tuber slices inoculated with each of the *S. scabies* T7SS mutant strains that were similar in size and severity to those produced by the wild-type strain 87.22. From this it can be concluded that the T7SS does not contribute to the ability of *S. scabies* to colonise potato tissue in this *in vitro* growth system.

4.7.6 Thaxtomin extraction and quantification

As discussed in Section 1.2.2.1.1, the primary virulence determinant produced by all scab-causing streptomycetes is thaxtomin A. This is produced and secreted into the culture medium of *S. scabies* strains grown in a liquid medium containing plant-derived material, two of which have been specifically developed to support production of thaxtomin (Johnson *et al.*, 2007).

Quantification of thaxtomin is routinely carried out during virulence testing of *S. scabies* mutant strains to ascertain if any decrease in virulence observed can be attributed either directly or indirectly to a reduction in thaxtomin levels. This assay was carried out as described in Section 2.8.6, following growth of strains 87.22, JKFSCAB1, JKFSCAB2, JKFSCAB3 and JKFSCAB4. The analysis and quantification of thaxtomin was kindly undertaken by Dr IM Francis. The results of this assay are shown in Figure 4.21.

It is clear from these assays that the *S. scabies* T7SS mutant strains are able to produce and secrete thaxtomin. Although production of thaxtomin by each of the *S. scabies* T7SS mutant strains appears reduced (by up to 54 %) when compared to the wild-type strain 87.22, it should be noted that the culture medium was inoculated with spores of the same OD_{600} which does not necessarily correlate with numbers of viable spores. Furthermore, no measurable reduction in virulence was observed for any of the mutant strains, which strongly suggests that there is not a serious defect in thaxtomin production.

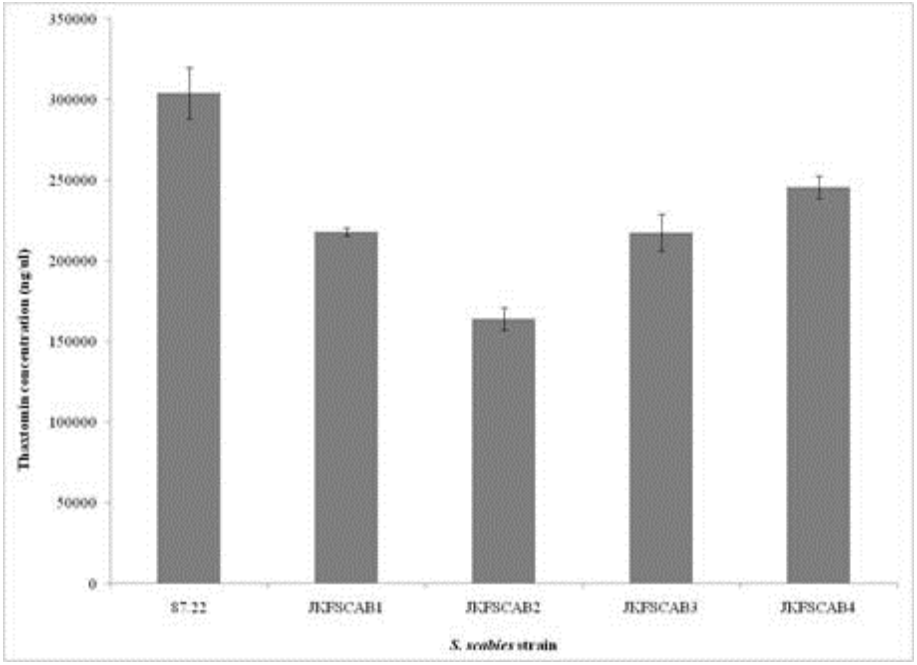

Figure 4.21: Thaxtomin production by *S. scabies* strains 87.22, JKFSCAB1, JKFSCAB2, JKFSCAB3 and JKFSCAB4 following seven days growth. A 50 ml culture of OBB medium was inoculating with an equal number of spores, as determined by measurement of their OD_{600} and incubated for one week at 28°C with shaking. The culture supernatant was then applied to conditioned C-18 extraction cartridge (Dionex) which bound thaxtomin. The cartridge was then washed with 3 ml of distilled H_2O followed by 3 ml of 25 % methanol. The thaxtomin was eluted in 3 ml of 50 % methanol, collected and analysed by HPLC. Thaxtomin was detected by absorbance between 215 and 400 nm and quantified by measuring absorbance at 380 nm and comparing to a pure thaxtomin standard. Error bars represent the standard error of the mean, where n=2.

4.8 Discussion

4.8.1 The WXG100 proteins of *S. scabies* contain an alternative FXXXG motif

Identification of genes encoding components of a putative T7SS in the genome of *S. scabies* was complicated by the fact that each of the WXG100 proteins do not contain the classical W-x-G motif. Instead in *S. scabies* this conserved motif is replaced with amino acid residues (F-X-X-X-G), which does not appear to alter the overall fold of these proteins as predicted using the Phyre algorithm. It should be noted that F and W are both aromatic amino acids. Although unusual, this is not an exceptional observation, since it was shown

that the W-x-G motif is not conserved in the *S. coelicolor* WXG100 protein Sco4509 (Chapter 3.2) and has been noted for WXG100 proteins from other bacteria (Callahan *et al.*, 2010). However, this highlights the possibility that multiple W-x-G family proteins may be encoded in the genome of *S. scabies*, or other organisms, that have so far eluded discovery due to their very short length and lack of sequence conservation.

4.8.2 The T7SS of *S. scabies* is not essential for viability

In this chapter strains carrying mutations in the genes coding for the T7SS FtsK/SpoIIIE ATPase protein, the putative pore and the WXG100 proteins were obtained easily, indicating that this system is not essential for viability of the organism. When various phenotypic analyses of these mutant strains were undertaken, using a range of growth media, the strain deleted for the gene encoding the putative pore protein displayed similar growth behaviour to the wild-type. This can be likened to the observations made for the corresponding mutation in *S. coelicolor*, and indeed Akpe San Roman *et al.* (2010) demonstrated that this hydrophobic protein is not required for the secretion of the *S. coelicolor* WXG proteins, making it an unlikely candidate for the secretion pore.

My studies demonstrated, however, that the *S. scabies* strain deleted for the gene encoding the FtsK/SpoIIIE ATPase protein displayed a subtle increase in aerial hyphae formation and an increased level of sporulation in a media-dependent manner. This differs from the observations made for corresponding mutations in *S. coelicolor* (Figure 3.6, Wang *et al.*, 2007; Akpe San Roman *et al.*, 2010). It is possible that the difference observed between *S. scabies* and *S. coelicolor* is reflective of the different nutritional requirements of these species.

4.8.3 The WXG100 proteins are required for normal development

Interestingly, I demonstrated that individual deletions of each of the genes encoding the WXG100 proteins led to a severe reduction in aerial hyphae formation and/or sporulation when strains harbouring these mutations were cultured on some growth media. This prompted me to examine spore chains of strains JKFSCAB3 and JKFSCAB4 (which harbour mutations in these genes) by SEM. This analysis revealed that the spores were irregularly sized and the spore chains did not develop normally, showing extensively reduced coiling when compared to wild type. This observation was also made by Akpe San Roman *et al.* (2010) when the genes encoding the WXG100 proteins from the most closely related T7SS in *S. coelicolor* were disrupted, indicating that the system likely performs the same physiological function in both organisms (and perhaps all streptomycetes). Interestingly, two BldB-like proteins and and a WhiJ orthologue are encoded in the genomic region of the T7SS in *S. scabies,* an association that has also been observed in *S. coelicolor* (Section 3.2, Akpe San Roman *et al.*, 2010). This strengthens the proposal that the T7SS contributes to developmental regulation (discussed in more depth in Section 4.8.6).

During complementation of the strains harbouring mutations in the genes encoding the WXG100 proteins a serendipitous discovery was made that expression of the genes encoding the WXG100 proteins *in trans* in the wild-type strain also slowed down aerial hyphae formation but appeared to induce hyper-sporulation. If the WXG100 proteins do interact with other proteins involved in developmental regulation, such as BldB or WhiJ, it is possible that the level of these proteins is critical to their function. Introduction of a second copy of the genes may have resulted in increased cellular levels of WXG100 proteins that accounts for the effects observed.

4.8.4 Are additional proteins required for WXG100 protein function?

Introduction of the pair of genes encoding the WXG100 proteins *in trans* into strains JKFSCAB3 and JKFSCAB4 (that harbour individual mutations in one or other of the genes encoding the WXG100 proteins) was only able to partially restore the developmental defect. This suggests that the total level and ratio of the two WXG proteins present in the cell may be critical to their function. A second possible explanation for the inability to fully complement either of these strains is that other factors are also required. For example, as RT-PCR analysis suggests that the genes *ssc58681* to *ssc58661* are co-expressed it is possible that the protein encoded by *ssc58661* is also required for full functionality of the WXG100 proteins. It should be noted, however, that the genome of *S. coelicolor* does not encode an Ssc58661 homolog in the genomic region of the T7SS. Any additional requirement for Ssc58661 could be addressed through introduction of a construct into the chromosome of JKFSCAB3 and JKFSCAB4 that includes a chromosomal fragment encoding the genes *ssc58681* to *ssc58661* for co-expression. Alternatively, it is possible that full complementation of the mutant strains may require the co-expression of all of the genes of the T7SS of *S. scabies*, as has been observed for complementation of RD-1 mutants of *M. tuberculosis* which require complementation with the entire *esx-1* locus (Pym *et al.*, 2003). This is supported by the results of RT-PCR analysis of the T7SS gene cluster of *S. scabies* that indicates all genes from *ssc58681* through to *ssc58621* are co-ordinately expressed.

4.8.5 A role for the WXG100 proteins of *S. scabies* in phage infection?

Interestingly, results presented in this chapter strongly suggest that the WXG100 protein components of the T7SS are required for lytic infection by the bacteriophage ΦC31 as strains JKFSCAB3 and JKFSCAB4 (which lack one or other of the genes encoding the WXG100 proteins) displayed resistance to lysis. This is in contrast to the wild type strain

87.22, and strains JKFSCAB1 (lacking the FSD protein) and JKFSCAB2 (lacking the hydrophobic 'pore' protein) which were susceptible to lysis by this phage (Figure 4.15). This is also in complete contrast to the observations made in Chapter 3, investigating the infection of *S. coelicolor* strains with ΦC31, where it was shown that the normally resistant *S. coelicolor* is rendered susceptible to lysis by this phage when the FSD protein Sco5734 was lacking. In Chapter 3 it was suggested a likely reason for the susceptibility of the *sco5734* mutant strains to lysis was due to a secondary mutation in the PGL system, which arise with high frequency in *S. coelicolor* (Bedford *et al.*, 1995). However, this cannot be the reason for the lysis-resistant phenotype of strains JKFSCAB3 and JKFSCAB4. Firstly, the phenotype is opposite to that seen for *S. coelicolor* mutant strains, i.e. from sensitivity to resistance rather than the other way round. Secondly, *S. scabies* does not contain genes coding for the PGL. Thirdly it should be noted that the phage-resistance phenotype is identical for strains JKFSCAB3 and JKFSCAB4 which were independently constructed from the same lysis-sensitive wild type strain. It would therefore be very unlikely to suppose that the same secondary mutation giving rise to phage resistance arose twice completely independently (contrast this to the phage sensitive *S. coelicolor* strain deleted for *sco5734/sco4508* which was constructed from the phage sensitive *sco5734* mutant strain; thus the same mutation need only have arisen once). It should be noted, however, that to fully confirm that the WXG proteins of *S. scabies* are required for lytic infection by ΦC31, complementation of these strains should be carried out. However this is unlikely to be straightforward given that introduction of the two genes encoding the WXG100 proteins did not fully restore the developmental phenotype of strains JKFSCAB3 and JKFSCAB4.

Assuming that the resistance of *S. scabies* mutant strains to lysis by phage ΦC31 is directly due to the lack of either of the WXG proteins, this raises some interesting suggestions. Firstly, since the strain lacking the FSD protein, which according to the

accepted dogma should be essential for the export of the WXG proteins is still sensitive to lysis, this strongly suggests that it is the intracellular presence of the WXG proteins that is essential to assist in lysis. This is reminiscent of the effects of these same mutations on development of *S. scabies*, where I demonstrated that strains JKFSCAB3 and JKFSCAB4 (lacking the WXG proteins) showed significant developmental defects that were not apparent in the strain lacking the FSD protein. It is also consistent with the findings of Akpe San Roman *et al.* (2010), who showed that the developmental defect associated with loss of the WXG proteins in *S. coelicolor* was not seen when the cognate FSD protein was absent. All of these observations point to a major role for the WXG proteins intracellularly rather than extracellularly. Possible models for the role of the WXG proteins and the T7SS in streptomycetes is presented in Figure 4.22.

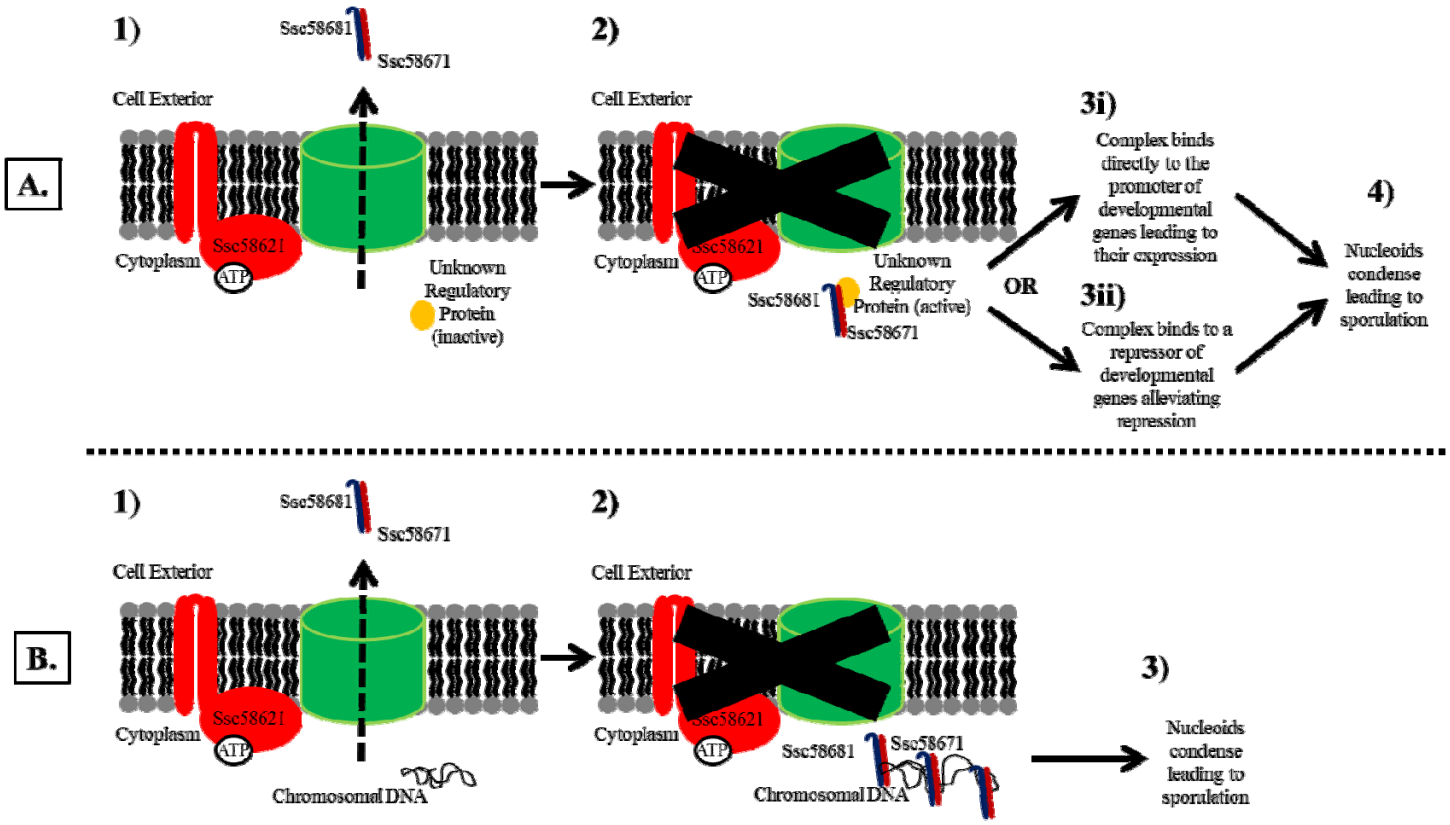

Figure 4.22: Possible models for operation of the T7SS in *Streptomyces scabies* either through binding of the WXG100 proteins to a regulatory protein (A) or through binding of the WXG100 proteins to chromosomal DNA (B). Following germination, a heterodimer formed by the WXG100 proteins Ssc58671 and Ssc58681 is secreted (A1) Secretion via the T7SS is blocked by an unknown mechanism leading to binding of the heterodimer to the unknown regulator (A2) This activates the regulator and induces expression of developmental genes either through binding directly to their promoter regions (A3i) or through binding a repressor alleviating repression of developmental genes (A3ii) Nucleoids condense and development proceeds to sporulation (A4). **OR** Following germination, a heterodimer formed by the WXG100 proteins Ssc58671 and Ssc58681 is secreted (B1) Secretion via the T7SS is blocked by an unknown mechanism leading to binding of the heterodimer to chromosomal DNA (B2) Nucleoids condense (facilitated by the WXG100 proteins) and development proceeds to sporulation (B3).

4.8.6 Proposed models for operation of the T7SS in *Streptomyces scabies*

According to the models, the major role of the WXG100 proteins is inside the cell with their export through the T7SS acting to prevent their intracellular function. It is proposed here that the WXG100 proteins are secreted through the T7SS during early growth (Figure 4.22, A1 & B1) but that they ultimately accumulate inside the cell by an unknown mechanism (Figure 4.22, A2 & B2). This hypothesis is supported by transcriptional expression data from *S. coelicolor* showing that both *sco4508* and *sco5734* (encoding the FSD proteins) are most highly expressed following germination and transcript levels drop steadily during the first 24 hours of growth (Dr Andrew Hesketh, personal communication). It may also explain why secretion of the WXG100 proteins in *S. coelicolor* was only observed during the first 24 hours following inoculation (Akpe San Roman *et al.*, 2010). As the intracellular levels of the WXG100 proteins increase, this allows them to perform their function within the cell.

Two possible modes of action are outlined in Figure 4.22. Akpe San Roman *et al.* (2010) proposed that a cytoplasmically-located heterodimer of the *S. coelicolor* WXG proteins Sco5724/Sco5725 sequesters protein factors that regulate nucleoid condensation such as DpsA (Facey *et al*., 2009) or BldB. The first model outlined in Figure 4.22A proposes that the heterodimer formed by the WXG100 proteins binds to an unknown regulatory protein thereby activating it which induces expression of developmental genes (Figure 4.22, A3). This may be through direct binding to the regulatory protein to the promoter region(s) of the developmental genes (Figure 4.22, A3i) or through alleviating repression of developmental genes (Figure 4.22, A3ii). Nucleoid condensation then takes place and development proceeds (Figure 4.22, A4).

The second mode of action proposes that the heterodimer formed by the WXG100 proteins binds to the chromosomal DNA (Figure 4.22, B2) perhaps promoting nucleoid condensation leading to sporulation (Figure 4.22, B3). This second hypothesis

could also account for the lysis resistance phenotype of the *S. scabies* strains lacking the WXG100 proteins, since it is known that host DNA binding proteins (such as *E. coli* integration host factor; Craig & Nash, 1984) are required for phage infection. It is possible that the WXG proteins could play a role in the stabilisation of ΦC31 DNA once it enters the cytoplasm, promoting recombination into the chromosome. However, it should be pointed out that recombination of plasmid constructs into the ΦC31 site on the chromosome of *S. scabies* was not noticeably affected by the loss of the WXG proteins. Any possible DNA binding activity of the WXG proteins would be relatively straight-forward to test following purification of the heterodimer.

4.8.7 An extracellular function for the WXG100 proteins of *S. scabies*?

Despite the intracellular role for the WXG100 proteins proposed in Figure 4.22, the possibility that the extracellular WXG proteins also have a role in *Streptomyces* biology cannot be excluded. Analysis of the phenotypes of *S. coelicolor* and *S. scabies* strains lacking the FSD proteins of the T7SS reveal some subtle phenotypes, but proteomic analysis of the cell wall washes of mutant strains did not lead to the identification of any candidate substrate proteins. Again it is possible that apart from the WXG proteins the major substrate for Type VII secretion is DNA, and that the WXG proteins may be required for holding DNA in an export-competent state, or for assisting its recognition as a secretion substrate. Clearly, this would form an interesting line of investigation for future studies.

4.8.8 The T7SS of *S. scabies* is not required for virulence

Whereas Gram-negative bacteria possess numerous specialized secretion systems, such as Type III, Type IV and the more recently described Type VI (see Section 1.3.1), for delivery of virulence factors directly into the host, protein secretion

in the Gram-positive bacteria was, until recently, thought to be rely solely on the Sec and Tat pathways (see Section 1.3.2). However, the discovery that Gram-positive bacteria also possess an alternative secretion system that contributes significantly to virulence of human pathogens, such as *M. tuberculosis* and *S. aureus* (Stanley *et al.*, 2003; Burts *et al.*, 2005; MacGurn & Cox, 2007; Millington *et al.*, 2011; Lerena & Colombo, 2011), led me to examine the potential role of the T7SS in the virulence of *S. scabies*. The results presented in the last part of this chapter are, to my knowledge, the first study of a T7SS in any plant pathogenic bacterium. As it has previously been shown that mutation in genes encoding components of the Tat translocase or individual Tat-substrate proteins in *S. scabies* are attenuated in virulence when compared to the wild-type strain (Joshi *et al.,* 2010) a similar approach was employed to assess the effect that mutation of genes encoding components of the T7SS in *S. scabies* has on virulence.

A number of virulence assays were carried out using the T7SS mutant strains of *S. scabies* showing that the secretion apparatus does not appear to contribute to the virulence of the organism. Although the virulence bioassays used here are generally accepted to be good models for crop infection in the field, it should be noted that these have been carried out in what is essentially artificial growth conditions. These assays are also largely qualitative in nature and statistical analysis of results is lacking. In addition, the assays may not be sensitive enough to reveal subtle differences that are masked by the strong effects of other virulence determinants such as thaxtomin. For example a study showing the effect of inactivation of the T6SS in *V. cholerae* was carried out in a Δ*hlyA*/Δ*hapA* background strain, deleted for two genes encoding virulence factors (Manning *et al.*, 1984; Wu *et al.*, 1996) and this revealed the contribution of the T6SS to the virulence of *V. cholerae* in a mouse intestine model (Ma & Mekalanos, 2010). A more in-depth analysis of the T7SS would, therefore, involve analysing T7SS mutant strains that had been constructed in a thaxtomin-deficient

background strain. Where putative virulence factors are expected to be secreted, consideration could also be given to performing assays using culture supernatant derived from *S. scabies* mutant strains that have been constructed in a thaxtomin-deficient background, as reported previously for examination of a CFA-like metabolite produced by *S. scabies* (Bignell *et al.*, 2010). Although the application of culture supernatant has previously been used to examine individual virulence factors (Joshi *et al.*, 2007a; Bignell *et al.*, 2010) in theory it could also apply to the study of a secretion system.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

In Gram-negative bacteria no fewer than eight transport systems exist for the export of proteins across one or both membranes of the cell envelope (Figure 1.6), and some of these systems are extremely complex, allowing secretion of proteins across three membranes and into the cytoplasm of a host cell. By contrast, protein secretion in Gram-positive bacteria has always been regarded as a simpler process, relying primarily on the activity of the 'housekeeping' Sec and the Tat pathways for export across the Gram positive cytoplasmic membrane. The recent discovery of the T7SS in *M. tuberculosis* and the observation that similar genes encoding components of putative T7SSs are present in a wide range of Gram-positive bacteria raised the prospect of a Gram-positive specific protein secretion system that has a major role in virulence. However, as demonstrated in this thesis, the T7SS is not required for the virulence of *Streptomyces scabies*, and it is still not clear whether the major substrate of this secretion system is protein or DNA.

One of the hottest topics in bacterial protein secretion is the identification and characterisation of bacterial effector proteins. These are proteins that are secreted primarily by the Gram negative Type III and Type VI protein secretion pathways into host cells. Effectors interfere with the normal biology of the host, for example by affecting signalling pathways (e.g. phosphorylation, acetylation or ubiquitination), subverting the host for the bacterium's own advantage (e.g. Kim *et al.*, 2005; Hentschke *et al.*, 2010; Selbach *et al.*, 2003; Bliska, 2006; Zhou *et al.*, 1999b). Although effector biology is largely studied in animal cells, effectors are also targeted into plant cells by the T3SS of Gram negative plant pathogens, where they similarly block various aspects of host cell function (e.g. Bos *et al.*, 2010; Kaffarnik *et al.*, 2009; Sugio *et al.*, 2007).

Since the protein secretion systems of Gram positive bacteria are less complex than those of Gram negative bacteria, they do not appear to encode components required

to mediate delivery across the host cell cytoplasmic membrane. Delivery of effectors to plant cells would also require crossing the additional barrier of the plant cell wall. This led Hogenhout *et al*. (2009) to broaden the definition of an effector to 'small molecules and proteins that alter host cell structure'. Thus proteins that have an extracellular target, such as the plant cell wall, can also be considered effectors. Interestingly, one of the initial aims of this thesis was to explore the role of the Tat protein export pathway of *S. scabies* in the secretion of plant cell effector proteins. As part of this work, I developed methods to examine the infection of *Arabidopsis* roots by *S. scabies* wild type and *tatC* mutant strains using confocal microscopy. To this end, a genetic construct, either pIJ8641 or pRFSRF16 (Table 2.5), was introduced into the wild-type and Δ*tatC* mutant strain to give constitutive expression of *egfp*. This allowed the GFP labelled bacterial hyphae to be visualised by laser scanning confocal microscopy (LSCM) during colonisation of *Arabidopsis* roots – some images of this are shown in Fig 5.1. It is clear from these images that the hyphae of the wild type *S. scabies* strain are able to penetrate deep into the root tissue, growing between the layers of cells and appear to be in intimate contact with individual cells. It is also readily apparent that the ability to infect *Arabidopsis* roots is severely compromised by loss of the Tat system.

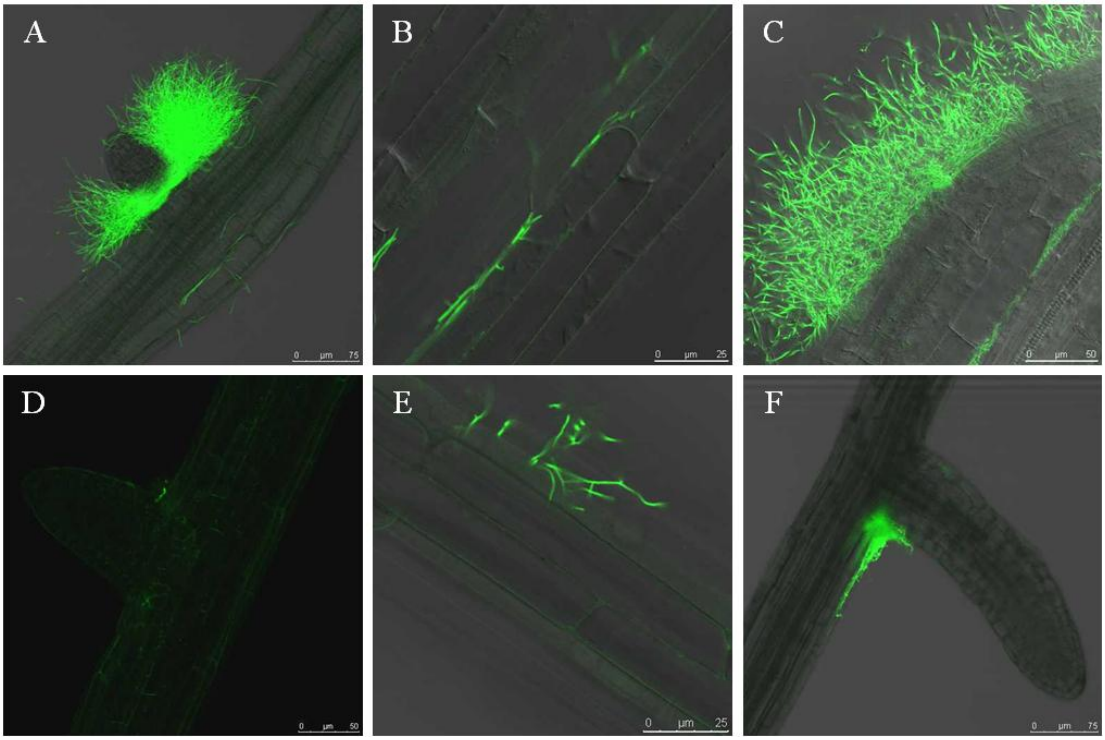

Figure 5.1: Confocal microscopy images showing colonisation of *A. thaliana* roots by EGFP-labelled *S. scabies* 87.22 and the *tatC* mutant strain. Hyphae were visualised during infection of *Arabidopsis* roots by fluorescence microscopy. Seedlings were incubated with shaking at 100 rpm and a 16 hour photoperiod prior to inoculation with with $1x10^6$ c.f.u. of a spore suspension of the *egfp* expressing 87.22 (A-C) or Δ*tatC* (D-F). Plants were incubated as before and visualised at 24 hour intervals post inoculation (hpi) by laser scanning confocal microscopy. The root surface is heavily colonised by the wild-type strain 87-22 at sites of lateral root emergence 48 hpi (A). Fluorescent hyphae of strain 87.22 can clearly be seen penetrating intercellularly at 48 hpi (B) and colonising the root tip 48 hpi (C). The *tatC* mutant strain begins to colonise at sites of lateral root emergence 48 hpi (D) and can be seen attaching to the root surface by 72 hpi (E). Colonisation with the *tatC* mutant strain at sites of lateral root emergence is firmly established by 96 hpi by which time the lateral roots have developed considerably (F)

Studies by members of the Palmer and Loria labs had identified Tat substrate proteins that had been shown to make individual contribution to virulence (Joshi *et al.*, 2010). Some of these proteins have domains that are strongly predicted to interact with the plant cell wall, perhaps to mediate anchoring of the proteins, or to facilitate their passage across the cell wall. It was an initial aim of this thesis to investigate the function of some of these virulence factors in more depth. To this end I characterised

four *S. scabies* strains harbouring mutations in genes encoding the Tat secreted virulence factors. Two of these virulence factors, Ssc03871 and Ssc10131 show common organisation and are predicted to contain a glycosyl hydrolase domain followed by ricin domain (the latter of which is predicted to bind to the plant cell wall). Ssc06471 which I also selected for examination is a putative α-*L*-fucosidase showing homology to an α-*L*-fucosidase produced by fungal plant pathogens. This enzyme is predicted to hydrolyse the α-1,6-link between fucose and N-acetylglucosamine of carbohydrate moieties in glycoproteins. Finally, Ssc77391 is a hypothetical protein containing a galactose-binding domain. Each of these proteins may conceivably act by binding to the surface of the plant cell wall and degrading components such as glycan, facilitating entry of the pathogen.

I was able to demonstrate that strains carrying individual deletions in each of the genes coding for these four virulence factors (constructed by Dr Madhumita Joshi) had no reduction in fitness, as determined by growth curve analysis (Figure S4 of Joshi *et al.*, 2010; presented in the appendix of this thesis). This allowed the important conclusion that the loss in virulence seen for these mutant strains arose due to loss of the protein function against the plant host, rather than due to a general effect on *S. scabies* physiology.

One of my original aims was to establish whether these proteins were extracellular effector proteins, or whether they were internalised into the plant cell and had an intracellular function. The way I chose to approach was with the idea of performing a confocal microscopy study whereby genetic fusions of GFP to the C-termini of Tat secreted virulence factors were constructed and expressed in *S. scabies*. The localisation of the virulence factors could then be visualised *in planta* following infection. As the periplasmic localisation of Tat substrates of *E. coli* had previously been shown using covalent fusions of GFP to the C-termini of Tat substrate proteins

(Bernhardt & de Boer, 2003; Tarry *et al*., 2009) and the components of the *E. coli* and *S. coelicolor* Tat systems have been shown to function interchangeably (Hicks *et al.*, 2006) it seemed reasonable to assume that functional GFP fusions could also be exported by the Tat system of *S. scabies.*

However, expression of a genetic fusion of GFP to the C-terminus of agarase (a natural Tat substrate in *Streptomyces* whose activity is only detected when secreted via the Tat system; Widdick *et al*, 2006; 2008) in both *S. scabies* and *S. lividans* failed to result in the detection of agarase activity. In addition, no fluorescence could be detected. Furthermore, secretion of an agarase signal sequence-GFP fusion protein could not be detected in the culture supernatant of *S. scabies* or *S. lividans* by western blot using an α-GFP antibody. As I was unable to show that the Tat system of *Streptomyces* can export GFP, the investigation into the localisation of the Tat-secreted virulence factors was not pursued further.

For future studies, consideration could be given to using different approaches to investigate the localisation of virulence factors secreted by bacterial secretion systems. One example might be to use an alternative fluorophore to GFP, derived from the light, oxygen or voltage-sensing (LOV) domain of the plant blue light receptor phototropin, that has been used to show viral infection of plants (Chapman *et al.*, 2008) which could be fused to the substrate protein. As the LOV protein has a lower molecular mass than GFP it is perhaps less likely to interfere with secretion and the normal function of the protein(s) being investigated. This is important as some proteins may not be compatible for secretion by all pathways, or assume their normal folded conformation following secretion, for example GFP is not active when exported via the Sec pathway in *E. coli* (Feilmeier *et al.*, 2000). Native proteins could also be detected by fluorescence or electron microscopy following immunolabeling however this requires sample preparation that may interfere with the ultrastructure of the plant.

As an alternative to microscopy-based methods other techniques could be used to identify the plant cell target of bacterial effectors, such as protein pull down assays using an immobilised virulence factor to probe plant extracts for interacting proteins. One example where this method has been used previously to confirm proteins from apple cells that interact with effectors from *Erwinia amylovora* is provided by Meng *et al.* (2006).

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APPENDIX

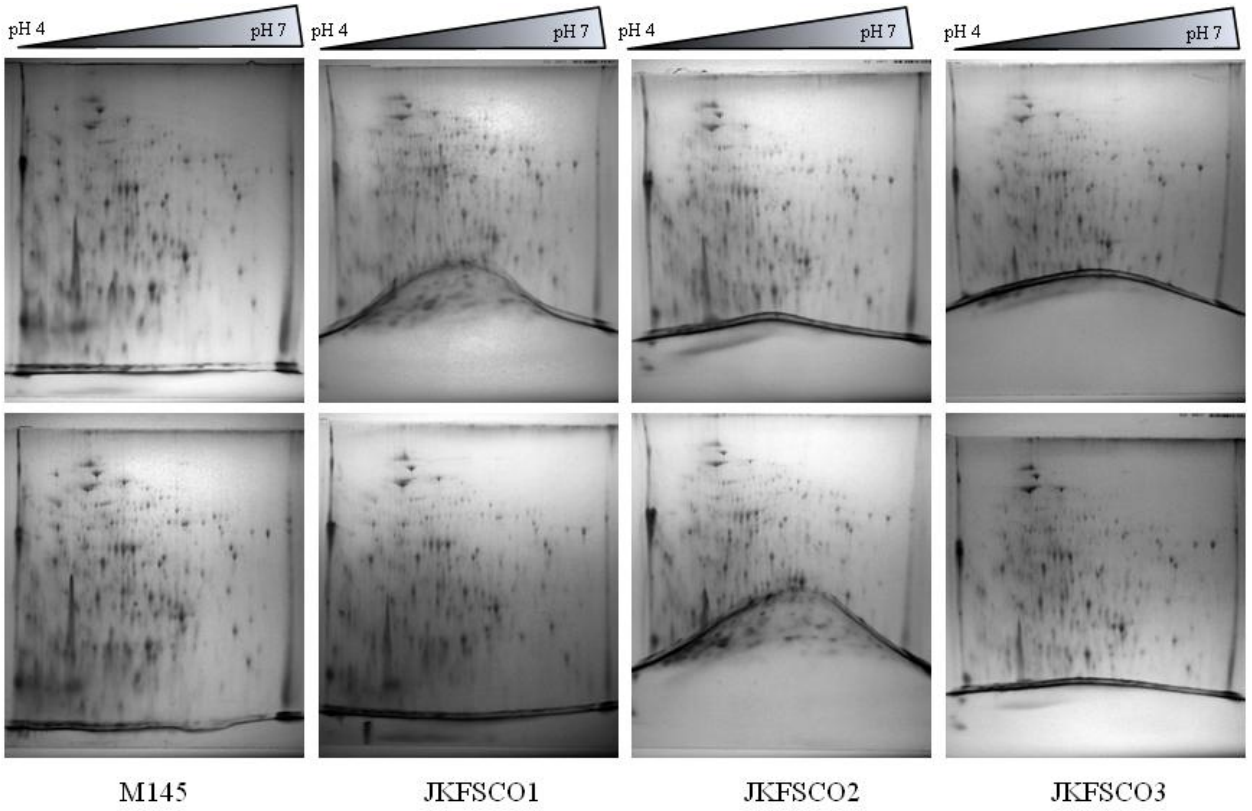

Figure A1: Duplicates of two-dimensional SDS PAGE separation of proteins present in cell wall washes of *S. coelicolor* strains M145, JKFSCO1, JKFSCO2 and JKFSCO3. Strains were cultured on SFM and incubated at 30°C for 20 hours. Cell wall protein samples were then prepared as described in Section 2.5.4. Protein pellets were resuspended in 2D sample buffer and the concentration estimated using a 2D Quant Kit (GE Healthcare). 900 µg of protein was separated by isoelectric focusing (pH gradient 4–7) followed by SDS PAGE. Proteins were stained with Coomassie blue prior to imaging. Images were analysed visually.

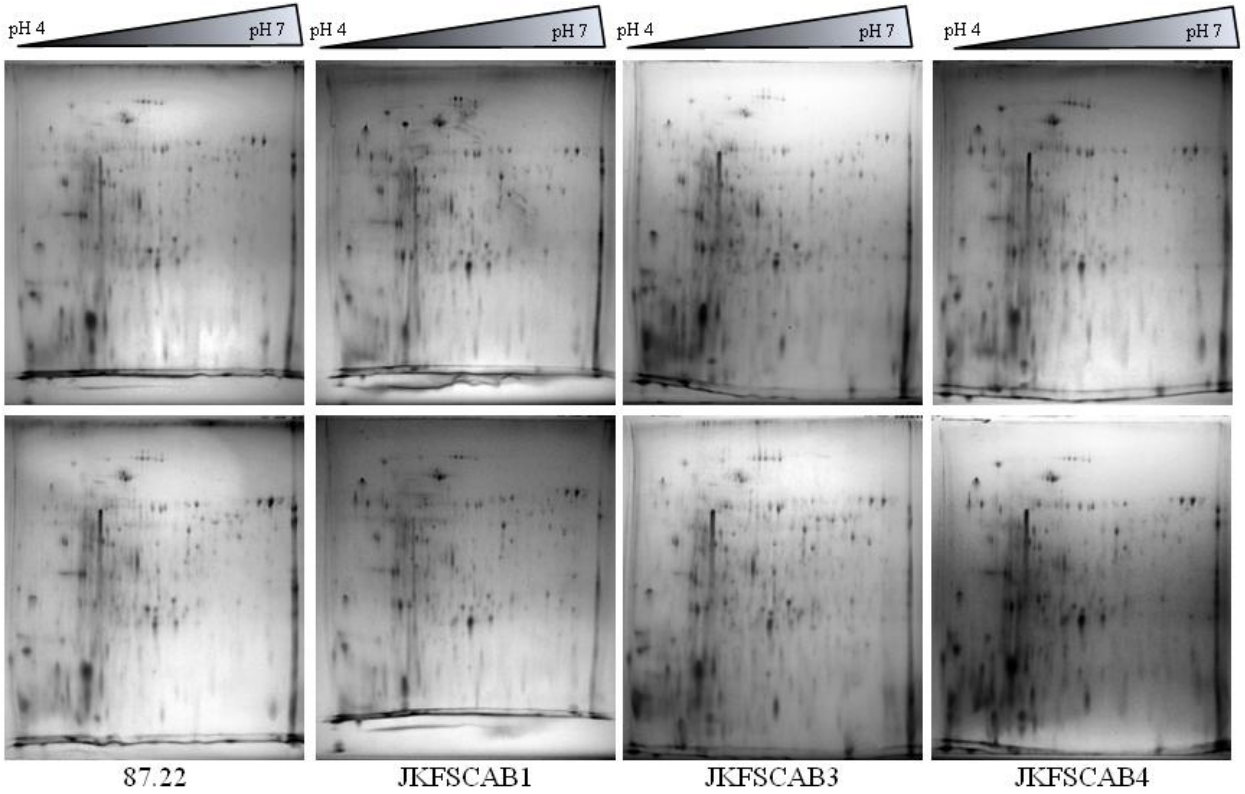

Figure A2: Duplicates of two-dimensional SDS PAGE separation of proteins present in cell wall washes of *S. scabies* strains 87.22, JKFSCAB1, JKFSCAB3 and JKFSCAB4. Strains were cultured on YME and incubated at 30°C for 20 hours. Cell wall protein samples were then prepared as described in Section 2.5.4. Protein pellets were resuspended in 2D sample buffer and the concentration estimated using a 2D Quant Kit (GE Healthcare). 900 µg of protein was separated by isoelectric focusing (pH gradient 4–7) followed by SDS PAGE. Proteins were stained with Coomassie blue prior to imaging. Images were analysed visually.

Figure A3: Virulence testing of *S. scabies* T7SS mutant strains using the radish seedling virulence assay. Radish seeds were surface sterilised for 5 minutes in 70 % ethanol followed by 10 minutes in 15 % bleach then rinsed ten times in 50 ml of sterile H_2O. Seeds were then allowed to germinate in the dark for 36-40 hours in a petri dish lined with filter paper that was pre-wet with 2 ml of sterile H_2O. Seedlings of equal size were transferred to each of six wells in 1.5 % water agar in a deep petri dish then inoculated with 200 µl of a freshly prepared spore suspension derived from the strain of interest (as indicated above each plant) adjusted to an OD_{600} of 0.5. Petri dishes were sealed with parafilm and incubated at 21(+/-2) °C with a 16 hour photoperiod for six days prior to excision of roots from the agar. Seedlings were assessed for disease symptoms and photographed. Scale bars = 1 cm.

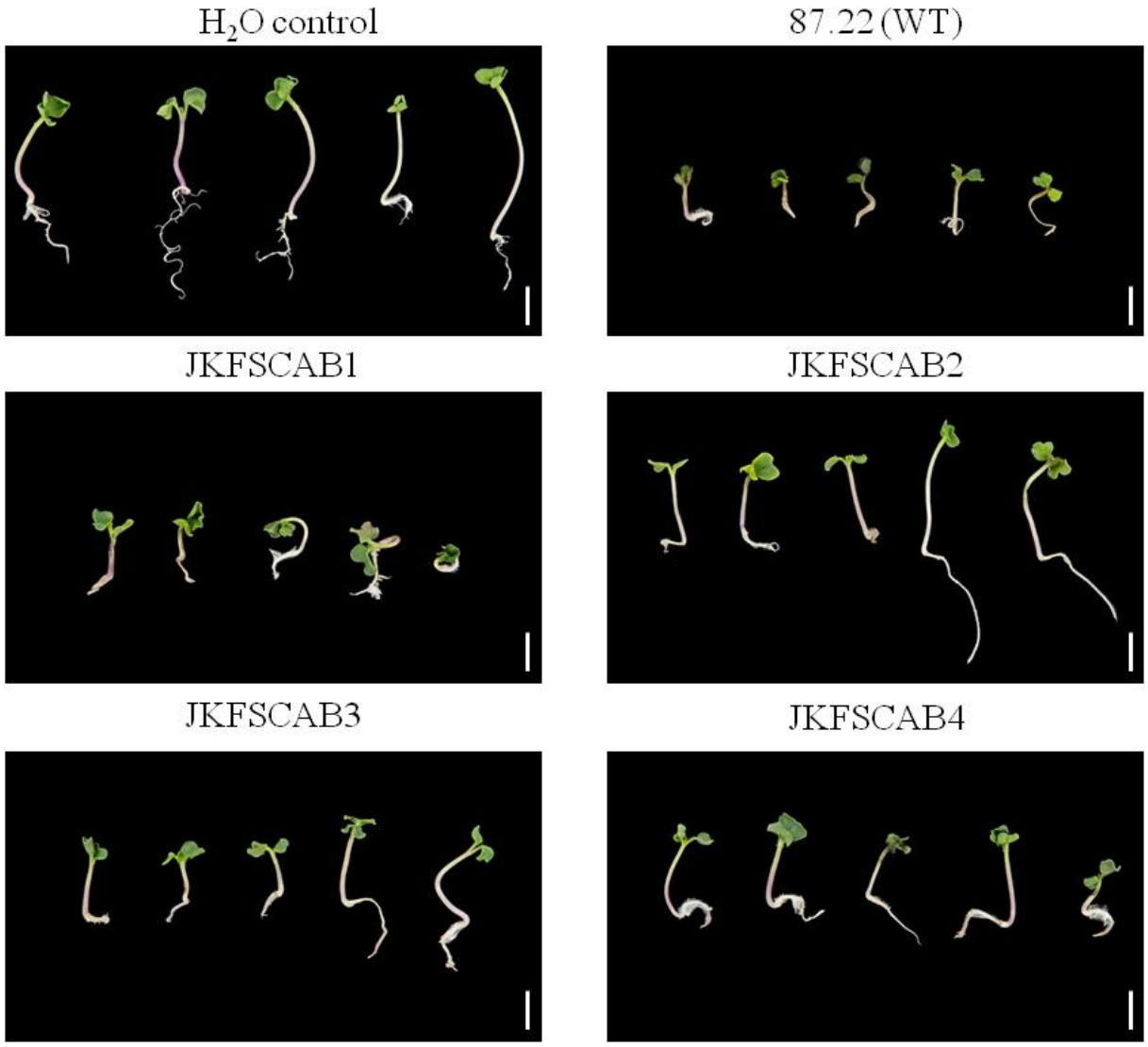

Figure A4: Virulence testing of *S. scabies* T7SS mutant strains using the radish seedling virulence assay. Radish seeds were surface sterilised for 5 minutes in 70 % ethanol followed by 10 minutes in 15 % bleach then rinsed ten times in 50 ml of sterile H_2O. Seeds were then allowed to germinate in the dark for 36-40 hours in a petri dish lined with filter paper that was pre-wet with 2 ml of sterile H_2O. Seedlings of equal size were transferred to each of six wells in 1.5 % water agar in a deep petri dish then inoculated with 200 µl of a freshly prepared spore suspension derived from the strain of interest (as indicated above each panel) adjusted to an OD_{600} of 0.5. Petri dishes were sealed with parafilm and incubated at 21(+/-2) °C with a 16 hour photoperiod for six days prior to excision of roots from the agar. Seedlings were assessed for disease symptoms and photographed. Scale bars = 1 cm.

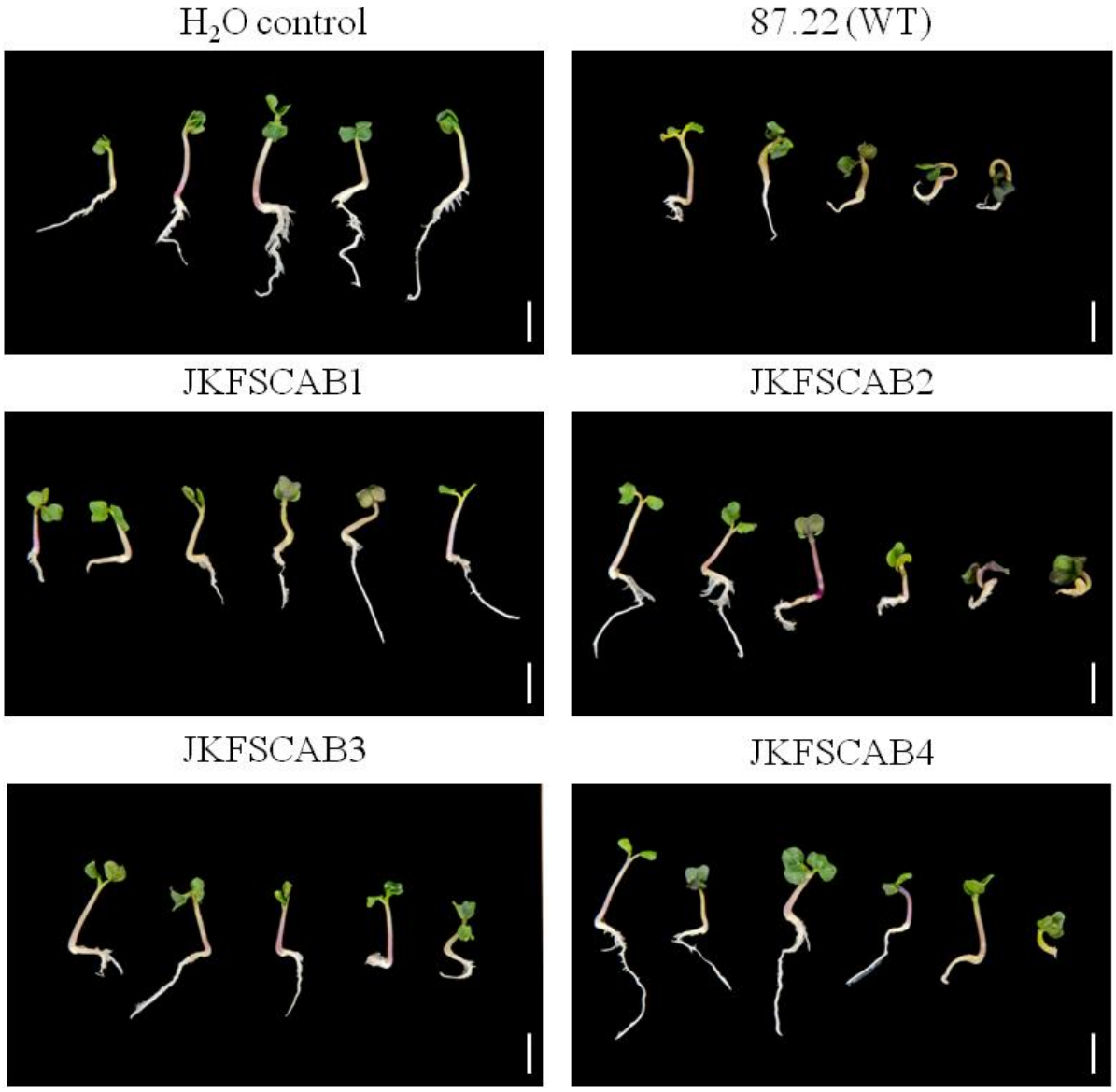

Figure A5: Virulence testing of *S. scabies* T7SS mutant strains using the radish seedling virulence assay. Radish seeds were surface sterilised for 5 minutes in 70 % ethanol followed by 10 minutes in 15 % bleach then rinsed ten times in 50 ml of sterile H_2O. Seeds were then allowed to germinate in the dark for 36-40 hours in a petri dish lined with filter paper that was pre-wet with 2 ml of sterile H_2O. Seedlings of equal size were transferred to each of six wells in 1.5 % water agar in a deep petri dish then inoculated with 200 µl of a freshly prepared spore suspension derived from the strain of interest (as indicated above each panel) adjusted to an OD_{600} of 0.5. Petri dishes were sealed with parafilm and incubated at 21(+/-2) °C with a 16 hour photoperiod for six days prior to excision of roots from the agar. Seedlings were assessed for disease symptoms and photographed. Scale bars = 1 cm.

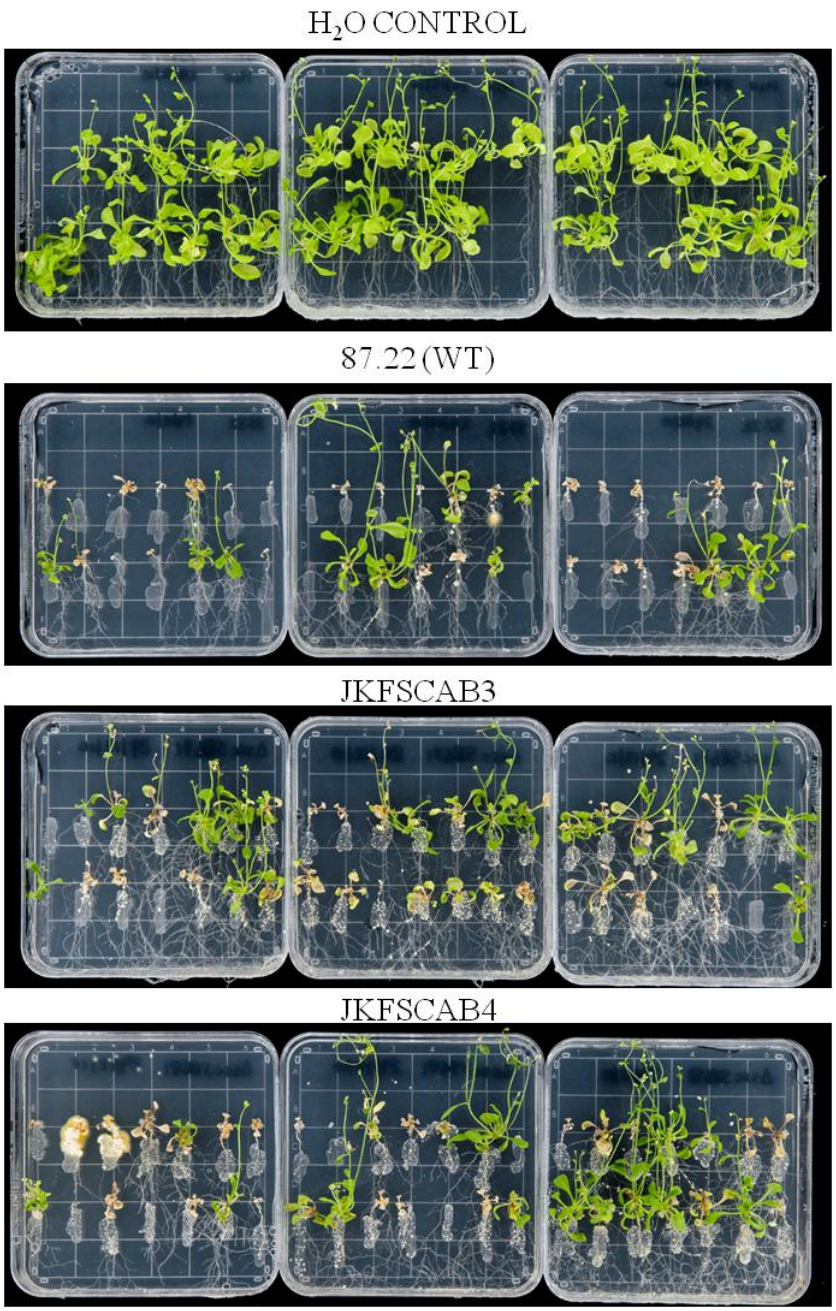

Figure A6: Virulence testing of *S. scabies* T7SS mutant strains using the *Arabidopsis* virulence assay. *Arabidopsis* seeds were surface sterilised for 3 hours by exposure to chlorine gas generated following the addition of 2 ml of 37 % HCl to 48 ml bleach. Seeds were stored in 1 ml of 0.1 % phytagar (Sigma) in the dark at 4°C in the dark for 2-5 days prior to being transferred onto MS/1 %sucrose/0.8 % agar in square petri dishes by pipetting. Plates were sealed with parafilm and incubated in an upright position at 21(+/-2) °C with a 16 hour photoperiod for four days. Root tips were then inoculated with 10 µl of a freshly prepared spore suspension derived from the strain of interest (as indicated above each panel) adjusted to an OD_{600} of 0.5. Petri dishes were sealed with parafilm and incubated in an upright position at 21(+/-2) °C with a 16 hour photoperiod for 4 weeks prior to being photographed.

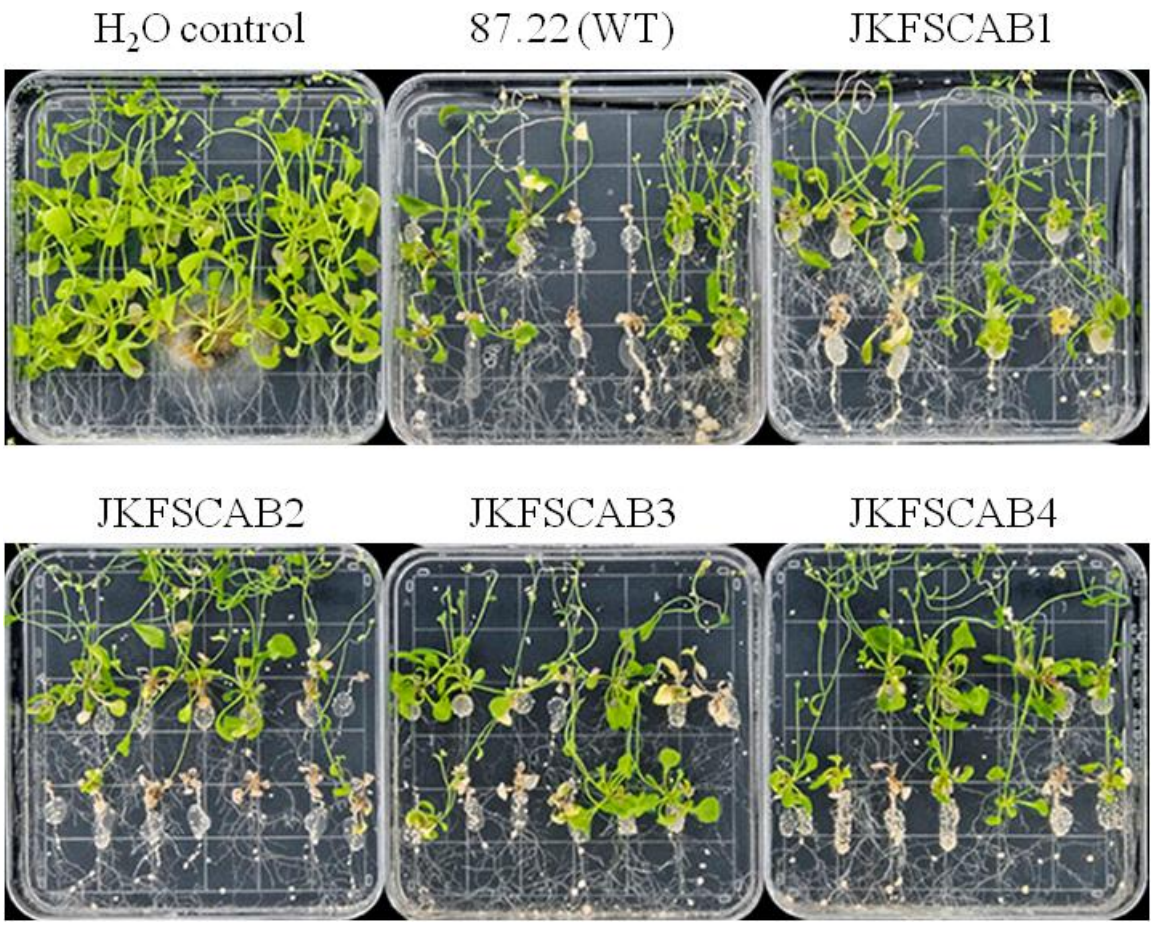

Figure A7: Virulence testing of *S. scabies* T7SS mutant strains using the *Arabidopsis* virulence assay. *Arabidopsis* seeds were surface sterilised for 3 hours by exposure to chlorine gas generated following the addition of 2 ml of 37 % HCl to 48 ml bleach. Seeds were stored in 1 ml of 0.1 % phytagar (Sigma) in the dark at 4°C in the dark for 2-5 days prior to being transferred onto MS/1 %sucrose/0.8 % agar in square petri dishes by pipetting. Plates were sealed with parafilm and incubated in an upright position at 21(+/-2) °C with a 16 hour photoperiod for four days. Root tips were then inoculated with 10 µl of a freshly prepared spore suspension derived from the strain of interest (as indicated above each panel) adjusted to an OD_{600} of 0.5. Petri dishes were sealed with parafilm and incubated in an upright position at 21(+/-2) °C with a 16 hour photoperiod for 39 days prior to being photographed.

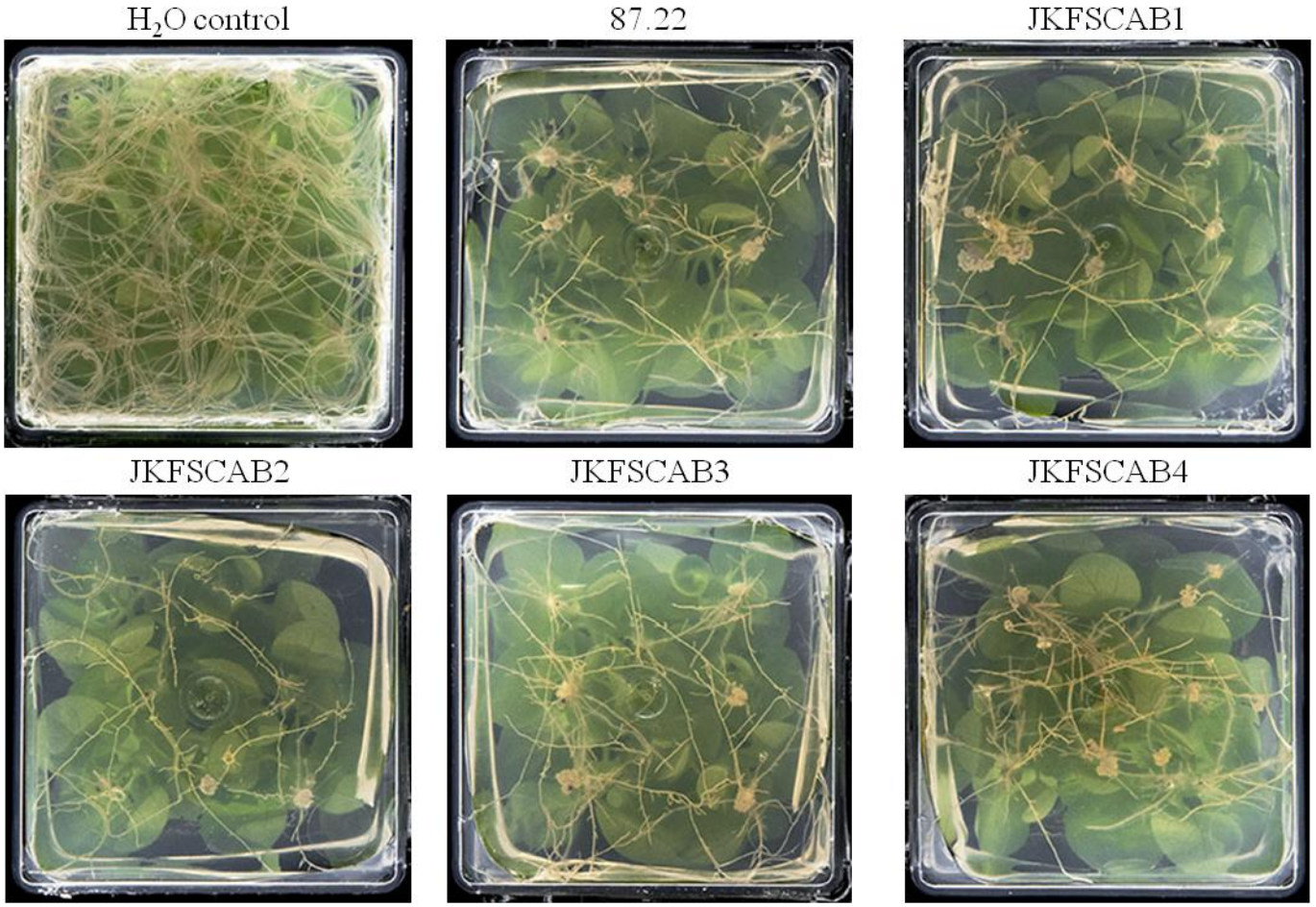

Figure A8: Close-up of roots following virulence testing of *S. scabies* T7SS mutant strains using the *N. tabaccum* virulence assay. Tobacco seeds were surface sterilised for 3 hours by exposure to chlorine gas generated following the addition of 2 ml of 37 % HCl to 48 ml bleach. Seeds were stored in 1 ml of 0.1 % phytagar (Sigma) in the dark at 4°C in the dark for 2-5 days then transferred onto MS/1 %sucrose/0.8 % agar in sterile magenta boxes. These were incubated in at 21(+/-2) °C with a 16 hour photoperiod for seven days prior to the surface of the agar in each box being flooded with 1 ml of sterile water containing approximately $5x10^7$ spores from a frozen spore stock derived from the strain of interest (as indicated above each group of plants). Plants were incubated at 21(+/-2) °C with a 16 hour photoperiod for 9 weeks prior to being photographed.

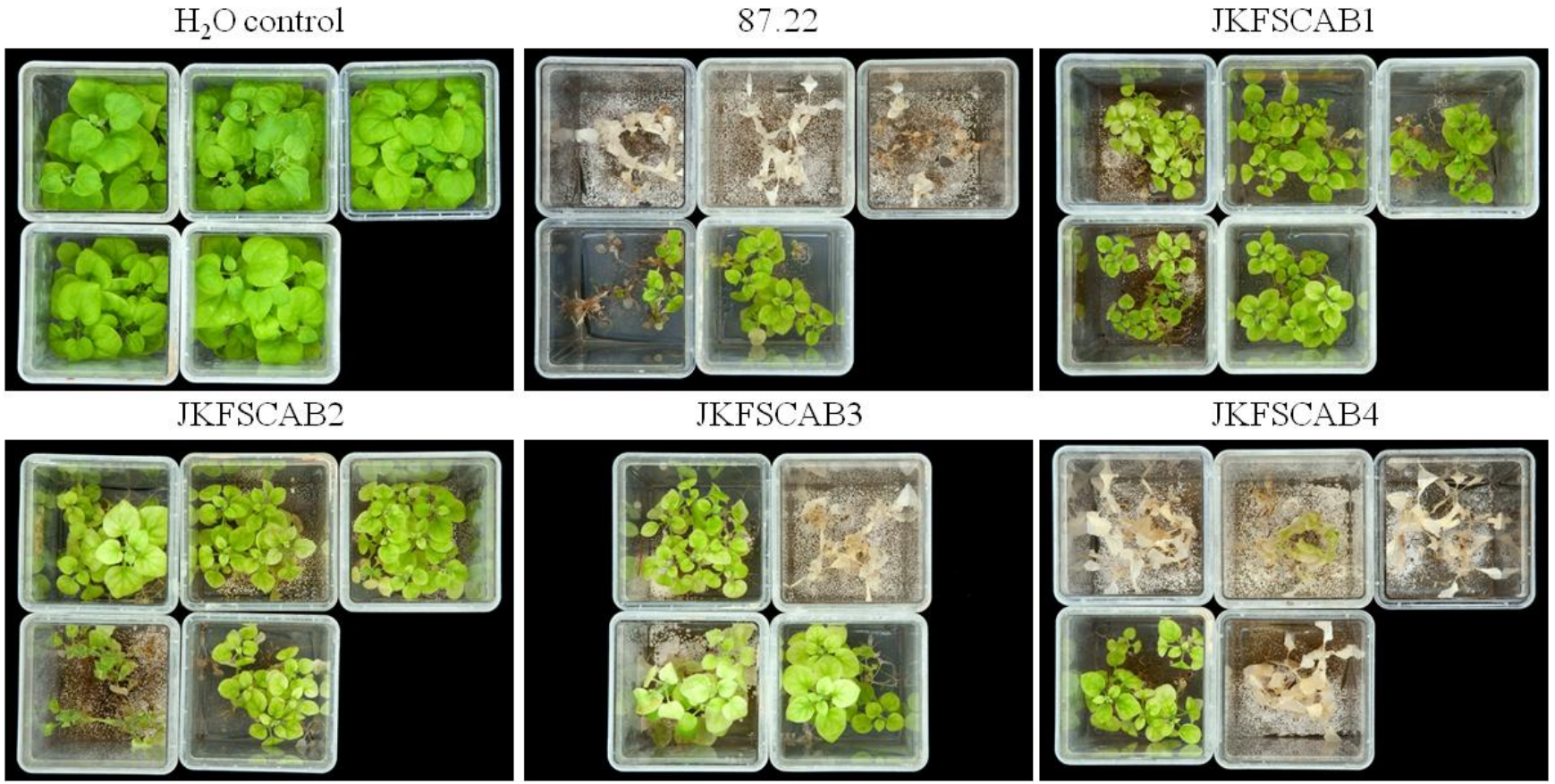

Figure A9: Virulence testing of *S. scabies* T7SS mutant strains using the *N. benthomiana* virulence assay. Seeds were surface sterilised for 3 hours by exposure to chlorine gas generated following the addition of 2 ml of 37 % HCl to 48 ml bleach. Seeds were stored in 1 ml of 0.1 % phytagar (Sigma) in the dark at 4°C in the dark for 2-5 days then transferred onto MS/1 %sucrose/0.8 % agar in sterile magenta boxes. These were incubated in at 21(+/-2) °C with a 16 hour photoperiod for seven days prior to the surface of the agar in each box being flooded with 1 ml of a freshly prepared spore suspension derived from the strain of interest (as indicated above each panel) adjusted to an OD_{600} of 10. Plants were incubated at 21(+/-2) °C with a 16 hour photoperiod for 53 days prior to being photographed from above.

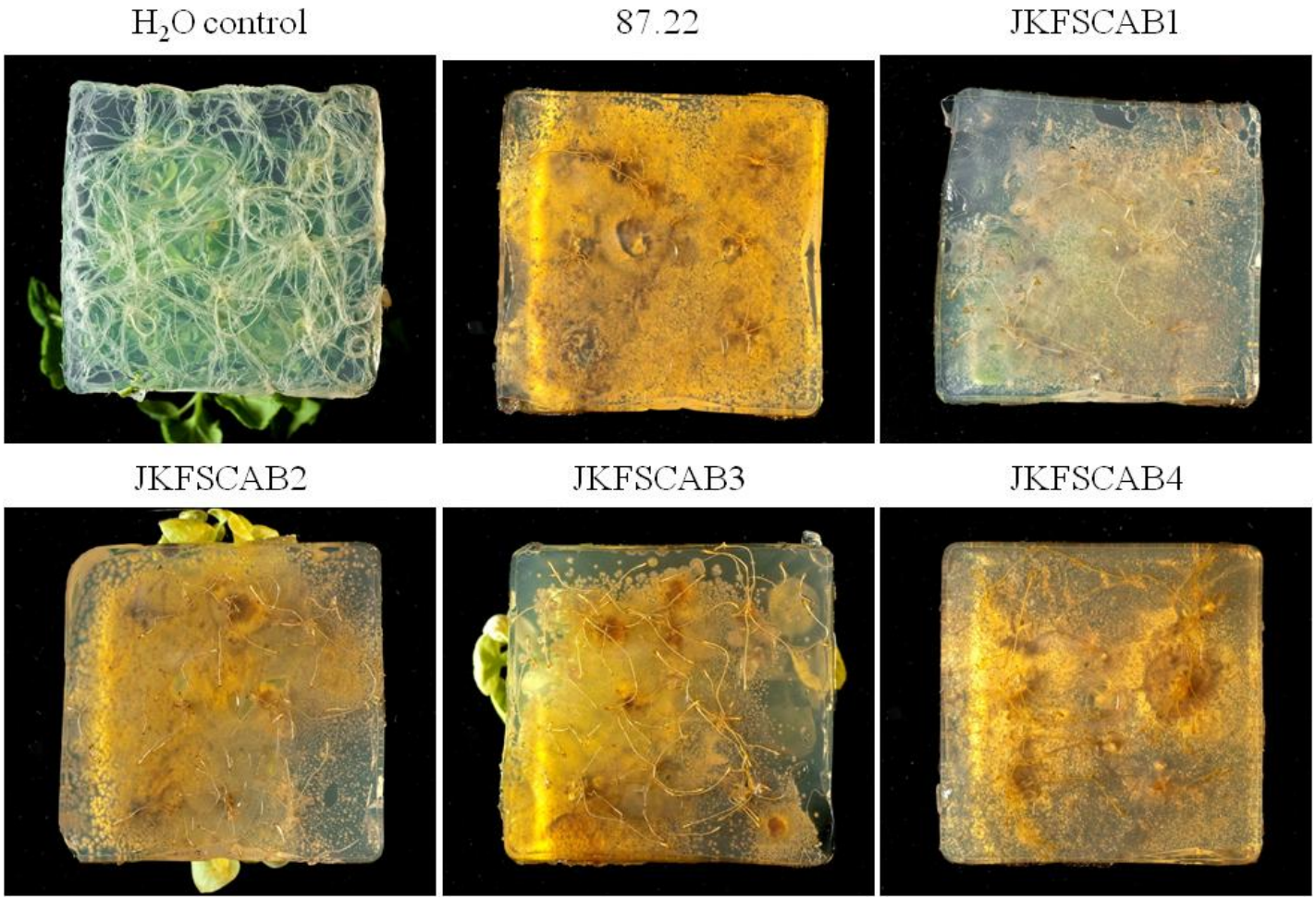

Figure A10: Close-up of roots following virulence testing of *S. scabies* T7SS mutant strains using the *N. benthomiana* virulence assay. Seeds were surface sterilised for 3 hours by exposure to chlorine gas generated following the addition of 2 ml of 37 % HCl to 48 ml bleach. Seeds were stored in 1 ml of 0.1 % phytagar (Sigma) in the dark at 4°C in the dark for 2-5 days then transferred onto MS/1 %sucrose/0.8 % agar in sterile magenta boxes. These were incubated in at 21(+/-2) °C with a 16 hour photoperiod for seven days prior to the surface of the agar in each box being flooded with 1 ml of a freshly prepared spore suspension derived from the strain of interest (as indicated above each panel) adjusted to an OD_{600} of 10. Plants were incubated at 21(+/-2) °C with a 16 hour photoperiod for 53 days prior to being photographed from below.

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The twin arginine protein transport pathway exports multiple virulence proteins in the plant pathogen *Streptomyces scabies*

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Summary

Streptomyces scabies **is one of a group of organisms that causes the economically important disease potato scab. Analysis of the *S. scabies* genome sequence indicates that it is likely to secrete many proteins via the twin arginine protein transport (Tat) pathway, including several proteins whose coding sequences may have been acquired through horizontal gene transfer and share a common ancestor with proteins in other plant pathogens. Inactivation of the *S. scabies* Tat pathway resulted in pleiotropic phenotypes including slower growth rate and increased permeability of the cell envelope. Comparison of the extracellular proteome of the wild type and Δ*tatC* strains identified 73 predicted secretory proteins that were present in reduced amounts in the *tatC* mutant strain, and 47 Tat substrates were verified using a Tat reporter assay. The Δ*tatC* strain was almost completely avirulent on Arabidopsis seedlings and was delayed in attaching to the root tip relative to the wild-type strain. Genes encoding 14 candidate Tat substrates were individually inactivated, and seven of these mutants were reduced in virulence compared with the wild-type strain. We conclude that the Tat pathway secretes multiple proteins that are required for full virulence.**

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Introduction

Potato scab is a polyphyletic disease caused by organisms in the genus *Streptomyces*, the best studied of which is *Streptomyces scabies* (Loria *et al.*, 2006). Like other streptomycetes, *S. scabies* is a mycelial bacterium that undergoes complex morphological differentiation involving the formation of aerial hyphae and spores (see Flärdh and Buttner, 2009 for a review of the complex biology of *Streptomyces*). The hyphal form of *S. scabies* infects plants through expanding plant tissue, including roots and tubers (Loria *et al.*, 2006; 2008).

One of the major pathogenicity determinants of *S. scabies* is thaxtomin, a nitrated dipeptide toxin that is a potent inhibitor of cellulose synthesis. The toxin induces plant cell hypertrophy in expanding plant tissues and likely facilitates penetration of plant tissue by the pathogen (King *et al.*, 1989; Healy *et al.*, 2000; Scheible *et al.*, 2003; Bischoff *et al.*, 2009). The mechanism by which thaxtomin inhibits cellulose synthesis is undefined but it apparently interacts with a highly conserved target, as the toxin affects all higher plants; this is congruent with the fact that thaxtomin-producing streptomycetes have an extremely wide host range. The thaxtomin biosynthesis genes are conserved in the plant pathogenic species, including *Streptomyces turgidiscabies* and *Streptomyces acidiscabies*, and are regulated by TxtR, an AraC family transcriptional regulator that binds cellobiose as a co-inducer (Kers *et al.*, 2005; Joshi *et al.*, 2007a). The *S. scabies* 87-22 genome contains a biosynthetic cluster that is highly similar in structure and organization to the coronafacic acid (CFA) clusters from the Gram negative plant pathogens *Pseudomonas syringae,* and *Pectobacterium atrosepticum* (Bignell *et al.*, 2010). CFA is the polyketide component of coronatine that acts as a jasmonate mimic during plant interactions; mutational studies demonstrate

that the cluster contributes to virulence in *S. scabies* 87-22 as it does in Gram negative pathogens. Unlike the thaxtomin biosynthetic cluster, the coronafacic acid-like cluster is not conserved in *S. turgidiscabies* or *S. acidiscabies*.

Secreted proteins are also critical determinants in the interaction between bacteria and eukaryotic hosts; virulence proteins are secreted either into the host environment or directly into host cells. Indeed, a secreted proteinaceous virulence factor, Nec1, lacks homologues outside of plant pathogenic streptomycetes, is required for colonization of the roots and may function to suppress plant defence responses (Bukhalid and Loria, 1997; Joshi *et al*., 2007b). Furthermore, the secreted protein TomA, is conserved among pathogenic streptomycetes (Kers *et al*., 2005) and is homologous to products of saponinase-encoding genes, which are important for host–pathogen interactions in some plant pathogenic fungi (Seipke and Loria, 2008).

The role of protein secretion in pathogenesis is particularly well characterized in the case of Gram negative bacteria, which have numerous systems dedicated to achieving the secretion of proteins across the complex double-membrane cell envelope (see, e.g. Christie *et al*., 2005; Büttner and He, 2009; Galán, 2009). In contrast, Gram positive bacteria have a simpler cell envelope, and secretion generally requires passage of proteins across only a single membrane. Therefore, it might be expected that the general protein transport machineries residing in the prokaryotic cytoplasmic membrane play more direct roles in the virulence of Gram positive organisms. In support of this it has been shown that many Gram positive bacteria have an accessory SecA protein, termed SecA2 that appears to contribute to bacterial virulence (e.g. Rigel and Braunstein, 2008).

The Tat system is, like Sec, a general protein export pathway that is found in the cytoplasmic membranes of some (although not all) bacteria and archaea. In Gram negative bacteria and in the Gram positive Actinobacteria, that include the streptomycetes, the Tat system is comprised of three essential components, TatA, TatB and TatC (Bogsch *et al*., 1998; Sargent *et al*., 1998; 1999; Weiner *et al*., 1998; Schaerlaekens *et al*., 2004; Hicks *et al*., 2006). By contrast, the Tat machineries in the low G + C Gram positive bacteria (exemplified by *Bacillus subtilis*) and in the archaea do not require a TatB protein and the Tat systems in these prokaryotes are made up only of TatA and TatC components (Jongbloed *et al*., 2004; Dilks *et al*., 2005).

Proteins are targeted to the Tat pathway by means of N-terminal signal peptides that superficially resemble Sec signal peptides, but that harbour a conserved S/T-R-R-x-F-L-K consensus motif, where the twin arginines are invariant and normally essential for efficient export by the Tat pathway (Berks, 1996; Stanley *et al*., 2000). The main distinguishing feature of the Tat system is that it transports fully folded proteins across the cytoplasmic membrane. In bacteria such as *Escherichia coli* and *B. subtilis*, relatively few proteins are exported by the Tat pathway and, in *E. coli* at least, the majority of them contain complex redox cofactors that are assembled into the substrate protein prior to transport across the membrane (reviewed in Palmer *et al*., 2005; Lee *et al*., 2006).

Although in most organisms the Tat pathway is generally held to be a relatively minor route of protein export, there is evidence that in some halophilic archaea, and in particular in *Streptomyces* bacteria, Tat exports significant numbers of proteins. Thus, bioinformatic predictions on the genome sequence of *Streptomyces coelicolor* suggested that as many as 189 proteins may be substrates of the Tat pathway (Rose *et al*., 2002; Dilks *et al*., 2003; Bendtsen *et al*., 2005). The validity of these prediction programmes were largely borne out by proteomic studies of *S. coelicolor* wild type (WT) and *tat* mutant strains coupled with testing of candidate Tat signal peptides in Tat-dependent reporter assays, where a total of 33 Tat substrate proteins have now been confirmed (Li *et al*., 2005; Widdick *et al*., 2006).

In spite of the fact that the Tat system is a general protein export system, Tat-secreted proteins contribute to virulence in some Gram negative and Gram positive bacteria (e.g. Ochsner *et al*., 2002; Ding and Christie, 2003; Pradel *et al*., 2003; Saint-Joanis *et al*., 2006). Given the apparent importance of the Tat pathway in protein secretion in the streptomycetes, we reasoned that this pathway may play an essential role in the virulence of *S. scabies*. Analysis of the recently available *S. scabies* 87-22 genome sequence (http://www.sanger.ac.uk/Projects/S_scabies/) with Tat signal peptide prediction programmes suggests that in excess of 100 proteins may be exported by the Tat pathway in this organism, including several that appear to share a common ancestor with homologues in other plant pathogens. Through proteomic studies coupled with reporter protein secretion assays we have verified 47 Tat substrates in this organism. Importantly, we show that a Δ*tatC* mutant of *S. scabies* is almost completely avirulent, and that the Tat pathway secretes multiple proteins that are required for full virulence.

Results

Bioinformatic analysis of the S. scabies *genome sequence indicates that it encodes many candidate Tat substrates*

Two generally available prediction programmes, TATFIND 1.4 and TatP, have been developed to identify candidate Tat-targeting signal peptides (Rose *et al*.,

2002; Bendtsen *et al*., 2005). When these programmes are applied to all of the open reading frames (ORFs) encoded by the genome sequence of *S. scabies*, TATFIND 1.4 predicts 154 likely Tat substrates, while TatP predicts 177 (the list of these proteins is available at http://www.lifesci.dundee.ac.uk/groups/tracy_palmer/links.html). However, both of these programmes generate a degree of false positive and false negative predictions; for example, when applied to the genome sequence of *E. coli*, both programmes overpredict Tat substrates by 20–25% (Dilks *et al*., 2003; Bendtsen *et al*., 2005). In general, the sets of candidate Tat substrates predicted by the two programmes show partial but not complete overlap. However to date, where tested, all of those predicted to be Tat substrates by both TATFIND 1.4 and TatP have been shown to have *bona fide* Tat-targeting signals (Widdick *et al*., 2006). This therefore gives confidence that the subset of proteins predicted to be Tat substrates by both programmes are highly likely to represent real substrates. For *S. scabies*, 82 proteins are predicted to be Tat substrates by each of the two programmes, and these are listed in Table S1.

It was noted from previous work with *S. coelicolor* that it is sometimes difficult to identify the correct N-terminus of a predicted protein and this can lead to mis-annotation of start codons (D.A. Widdick, G. Chandra and T. Palmer, unpublished). Therefore, all of the ORFs of *S. scabies* were re-analysed to take account of all potential start codons; these modified ORFs were also analysed by TATFIND 1.4 and TatP, leading to the identification of a further 21 potential Tat substrates, which are also listed in Table S1. Additionally, it is known that some Tat signals have very long n-regions that preclude their identification by TATFIND 1.4 or TatP; these programmes have maximum preferred lengths for signal peptide n-regions. Therefore all of the *S. scabies* ORFs were truncated *in silico* by 30 or 60 amino acids and re-analysed by TATFIND 1.4 and TatP resulting in the identification of an additional five candidate Tat substrates (Table S1). Finally, all of the ORFs that were predicted to have Tat signal peptides by only one of the two Tat signal peptide prediction programmes were sorted manually for those that were likely to be true Tat substrates on the basis of binding a complex cofactor, showing high homology to confirmed Tat substrates from other organisms, or by virtue of the fact that the twin arginine motif was highly conserved across bacterial homologues. This added a further 14 substrates to the manually curated list of likely Tat substrates (Table S1). An additional four proteins were added as a result of the outcome of the Tat-dependent reporter experiments described below and this curated list of likely Tat substrates in *S. scabies* (Table S1) therefore contains a total of 126 proteins.

Phenotype of a S. scabies *Δ*tatC *deletion strain*

In order to test the *in silico* predictions that there are many Tat substrates encoded in the genome of *S. scabies*, it was necessary to inactivate the Tat pathway. Inspection of the genome sequence of *S. scabies* reveals that, like other streptomycetes, it encodes the *tatA* (*SCAB73591*) and *tatC* (*SCAB73601*) genes in close proximity (separated by 51 bp) and a probable *tatB* gene (*SCAB31121*) at a distant location. Because the *tatC* gene encodes an essential component of the Tat transporter (e.g. Bogsch *et al*., 1998), we constructed a marked deletion of *tatC* as described in Experimental Procedures.

Inactivation of the Tat pathway in *S. coelicolor* and *Streptomyces lividans* is associated with a number of phenotypic changes including a dispersed growth in liquid culture (rather than the mycelial pellets that are seen for the WT strains), failure to sporulate on solid media containing sucrose and increased fragility of the hyphae that may reflect a cell wall defect (Schaerlaekens *et al*., 2004; Widdick *et al*., 2006). To ascertain whether inactivation of the Tat pathway had similar pleiotropic effects on *S. scabies*, we initially assessed the growth rate of the *S. scabies* WT and isogenic Δ*tatC* strains. It should be noted that *Streptomyces* growth rates cannot be measured by spectroscopic analysis as the presence of mycelial clumps in liquid culture, coupled with the production of cellular debris by programmed cell death, distorts results obtained by spectroscopy (Miguélez *et al*., 1999). Therefore to assess growth, total cytosolic protein was extracted from living cells and measured, as described in Experimental Procedures.

As shown in Fig. 1A, when cultured in tryptone soya broth (TSB) medium, the Δ*tatC* mutant strain does not grow as well as the WT strain. While both strains reach a peak of growth at around 28 h post inoculation (hpi), the mutant strain showed only about half of the total protein content per ml of culture as the WT strain. Following the growth peak, both strains showed a subsequent decline in the total protein content that may be related to programmed cell death (Miguélez *et al*., 1999). The slower growth rate of the Δ*tatC* strain was also apparent on solid growth media; for example, the *tatC* mutant strain formed smaller colonies on yeast extract malt extract (YEME) agar plates (Fig. 1B). It is also striking to note in Fig. 1B that the Δ*tatC* strain lacks the brown colouration of the medium and the hyphae that is associated with the WT strain. This may reflect a lack of production of melanin, which is catalysed by tyrosinase, a cofactor-containing Tat substrate (Schaerlaekens *et al*., 2001). Two probable tyrosinase enzymes are encoded in the *S. scabies* genome, SCAB85681/85691 and SCAB59231/59241, and the MelC1 components of both of these have candidate twin arginine signal peptides (see Table S1). It should be noted

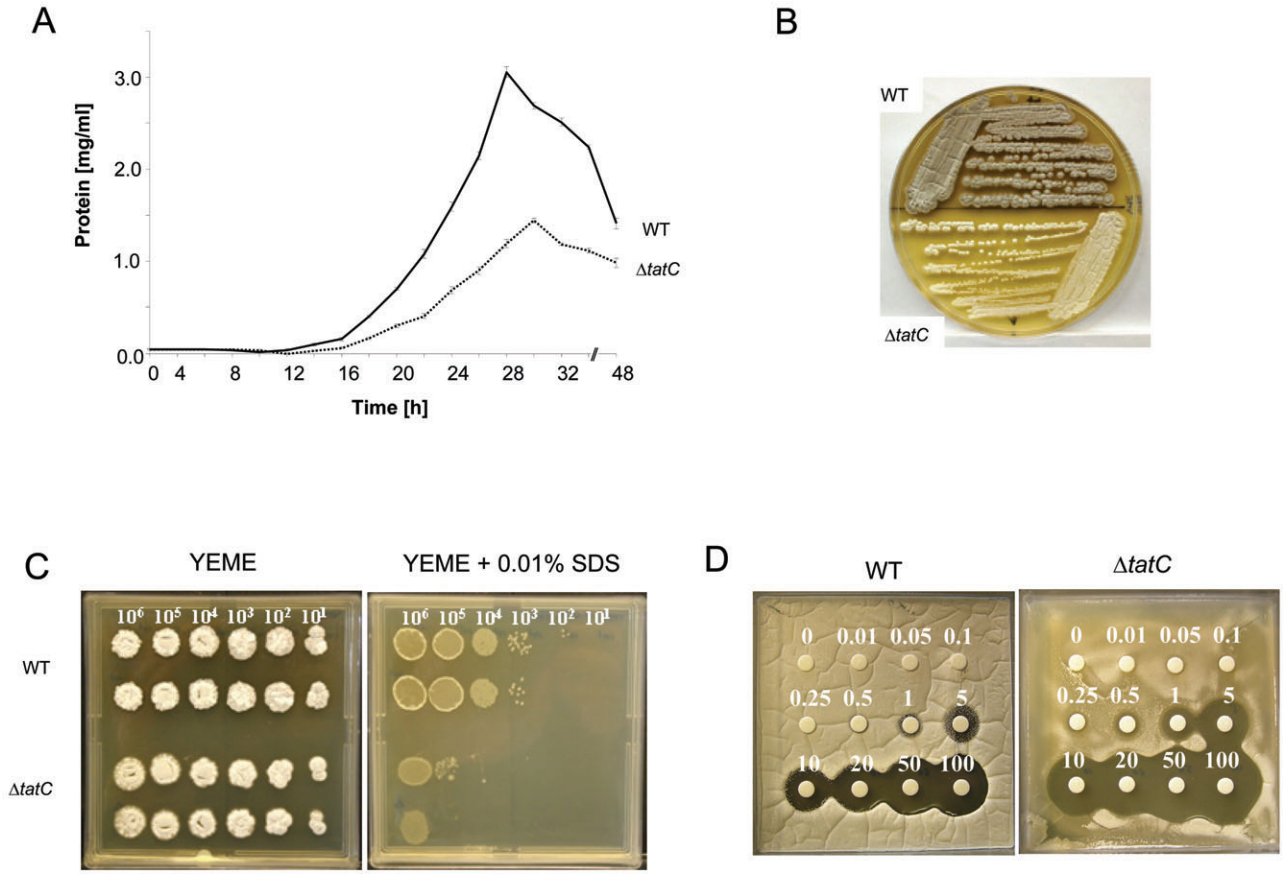

Fig. 1. Inactivation of the Tat pathway in *S. scabies* results in pleiotropic growth phenotypes.
A. 100 ml of TSB medium was inoculated with either *S. scabies* WT or Δ*tatC* strains, at a concentration of 100 000 spores per ml and incubated at 30°C with shaking. At the indicated time points, 3 × 1 ml samples were removed from each culture, the hyphae pelleted and total protein content was determined using the Biorad DC Protein Assay. The error bars represent the standard error of the mean of samples taken at indicated time points, where $n = 3$.
B. Spores of the indicated strains were streaked out from a fresh solid medium culture with an inoculation loop onto YEME medium and incubated for 7 days at 30°C.
C. Spores dilutions of each strain were plated onto YEME medium and onto the same medium containing 0.01% SDS. The plates were incubated at 30°C for 7 days.
D. Approximately 10^7 spores of each strain were spread on 144 cm^2 DNA medium. Antibiotic discs with of 6 mm diameter were imbued with one of the following different amounts of vancomycin in μg as indicated and plates were incubated at 30°C for 7 days.

that although the phytotoxin thaxtomin is also pigmented, thaxtomin production was not affected by inactivating *tatC* (Fig. S1). Complementation of the *S. scabies* Δ*tatC* strain with an integrative plasmid harbouring the *S. scabies tatAC* genes restored production of the brown pigment, and also reversed the slow growth rates seen on solid media, indicating that these phenotypes are directly linked to the *tatC* mutation (Fig. S2).

Inactivation of the Tat pathway is often associated with a decrease in the integrity of the bacterial cell envelope, and in some organisms at least this is linked to an inability to export Tat-dependent proteins involved in cell wall remodelling (Ize *et al*., 2003; Caldelari *et al*., 2006). In *E. coli* and *P. syringae* this has been observed as an increased sensitivity to the presence of the detergent SDS when the Tat pathway is inactivated. As shown in Fig. 1C, deletion of the *S. scabies tatC* gene also results in an increased sensitivity to killing by SDS, which might also be suggestive of a Tat-linked cell envelope defect in this organism. A possible underlying defect in the *S. scabies* cell wall linked to inactivation of the Tat pathway was investigated by determining the sensitivity of the WT and Δ*tatC* strains to the cell wall-directed antibiotic vancomycin. As shown in Fig. 1D, the Δ*tatC* strain was clearly significantly more sensitive to killing by vancomycin than the WT strain, and a similar result was also seen with a different cell wall-directed antibiotic, bacitracin (data not shown). These observations suggest that the cell wall of the *tatC* mutant strain differs from that of the WT.

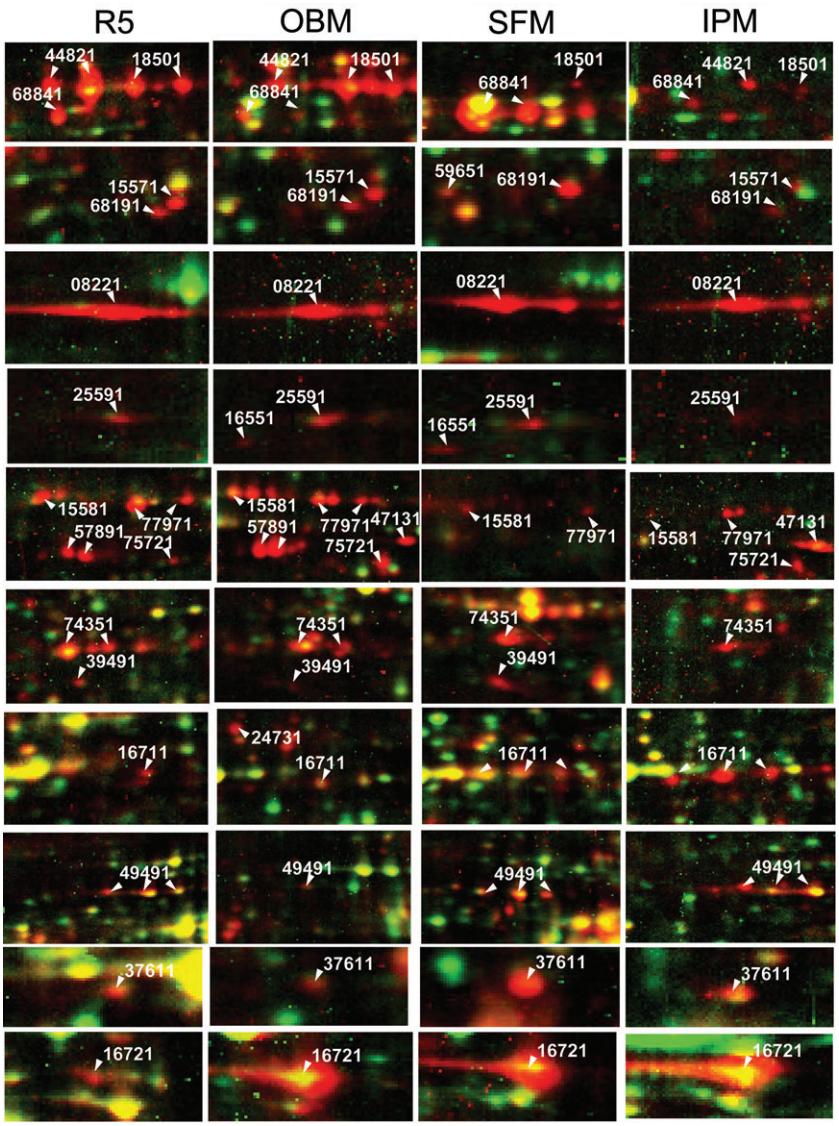

Fig. 2. Close-up of protein spots that are predominantly present in the WT extracellular proteome in all four types of growth media. The *S. scabies* WT and Δ*tatC* strains were grown on all four of the following growth media; IPM, OBM, R5 and SFM, and extracellular proteins were harvested from cell wall washes, TCA precipitated and subjected to 2D-PAGE as described in Experimental Procedures. Depicted are enlarged sections of the dual channel images of the extracellular proteomes of *S. scabies* WT (red colour) in comparison to the *tatC* mutant strain (green colour). The red-appearing proteins are labelled by the SCAB number and include 28 proteins that are strongly exported in more than one growth medium in the WT and decreased or absent in the Δ*tatC* mutant. The ratios for all identified proteins exported at lower amounts by the Δ*tatC* mutant are given in Table S2.

Analysis of the exported proteome of the S. scabies *WT and* Δ tatC *strains*

We next chose to examine the Tat-dependent proteome of *S. scabies* by comparison of the extracellular proteins produced by the WT and the *tatC* mutant strains under different growth conditions. As noted previously, the complex life cycle of streptomycetes results in significant lysis of the hyphae in liquid culture, and release of many cytoplasmic proteins into the growth medium. Therefore, we followed the procedure of Widdick *et al*. (2006), which involves growing *Streptomyces* on solid media on top of cellophane discs and washing the biomass with lithium chloride to release cell wall-associated proteins. The strains were cultured on four standard *S. scabies* growth media – oat bran medium (OBM), instant potato mash medium (IPM), soy-flour mannitol medium (SFM) and R5 medium (which is a more defined medium than the other three and lacks plant-derived material), and proteins from the cell wall washes were analysed by two-dimensional gel electrophoresis. For each growth medium analysed, the proteomes of the WT and *tatC* mutant strain were compared using the Decodon Delta 2D software with the WT proteins false coloured in red and the *tatC* proteins in green to allow differences to be visualized. The results of the proteome comparison for each type of growth media are shown in Fig. S3, and close-ups of some abundant red protein spots that are detected in the WT but are absent or decreased in the Δ*tatC* strain in at least two different growth media are presented in Fig. 2.

It is clear from inspecting the gels shown in Fig. S3 and the panels in Fig. 2 that many proteins are exported at lower amounts in the *tatC* mutant strain. The red-appearing protein spots were identified using tryptic

digestion and MALDI-ToF mass spectrometry. In total, 103 proteins were identified – these are indicated on Figs. 2 and S3, and are listed in Table 1. The ratios of the protein amounts for all red spots that are more highly exported in the WT were quantified in relation to the Δ*tatC* strain and presented in Table S2. The sequence coverages and protein scores for all identified proteins are given in Table S3.

Among the 103 proteins we identified, 73 proteins were predicted to have N-terminal signal peptides. Of these, 14 were predicted to be Tat substrates by both TATFIND and TatP (and this was increased to 19 if alternative start sites were selected), and a further nine were predicted to be Tat substrates by one of the two programmes. Interestingly, these 28 predicted Tat substrates include several enzymes that are related to phosphate starvation conditions, such as alkaline phosphatases (SCAB77971, SCAB68191) glycerophosphoryl diesterase (SCAB74351), and 5′-nucleotidases (SCAB68841, SCAB49491), which are abundantly exported proteins in WT cells (Fig. 2). Furthermore, several glycosyl hydrolases (SCAB16771, SCAB17721), cellulases (SCAB25591) and mannosidases (SCAB16551) were exported strongly in WT cells but at significantly reduced levels in the *tatC* mutant strain.

In addition to these 28 predicted Tat substrates, there were 50 signal peptide-bearing proteins that were reduced in the extracellular proteome of the *tatC* mutant but were not predicted to be Tat substrates and many of these did not have consecutive arginines in their signal peptides. It should be noted that the majority of exported proteins identified as being differentially missing from the extracellular proteome of the *S. coelicolor* Δ*tatC* strain were also not Tat substrates, and it was concluded that they were simply not produced in the Δ*tatC* strain because of the pleiotropic effects of the mutation (Widdick *et al*., 2006). It is possible that some of these differences also relate to the different growth rates of the *S. scabies* WT and Δ*tatC* strains. Finally, 30 proteins were identified in the cell wall wash of the *S. scabies* WT strain that did not appear to have N-terminal signal peptides (Table 1). It is likely that most of these proteins are cytoplasmic and their presence in the cell wall is due to contamination, with the notable exception of tyrosinase (SCAB85681) which is a known Tat substrate that lacks a signal peptide and is transported through the Tat system by forming a complex with a signal peptide-bearing partner protein (Chen *et al*., 1992; Leu *et al*., 1992; Schaerlaekens *et al*., 2001).

Verification of S. scabies *Tat-targeting signal peptides using the agarase reporter assay*

Using a combination of bioinformatic and proteomic approaches described above we identified a large number of candidate Tat substrates in *S. scabies.* A common method for ascertaining whether a given protein is likely to be a Tat substrate is to fuse its signal peptide to a reporter protein and determine whether it is able to mediate export by the Tat pathway. Recently, Widdick *et al*. (2006; 2008) described the agarase reporter system as a convenient and reliable reporter to test for Tat-dependent export. Agarase is a Tat-exported extracellular enzyme produced by *S. coelicolor* whose extracellular activity can be readily detected by the formation of halos on agar plates after staining with iodine. Agarase cannot be exported in an active form by the Sec pathway, but has been shown to be exported by the *Streptomyces* Tat pathway when fused to Tat signal peptide sequences from a wide range of organisms including Gram negative bacteria, archaea and even eukaryotes (Widdick *et al*., 2006; 2008). Because the size of the halo is related to the amount of agarase secreted, the assay is also semi-quantitative.

We therefore selected 35 proteins that had been identified from the extracellular proteome of the WT strain, including 13 from Table 1 that were predicted to be Tat substrates by both TATFIND 1.4 and TatP, three identified by TATFIND 1.4 only, two identified by TatP only, a further 16 that had recognizable signal peptides that were not predicted to be Tat-targeting by either prediction programme, and one that as presented had no apparent signal peptide but if an upstream start site was used it had a good candidate Tat signal peptide. The signal peptides from each of these proteins were fused in-frame to the mature region of agarase. The constructs are designed such that each clone carries the *dagA* ribosome binding site, with identical spacing between the ribosome-binding site and the start codon (Widdick *et al*., 2006; 2008). The recombinant proteins were expressed in both *S. lividans* WT and Δ*tatC* strains, and scored for agarase activity. In total, 14 of the 35 signal peptides were able to mediate Tat-dependent export of agarase (Fig. 3A) and these proteins are listed in Table 2.

Of the proteins that had signal peptides that could export agarase, ten of them were predicted to have Tat signal peptides by both programmes, and a further two by one or other programme. The remaining two (SCAB15581 and SCAB68191) did not have Tat signal peptides on the ORFs as called, but if plausible upstream start codons were used both had very good signal peptides that were recognized as Tat-targeting by both TATFIND 1.4 and TatP. This observation strongly suggests that the start codons of these two proteins have been mis-annotated (http://www.sanger.ac.uk/Projects/S_scabies/).

Interestingly, for two of the signal peptides that were able to mediate export of agarase, no agarase activity was detected if alternative versions of these signal peptides were tested. For SCAB15581, if the n-region of the signal peptide was truncated by selecting an alternative start codon closer to the twin arginine motif, no agarase

Table 1. Proteins that are predominantly found in the extracellular fraction of the *S. scabies* wild-type strain following 2D gel analysis.

Protein	Growth medium detected	Putative function	Agarase test[a]
Pass TATFIND 1.4 and TatP			
SCAB08951	R5	ABC-type $Fe3^{+}$ transport system, periplasmic component	Pass
SCAB15571	IPM, OBM, R5	Putative secreted phosphoesterase	Pass
SCAB16551	OBM, SFM	Putative mannosidase	ND
SCAB16651	IPM	Putative secreted protein	Pass
SCAB25591	IPM, OBM, R5, SFM	Putative secreted cellulase	Pass
SCAB38731	OBM	Putative secreted beta-lactamase	Pass
SCAB47131	IPM, OBM	Peptidase family M23/M37 protein	Fail[b]
SCAB59671	R5	Putative hydrolase	Pass
SCAB63891	IPM, SFM	Putative secreted transport-associated protein	Fail[b]
SCAB68841	IPM, OBM, R5, SFM	Putative 5′ nucleotidase	Pass
SCAB74351	IPM, OBM, R5, SFM	Glycerophosphoryl diester phosphodiesterase	Pass
SCAB75721	IPM, OBM, R5, SFM	Putative secreted protein	Pass
SCAB77971	IPM, OBM, R5, SFM	Putative secreted phosphatase (fragment)	Fail[b]
SCAB81841	OBM	Putative secreted hydrolase	Pass
Pass TATFIND 1.4 only			
SCAB18501	IPM, OBM, R5, SFM	Alpha-N-acetylglucosaminidase	Pass
SCAB27931	SFM	Putative neutral zinc metalloprotease	Fail[b]
SCAB37611	IPM, OBM, R5, SFM	Putative secreted aminopeptidase	Fail
SCAB57891[c]	OBM, R5	Conserved hypothetical protein	ND
SCAB82451	SFM	Putative secreted protein	ND
Pass TatP only			
SCAB08221	IPM, OBM, R5, SFM	Conserved hypothetical protein	Pass
SCAB64081	IPM, SFM	Putative secreted protein	Fail
SCAB78761	OBM	Putative secreted protein	ND
SCAB84861	R5	Putative secreted amidase	ND
Pass Signal P but not TATFIND 1.4 or TatP			
SCAB01191	IPM	Putative secreted protein	ND
SCAB04761	R5	Putative secreted protein	ND
SCAB05351	R5	Substrate-binding component of ABC transporter	Fail
SCAB07651	IPM	Neutral zinc metalloprotease	Fail
SCAB08871	IPM, OBM	Secreted endoglucanase	ND
SCAB13321	R5	S15 non-peptidase homologue family	ND
SCAB13551	SFM	Conserved hypothetical protein	Fail
SCAB14911	SFM	Putative secreted protein	ND
SCAB16431	OBM	Putative possible cellulase CELA1	ND
SCAB16711	IPM, OBM, R5, SFM	Putative secreted glycosyl hydrolase	Fail[b]
SCAB16721	IPM, OBM, R5, SFM	Putative secreted glycosyl hydrolase	Fail
SCAB17001	IPM, SFM	Secreted cellulase	ND
SCAB17571	IPM	Putative secreted peptidase	Fail
SCAB18081	IPM, SFM	Gamma-glutamyltranspeptidase	ND
SCAB18661	SFM	Putative serine protease	ND
SCAB19481	IPM	RTX-family exoprotein	ND
SCAB19491	SFM	Putative probable exported protein	ND
SCAB19941	SFM	Cyclase	ND
SCAB24731	OBM	Putative secreted aminopepetidase	ND
SCAB24891	SFM	Glutamate-binding protein	ND
SCAB26841	SFM	Putative serine protease	ND
SCAB27771	OBM	Putative secreted protein	ND
SCAB31531	IPM, R5	Putative BldKB-like transport system extracellular solute-binding protein	ND
SCAB33981	SFM	Hypothetical protein 2SCK36.08	
SCAB36371	IPM	Putative xylanase/cellulase	Fail
SCAB41181	SFM	Conserved hypothetical protein	ND
SCAB43901	IPM	Putative secreted hydrolase	Fail
SCAB44001	SFM	Putative secreted protein	ND
SCAB44161	R5, SFM	Hydrolase	ND
SCAB44541	IPM, OBM	Putative secreted protein	ND
SCAB44691	IPM, OBM, SFM	Putative secreted protein	Fail
SCAB45141	SFM	D-alanyl-D-alanine carboxypeptidase	ND
SCAB47841	R5, SFM	Lipoprotein	ND
SCAB49491	IPM, OBM, R5, SFM	Putative secreted 5′-nucleotidase	ND
SCAB56441	IPM	Putative secreted protease	ND
SCAB58251	SFM	Putative secreted tripeptidyl aminopeptidase	ND
SCAB59651	SFM	Putative secreted serine protease	Fail
SCAB66031	SFM	Putative glycosyl hydrolase	ND
SCAB68191[c]	IPM, OBM, R5, SFM	Putative secreted alkaline phosphatase	Pass[b]

Table 1. *cont.*

Protein	Growth medium detected	Putative function	Agarase test[a]
SCAB68931	SFM	Branched chain amino acid-binding protein	ND
SCAB69691	IPM	Zinc-binding carboxypeptidase	Fail
SCAB72441	IPM, SFM	Putative membrane protein	Fail[b]
SCAB72781	IPM	Penicillin acylase	ND
SCAB74081	IPM, OBM, R5	Putative secreted protein	ND
SCAB78431	SFM	Secreted tripeptidylaminopeptidase	Fail
SCAB80071	SFM	Putative secreted protein	ND
SCAB84971[c]	IPM	Conserved hypothetical protein	Fail
SCAB85291	IPM	Conserved hypothetical protein	ND
SCAB90091	IPM, OBM, SFM	Secreted cellulase	fail
SCAB90101	SFM	Secreted cellulase	ND
No signal peptide			
SCAB15581[c]	IPM, OBM, R5, SFM	Conserved hypothetical protein	Pass[b]
SCAB17551	IPM	Conserved hypothetical protein	ND
SCAB20121	R5, SFM	Putative germacradienol synthase	ND
SCAB21191	IPM, SFM	Hypothetical protein	ND
SCAB25251	OBM	Guanosine pentaphosphate synthetase	ND
SCAB26011	OBM	Elongation factor Ts	ND
SCAB31831	R5	Cytochrome P-450 hydroxylase	ND
SCAB36931	R5	50S ribosomal protein L4	ND
SCAB37201	OBM	50S ribosomal protein L7/L12	ND
SCAB39491	OBM, R5, SFM	Clp-family ATP-binding protease	ND
SCAB39811	R5	Conserved hypothetical protein	ND
SCAB41891	R5	Adenylosuccinate synthetase	ND
SCAB44821[c]	IPM, OBM, R5, SFM	Conserved hypothetical protein	ND
SCAB45751	R5	DNA gyrase subunit A	ND
SCAB50441	R5	Chaperonin 2	ND
SCAB51341	OBM, R5	Citrate synthase	ND
SCAB51541	SFM	Calcium binding protein	ND
SCAB54151	OBM	Ribosomal L25p family protein	ND
SCAB55881	IPM	Transcriptional regulator	ND
SCAB57721	OBM	Cellobiose hydrolase	ND
SCAB58791	R5	Citrate synthase	ND
SCAB59311	R5	ABC transporter ATP-binding protein	ND
SCAB59871	SFM	GntR family DNA-binding regulator	ND
SCAB62141	SFM	Pyruvate phosphate dikinase	ND
SCAB64141	SFM	Acyl carrier protein	ND
SCAB64311	SFM	Conserved hypothetical protein	ND
SCAB67061	SFM	Dihydrolipoamide dehydrogenase	ND
SCAB69391	R5	Conserved hypothetical protein	ND
SCAB71391	OBM	Conserved hypothetical protein	ND
SCAB85681[d]	R5	Putative tyrosinase MelC2	ND

a. Signal peptide sequences (listed in Table S5) of the indicated proteins were fused to the mature region of agarase and their ability to export agarase in a Tat-dependent manner was determined as described in the text.
b. Two variants of each of these signal peptides were tested (see Table S5).
c. The N-termini of these proteins are recognized as Tat signal peptides by both TATFIND 1.4 and TatP if alternative start sites are selected.
d. This protein lacks an N-terminal signal peptide but is a Tat substrate by virtue of interacting with a Tat signal peptide-bearing partner protein, MelC1 (Leu *et al.*, 1992; Schaerlaekens *et al.*, 2001).
ND, not determined.

activity was detected. For SCAB68191, which does not have a twin arginine motif in the ORF as called, two alternative start codons were selected that both produced peptides containing a twin arginine motif but with different lengths of n-region. Again only the peptide with the longer n-region gave any detectable agarase activity. This indicates that the choice of start codon may be critical to whether a given signal peptide is able to mediate agarase export.

Surprisingly, three of the signal peptides in Table 2 that were predicted to be Tat-targeting by both TATFIND 1.4 and TatP did not mediate detectable export of agarase. The reason for this is not clear; however, given the observation that choice of start site can significantly affect the extracellular agarase activity, it is possible that incorrect start sites were selected for these signal peptides. This may be particularly relevant for SCAB77971, which is a predicted PhoD family phosphatase that are known to have very long and variable signal peptide n-regions (Widdick *et al.*, 2006; 2008). Alternatively, it is possible that these are either not Tat-targeting signals or that they are genuine Tat-targeting signals, but that they do not

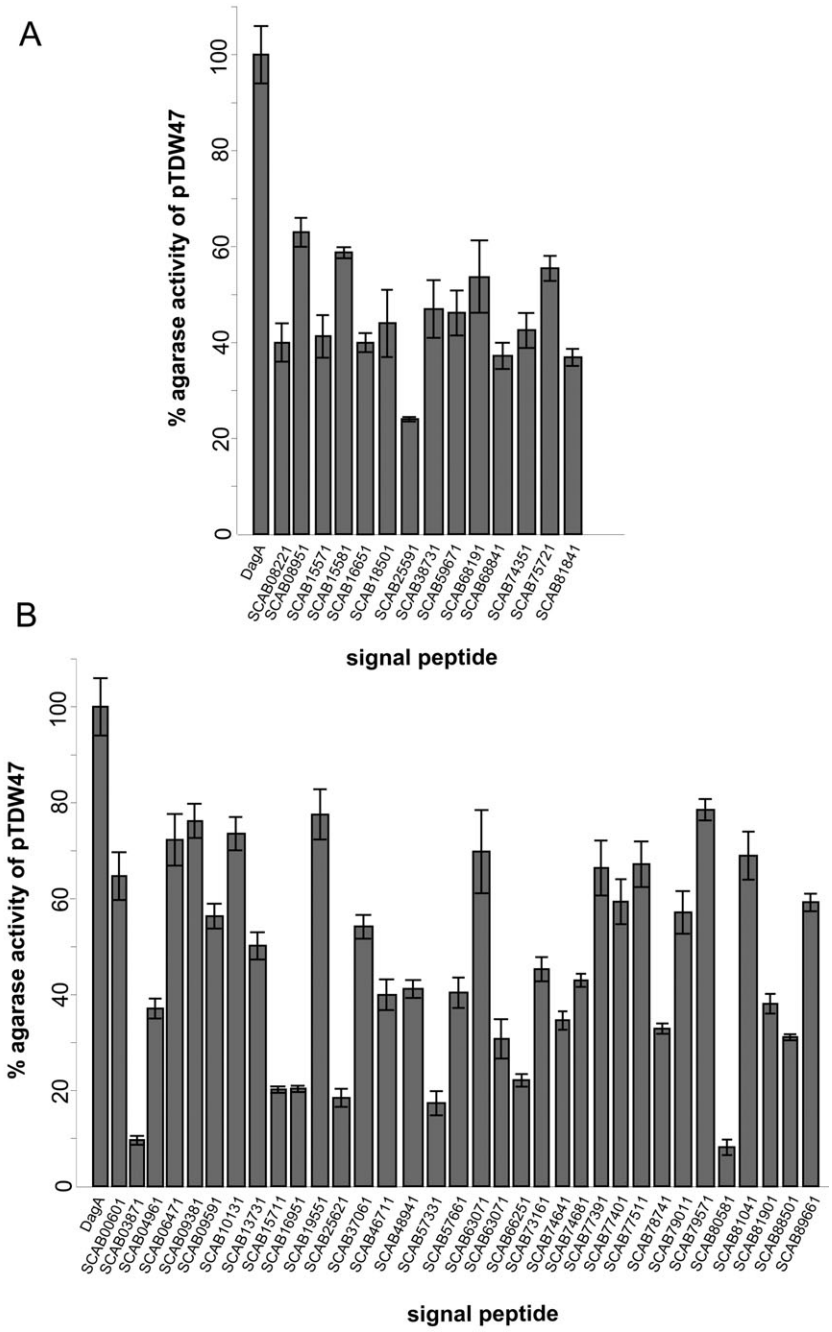

Fig. 3. Export of agarase mediated by *S. scabies* signal peptides. The x-axis shows a range of signal peptides from *S. scabies* from either (A) cell wall-associated proteins that were differentially absent from the Δ*tatC* strain (selected from the list in Table 2) or (B) that were predicted to be Tat signal peptides by bioinformatic analysis (listed in Table S1). The y-axis gives a measure of agarase export from each fusion protein, compared with agarase bearing its native signal peptide (DagA) cloned in the same manner (set at 100%). Each construct carries the native agarase promoter and ribosome binding site. The error bars represent the standard error of the mean, where $n = 3$.
A. Signal peptides from the following proteins listed in Table 1 were also tested and found to be negative in this assay: SCAB07651, SCAB13551, SCAB16711 (two variants), SCAB16721, SCAB17571, SCAB27931 (two variants), SCAB36371, SCAB37611, SCAB43901, SCAB44691, SCAB47131 (two variants), SCAB59651, SCAB63891 (two variants), SCAB64081, SCAB69691, SCAB72441 (two variants), SCAB77971 (two variants), SCAB78431, SCAB84971, SCAB90091.
B. Two variants of the SCAB63071 signal peptide were able to mediate export of agarase to differing degrees – both are shown in the Figure. Signal peptides from the following proteins listed in Table S1 were also tested and found to be negative in this assay: SCAB08301, SCAB31461, SCAB34181, SCAB70581 and SCAB78851. The exact amino acid sequences of each of the sequences tested in these assays can be found in Table S5.

direct the Tat-dependent export of agarase for some unknown reason.

In addition to testing signal peptides from proteins shown in the proteomic study to be predominantly present in the extracellular protein fraction of the WT strain, we also tested a further 38 signal peptides that were predicted to be Tat-targeting based on the bioinformatic analysis of the *S. scabies* genome sequence described above (and listed in Table S1). These 38 proteins are made up of 28 proteins that were predicted to have Tat signal peptides based on the fact that they were recognized by TATFIND 1.4 and TatP, six that were recognized by TATFIND 1.4 and TatP following reassignment of the start codon, two that were recognized by both prediction programmes after *in silico* truncation of the N-terminal part of the ORF, one protein that was predicted to have a Tat signal peptide by TATFIND 1.4 only, and one that was predicted by TatP only.

As shown in Fig. 3B, signal peptides derived from 33 of the proteins tested were able to mediate export of agarase and we therefore conclude that these proteins are Tat substrates. The proteins that bear these signals

Table 2. Proteins from *S. scabies* with signal peptides that are able to mediate the Tat-dependent export of agarase.

Signal peptide tested	Putative function
Protein detected by proteomics	
SCAB08221	Putative phosphoesterase
SCAB08951	ABC-type Fe^{3+} transport system, periplasmic component
SCAB15571	Putative secreted phosphoesterase
SCAB15581	Possible histidinol phosphatase (PHP family)
SCAB16651	Putative L-xylulose-5-phosphate 3-epimerase
SCAB18501	Alpha-N-acetyl glucosaminidase
SCAB25591	Endo-1,4-beta-glucanase
SCAB38731	Beta-lactamase
SCAB59671	Cyclic 3′,5′-adenosine monophosphate phosphodiesterase
SCAB68191	Phospholipase D precursor
SCAB68841	2′,3′-cyclic-nucleotide 2′-phosphodiesterase
SCAB74351	Glycerophosphoryl diester phosphodiesterase
SCAB75721	A subfamily of peptidase family C39
SCAB81841	Alpha-L-rhamnosidase
Proteins predicted by bioinformatics	
SCAB00601	Hypothetical protein
SCAB03871	Glycosyl hydrolase domain followed by ricin domain
SCAB04961	Endo-xylanase
SCAB06471	Putative alpha-L-fucosidase
SCAB09381	Hypothetical protein
SCAB09591	Alpha-L-fucosidase
SCAB10131	Glycosyl hydrolase domain followed by ricin domain
SCAB13731	Phospholipase C
SCAB15711	Putative endo-1,3-beta-glucanase
SCAB16951	Hypothetical protein
SCAB19551	Rhamnogalacturonase B precursor
SCAB25621	Lipoprotein
SCAB37061	Putative secreted protein
SCAB46711	Protocatechuate dioxygenase
SCAB48941	ATP-dependent nuclease subunit B-like
SCAB57331	No homologues in database
SCAB57661	Putative multiple sugar ABC transporter solute-binding protein
SCAB63071	Putative gluconolactonase
SCAB66251	Iron-dependent peroxidase
SCAB73161	Gluconolactonase
SCAB74641	Lipoprotein
SCAB74681	Hypothetical protein
SCAB77391	Hypothetical Protein
SCAB77401	Glycosyl hydrolase
SCAB77511	Beta-galactosidase
SCAB78741	Putative secreted protein
SCAB79011	Lipase/acylhydrolase, putative
SCAB79571	Large secreted protein
SCAB80581	Amine oxidase
SCAB81041	Putative ABC transporter periplasmic binding protein
SCAB81901	Peptide ABC transporter peptide-binding protein
SCAB88501	Rhamnogalacturonase B precursor
SCAB89661	Putative factor C protein precursor

The amino sequences of each signal peptide tested are given in Table S5.

are also listed in Table 2. Of the signal peptides tested in this section, two variants of the SCAB00601 signal peptide were tested, of which only the clone that encoded a signal peptide with the longer n-region (pSM29b, see Table S5), gave any detectable activity. Likewise, three variants of the SCAB63071 signal peptide were tested. Of these, the agarase-producing clone that encoded the longest n-region (pSM35a) failed to give detectable agarase activity while the remaining two both gave detectable agarase export but at different levels. This indicates that the features of the cloned DNA sequence and/or the sequence of the signal peptide tested in this assay may have a profound effect on the outcome of the reporter assay.

Five of the signal peptides identified as Tat-targeting by bioinformatic means (SCAB08301, SCAB31461, SCAB34181, SCAB70581 and SCAB78851) did not promote the export of agarase. Again the reasons for this is not clear – it may be related to the fact that inappropriate start codons were selected for these signal peptides,

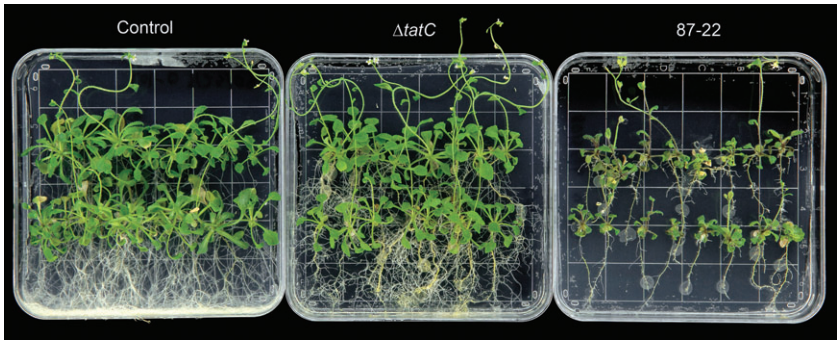

Fig. 4. Reduced disease severity of Arabidopsis seedlings inoculated with the Δ*tatC* mutant strain. Arabidopsis seedlings (7 days old) were inoculated with spores (corresponding to 1×10^6 cfu) of the *S. scabies* WT (87.22) and Δ*tatC* mutant (Δ*tatC*) strains, keeping un-inoculated seedlings as controls. The plants were grown for a further 21–28 days, and the images are taken at 28 days post inoculation.

or that they were genuine Tat signal peptides but were unable to mediate detectable export of agarase. Alternatively, it is possible that despite the fact they were predicted to be Tat signal peptides by both TATFIND and TatP they are actually Sec-targeting. In this context it is interesting to note that SCAB31461 is the *S. scabies* homologue of *S. coelicolor* BldKB that has a KR rather than an RR motif in its signal peptide and is also unable to mediate export of agarase (Widdick *et al*., 2006). This would be consistent with the idea that this protein may be a Sec substrate in *Streptomyces*.

In total, the agarase reporter assay has identified 47 Tat-targeting sequences, four of which were predicted to be Tat-targeting by only one of the two prediction programmes, and we conclude that it is highly likely that these proteins are Tat substrates in *S. scabies*.

The Tat secretion system contributes to the virulence of S. scabies

The demonstrated role of Tat-secreted proteins in the virulence of other microbial pathogens (reviewed in De Buck *et al*., 2008) and the existence of proteins in the *S. scabies* genome that have homologues in pathogenic microbes, suggested a likely role for Tat secretion in virulence. We therefore investigated the virulence of the Δ*tatC* mutant, relative to the WT strain, on the model plant Arabidopsis. *S. scabies* is a broad host range pathogen that causes root rot on both model plant species and agricultural crops (reviewed in Loria *et al*., 2006). The root tips of Arabidopsis (Col-O) seedlings were inoculated with *S. scabies* spores from either the WT (WT; 87-22) or the Δ*tatC* mutant, and seedling growth was monitored weekly.

As shown in Fig. 4, infection of seedlings by the WT strain caused root stunting and necrosis; secondary roots were killed soon after emergence from the taproot. In addition, the leaves and shoots were stunted, chlorotic and necrotic; all of the plants inoculated with the WT strain were dead within 21–30 days post inoculation. By contrast, plants inoculated with the Δ*tatC* mutant strain grew vigorously and were similar to the un-inoculated control in size and colour (Fig. 4). Interestingly, the Δ*tatC* mutant grew extensively on the roots as yellow substrate mycelium, without noticeable sporulation, but did not appear to necrotize the colonized tissue. Plants inoculated with the Δ*tatC* mutant did, however, differ from the un-inoculated control in root morphology, particularly root-branching pattern (Fig. 4).

Given the dramatic virulence phenotype of the Δ*tatC* mutant, we carried out a time course study of Arabidopsis root colonization using confocal scanning microscopy and enhanced green fluorescent protein (EGFP)-labelled *S. scabies* WT and Δ*tatC* strains. To facilitate observation of microbial growth, plants were grown hydroponically. The WT strain attached to the root tip and began to colonize plant tissues within 24 h after spores were added to the hydroponic medium (Fig. 5A). Within 48 hpi the WT strain was aggressively colonizing the root at the zone of cell differentiation (Fig. 5B) and at lateral meristems (Fig. 5C). Intercellular colonization was evident at the root tip and at the point of secondary root emergence by 72 hpi (Fig. 5D). By contrast, colonization of root tissue by the Δ*tatC* mutant was greatly delayed and limited in scope. The mutant was not able to attach to the root tip during the first 48 hpi; however, there were loosely attached colonies at the root elongation zone (Fig. 5E), and minimal colonization and restricted growth at the differentiation and elongation zones (Fig. 5F–G), and at lateral meristems (Fig. 5H) after 72–96 hpi. Intercellular colonization was delayed until 96–120 hpi (Fig. 5I–J) and was limited in scope.

Individual Tat-secreted proteins contribute to the virulence of S. scabies

Because the Δ*tatC* strain was essentially avirulent on Arabidopsis seedlings, we sought to address the contribution of individual candidate Tat substrates to host–pathogen interactions. Fourteen individual strains were constructed that were deleted for genes encoding the putative or confirmed Tat substrate proteins listed in Table 3. The strains were then assessed for virulence using Arabidopsis seedlings as a host. To this end, Arabidopsis seedlings were grown on Murashige and Skoog

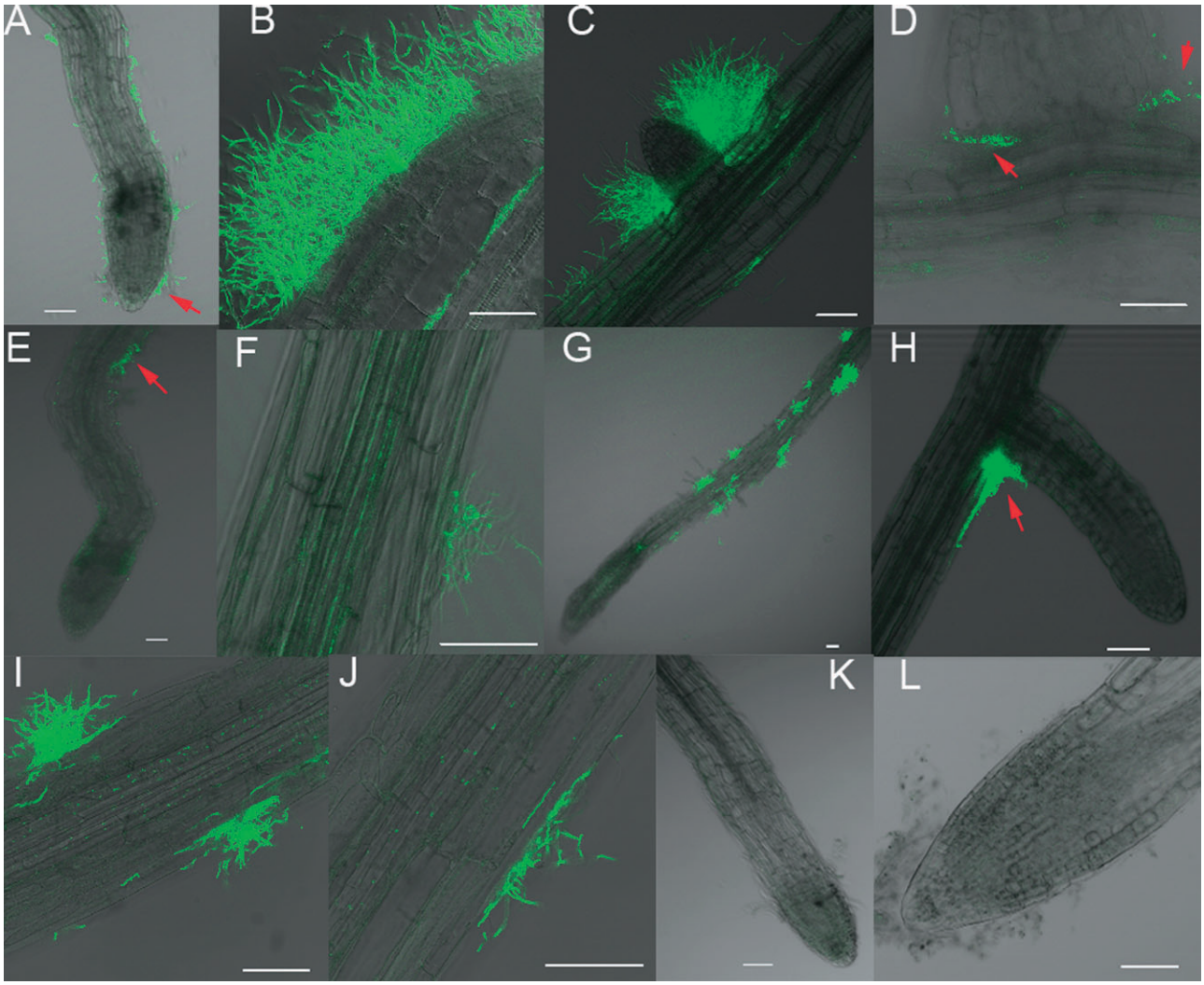

Fig. 5. Micrographs of Arabidopsis roots colonized by EGFP-labelled *S. scabies* strains. Colonization and disease progression was monitored on hydroponically grown Arabidopsis roots after inoculation with EGFP labelled *S. scabies* strains. (A–D) Inoculation with the WT strain 87-22, and (E–J) inoculation with the Δ*tatC* mutant strain. Colonies of the WT strain are shown (A) 24 h post inoculation (hpi) showing cells at and near the root tip (red arrow); (B and C) 48 hpi showing colonization of (B) the root at the zone of cell differentiation and (C) at a lateral meristem; and (D) 72 hpi at a lateral root meristem – intercellular growth is indicated by the red arrows. Colonies of the *tatC* mutant strain are shown (E) at the root tip zone during the first 24–48 hpi; the arrow indicates loosely attached mycelium at the elongation zone; (F–H) after 72–96 hpi; (F) at the differentiation zone (G) at elongation zones and (H) at a lateral meristem; and (I and J) at 96–120 hpi. (K and L) Un-inoculated control seedlings showing (K) normal root growth, and (L) loose cells surrounding the root cap region. Images were taken using a Leica TCS SP5 confocal microscope. Size bars represent 50 μm.

(MS) medium and inoculated with spore suspensions of the WT or with mutant strains containing deletions in genes encoding candidate Tat substrates using methods just described. Of the 14 individual mutant strains tested, seven were reduced in virulence relative to the WT strain (Fig. 6).

Gene deletions in *SCAB03871* and *SCAB10131* were among those coding for Tat substrates that have a reduced virulence phenotype in the Arabidopsis seedling assay. Interestingly, these genes encode paralogues with predicted glycosyl hydrolase and ricin-like domains. In spite of the similarity in their amino acid sequences, each of these proteins appears to make a large and unique contribution to virulence, based on the phenotypes of the individual mutants (Fig. 6). Furthermore, proteins encoded by *SCAB03871* and *SCAB10131* have homologues in fungal plant pathogens such as *Chaetomium globosum* (CHGG_02005) and *Phaeosphaeria nodorum* (SNOG_01152) respectively.

SCAB06471 encodes a putative alpha-L-fucosidase; the cognate deletion mutant was compromised in virulence (Fig. 6). Alpha-L-fucosidases are responsible for processing of fucosylated glycoconjugates that play a role in a wide variety of biological processes. Interestingly,

Table 3. Potential Tat-dependent virulence factors encoded in the genome of *S. scabies*.

Gene ID	Putative function	Homologs with highest similarity (identity/similarity)	Locus_tag	Agarase test[a]	Virulence phenotype[b]
SCAB03871	Glycosyl hydrolase domain followed by ricin domain	*Streptomyces sviceus* (89/93) *Micromonospora aurantiaca* (76/85) *Actinosynnema mirum* (71/81)	SSEG_02060 MicauDRAFT_3913 Amir_3107	Yes	Yes
SCAB06471	Putative alpha-L-fucosidase	*Streptomyces sviceus* (82/90) *Streptomyces viridochromogenes*(83/89) *Streptomyces hygroscopicus* (57/70)	SSEG_02413 SvirD4_010100003282 ShygA5_010100010179	Yes	Yes
SCAB08951	ABC-type $Fe3^{+}$ transport system, periplasmic component	*Acidovorax delafieldii* (60/74) *Phytophthora infestans* (51/67) *Pectobacterium carotovorum* (48/63)	AcdelDRAFT_2816 PITG_03258 PC1_1331	Yes	No
SCAB10131	Glycosyl hydrolase domain followed by ricin domain	*Streptomyces sviceus* (84/92) *Micromonospora* sp. L5 (75/83) *Actinosynnema mirum* (70/80)	SSEG_02060 ML5DRAFT_2741 Amir_3107	Yes	Yes
SCAB18791	Hypothetical secreted protein	*Bacteroides cellulosilyticus* (39/57) *Pectobacterium carotovorum* (34/50) *Ralstonia solanacearum* (34/49)	BACCELL_04049 PC1_0414 RSIPO_04319	ND	No
SCAB58511	Hypothetical protein.	*Pseudomonas putida* (27/45) *Nitrobacter winogradskyi* (27/43) *Clostridium botulinum A3* (25/42)	PP_2006 Nwi_0886 CLK_A0070	ND	No
SCAB58531	Hypothetical protein	*Nitrobacter winogradskyi* (29/45) *Clostridium botulinum* Ba4 (26/43) *Pseudomonas putida* (29/45)	Nwi_0886 CLJ_0010 PP_2006	ND	No
SCAB70581	Hydrolase of the alpha/beta superfamily	*Streptomyces sviceus* (76/82) *Streptomyces griseoflavus* (76/83) *Streptomyces ghanaensis* (76/83)	SSEG_08785 SgriT_010100028100 SghaA1_010100027639	ND	No
SCAB76661	Rare lipoprotein A	*Streptomyces sviceus* (54/65) *Micromonospora* sp. (66/79) *Streptomyces viridochromogenes* (72/82)	SSEG_04607 MCAG_02629 SvirD4_010100009886	ND	No
SCAB77391	Conserved hypothetical protein containing a galactose-binding domain	*Bacteroides cellulosilyticus* (36/53) *Pectobacterium carotovorum* (30/47) *Ralstonia solanacearum* (29/44)	BACCELL_04049 PC1_0414 RRSL_03557	Yes	Yes
SCAB77401	Putative glycosyl hydrolase	*Bacteroides ovatus* (45/63) *Dickeya dadantii* (36/52) *Ralstonia solanacearum* (36/52)	BACOVA_01686 Dd586_1768 RSMK02989	Yes	No
SCAB80581	Putative FAD-containing amine oxidase	*Streptomyces pristinaespiralis* (78/87) *Streptomyces* sp. (76/85) *Nocardia farcinica* (54/65)	SSDG_02025 StreC_010100030349 nfa3040	Yes	Yes
SCAB81041	Putative spermidine/putrescine transporter peptide-binding protein	*Streptomyces sviceus* (58/72) *Streptomyces hygroscopicus*(56/72) *Sphaerobacter thermophilus* (36/53)	SSEG_09980 ShygA5_010100044540 Sthe_3297	Yes	Yes
SCAB81841	Alpha-L-rhamnosidase	*Clostridium leptum* (35/54) *Victivallis vadensis* (37/51) *Lactobacillus rhamnosus* (33/48)	CLOLEP_02977 Vvad_PD1953 LRH_00532	Yes	Yes

a. Signal peptide sequences (listed in Table S5) of the indicated proteins were fused to the mature region of agarase and their ability to export agarase in a Tat-dependent manner was determined as described in the text.
b. Virulence phenotype of knock-out strains tested using the *Arabidposis thaliana* infection model.
ND, not determined.

SCAB06471 has homologues in the fungal plant pathogens such as *Gibberella zeae* (FG11254.1) and *Magnaporthe grisea* (MGG_03257). Furthermore, all three of these proteins are putative glycosidases and the cognate mutants have similar virulence phenotypes; in all cases plant growth was substantially greater than those inoculated with the WT strain, root necrosis was lacking, and the mutant strains grew luxuriously on Arabidopsis roots (Fig. 6). It is tempting to speculate that these proteins have a role in penetration of the plant cell wall.

The *SCAB77391* mutant strain was slightly reduced in virulence, relative to WT (Fig. 6). The encoded protein is predicted to contain a galactose-binding domain. It is possible that this domain recognizes specific carbohydrate moieties on the host cell surface; in that way the protein might function as a lectin, which could enhance host binding or recognition. The limited virulence phenotype, however, suggests a function that is either redundant in the genome or has only a minor role in host–pathogen interactions.

The *SCAB81841* mutant strain has a dramatic avirulence phenotype; plants inoculated with this mutant were comparable to the non-inoculated control, except for leaf chlorosis and a delay in flowering. The predicted function

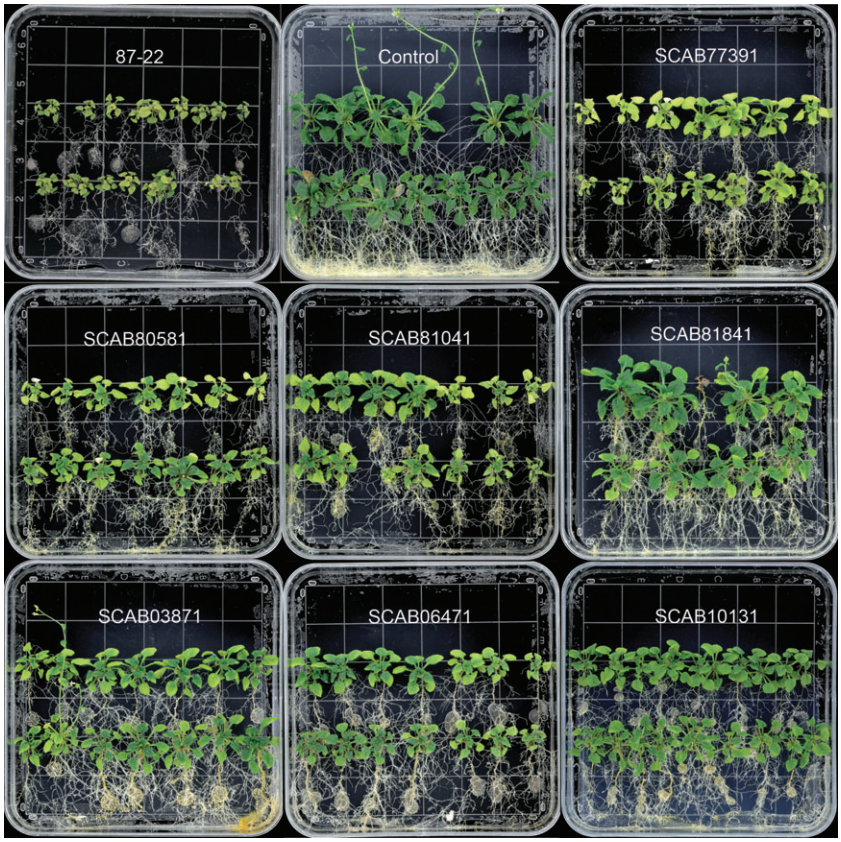

Fig. 6. Tat substrates are essential for the virulence of *S. scabies*. Deletion mutants of putative Tat-secreted virulence proteins are constructed using the ReDirect gene disruption protocol and compared to WT 87-22 for disease severity. Mutants SCAB77391, SCAB80581, SCAB81041, SCAB81841, SCAB03871, SCAB06471 and SCAB10131 show suppression of disease severity in terms of root necrosis and aerial growth.

for the encoded protein is an alpha-L-rhamnosidase, which hydrolyses the terminal non-reducing alpha-L-rhamnose residues in alpha-L-rhamnosides. Given the severe defect in virulence, it seems unlikely that this enzyme's primary function is to hydrolyse plant biomass. Some rhamnosides are biologically active, including antimicrobial saponins (Morrissey *et al*., 2000). Interestingly, the *SCAB81841* mutant grew poorly on Arabidopsis roots (Fig. 6), consistent with a role in the degradation of an antimicrobial molecule.

The *SCAB81041* deletion mutant showed a moderate virulence phenotype. The cognate protein encodes a putative spermidine/putrescine transporter peptide-binding protein (Table 3).

SCAB80581 encodes a putative FAD-containing amine oxidase. Proteins in this family additionally contain a second domain that is responsible for specifically binding a substrate and catalysing a particular enzymatic reaction. The deletion mutant has a moderate phenotype as shown in Fig. 6.

Discussion

In this study we have characterized the Tat secretome of the plant pathogen, *S. scabies*. In keeping with analyses of other streptomycete genomes, *in silico* analysis using Tat substrate prediction programmes TATFIND 1.4 and TatP predicts many candidate Tat substrates are encoded by *S. scabies*. Using the agarase reporter assay, we show here that the signal peptides of 47 candidate *S. scabies* Tat substrates were able to mediate Tat-dependent export, strongly suggesting that these represent *bona fide* Tat substrates. Interestingly, two of the Tat signal peptides identified in this study were not recognized by TATFIND 1.4 because they have hydrophobic amino acids in the +1 position of the twin arginine motif. Stanley *et al*. (2000) previously noted that the amino acid residue at this position is usually polar, but the work presented here shows that valine and leucine can both be tolerated at this position. Furthermore, additional highly likely Tat-dependent proteins listed in Tables S1 and S2 also have signal peptides that are not recognized by TATFIND 1.4 due to the presence of His, Ile or Phe at the +1 position. Further work is required to ascertain whether these additional 'allowed' residues at the +1 position are specific to *Streptomyces* proteins, or whether they reflect a general tolerance for the nature of the amino acid at this position of Tat signal peptides. None-the-less, taken together our analysis supports the contention that there are well in excess of 100 Tat substrates in *S. scabies*.

Consistent with the notion that the *S. scabies* Tat pathway is a major route of protein secretion, inactivation

of the Tat pathway was associated with a number of phenotypes. In particular, growth rate of the Δ*tatC* strain was reduced relative to WT and the mutant displayed a cell wall defect, a phenotype that is also linked to Tat pathway inactivation in other bacteria (e.g. Ize *et al*., 2003; Caldelari *et al.,* 2006; Widdick *et al*., 2006). The Δ*tatC* strain was also slow to sporulate on some types of solid media. Interestingly, the signal peptide of the *S. scabies* homologue of the extracellular signalling protein factor C (SCAB89661), that is involved in cellular differentiation in some species of *Streptomyces* (Birkó *et al*., 1999) was shown to mediate Tat-dependent export of agarase (Fig. 3B). This indicates that the *S. scabies* factor C homologue is a Tat substrate. The twin arginine signal peptides of streptomycete factor C proteins are highly conserved and the mature portion of the protein shows strong sequence identity to secreted proteins encoded by mycelial fungi, raising the possibility that this DNA coding this protein was acquired by lateral gene transfer (Chater *et al*., 2010).

Analysis of the extracellular proteomes of the WT and Δ*tatC* strains showed that in total 73 predicted secreted proteins were present at decreased amounts or absent in the extracellular proteome of the Δ*tatC* mutant. Using the agarase reporter assay, 14 predicted Tat substrates detected by proteomics as being abundantly exported in more than one growth condition in WT cells and strongly reduced in the Δ*tatC* mutant were verified to have Tat-targeting signal peptides. However, the majority of proteins that were apparently absent from the Δ*tatC* cell wall wash fraction are unlikely to be Tat substrates, again underscoring the pleiotropic nature of the mutation.

Bacteria in symbiotic associations with eukaryotes rely on secreted molecules to manipulate host cell physiology. In the broadest definition, the small molecules and proteins that alter host cell structure and function are referred to as 'effectors' (Hogenhout *et al*., 2009). In *S. scabies* thaxtomin and a coronatine-like molecule meet the criteria of small molecule effectors. Thaxtomin acts as a cellulose synthesis inhibitor, through an undefined mechanism, and is believed to aid in penetration of plant tissue (Scheible *et al*., 2003; Bischoff *et al*., 2009). A coronatine-like molecule likely serves as a molecular mimic of the plant defence signalling molecule, jasmonic acid (Bignell *et al*., 2010). The secreted proteins Nec1 and TomA are authentic and putative effectors, respectively, in *S. scabies* but other effector proteins had not previously been identified.

Effector proteins interact with host cells in many ways and the host cell targets of most effectors are not known (Cunnac *et al*., 2009); however, molecular mimicry and modification of host cell molecules are two broad categories of virulence mechanisms. Activity, abundance and localization of eukaryotic proteins, including those involved in host defence, can be regulated through attachment of ubiquitin or ubiquitin-like molecules. Many pathogens produce proteins that mimic components of the host ubiquitination machinery (reviewed in Spallek *et al*., 2009). Other defence and development signalling cascades are targeted by effector proteins, such as Rho GTPase regulators, cytoskeletal modulators and host innate immunity (Shames *et al*., 2009). Under the broad definition of protein effectors suggested by Hogenhout *et al*. (2009), secreted enzymes involved in degradation of host cell molecules, particularly plant cell wall components, are also considered to be effector proteins.

Microbial pathogens can undergo rapid evolution through the acquisition of pathogenicity islands (PAIs), which are regions in the genome encoding multiple virulence-associated genes (Lovell *et al*., 2009). In Gram negative pathogens, PAIs often have a modular configuration and include genes encoding both the type III secretion apparatus and effector proteins that use that pathway (Lindeberg *et al*., 2009). The type III secretion apparatus provides a very effective mechanism for introduction of effector proteins directly into host cells, but is lacking in Gram positive bacteria. Gram positive pathogens, such as *S. scabies*, have PAIs (Kers *et al*., 2005) but must rely on general protein secretion systems, such as Tat and Sec, which deliver effector proteins outside of the bacterial cell; genes encoding effector proteins are not typically clustered with those encoding protein secretion machineries in these pathogens.

Translocation of putative virulence factors is Tat-dependent in many microbial pathogens including those that contain type III secretion systems (reviewed in De Buck *et al*., 2008). Given the importance of the Tat system in the genus *Streptomyces*, it was not surprising to find that inactivation of the Tat secretion machinery in *S. scabies* resulted in an essentially avirulent phenotype (Figs 4 and 5). The *tatC* mutant strain was slow to colonize and invade rapidly expanding root tissue, relative to the WT strain, which likely was the result of several deficiencies. However the avirulence phenotype of the *S. scabies tatC* mutant strain was not due to a deficiency in thaxtomin production (Fig. S1). Regardless, the dramatic virulence phenotype of the Δ*tatC* mutant did suggest that a subset of Tat substrates has virulence functions. Furthermore, the combination of bioinformatic and proteomic analysis identified a large number of putative Tat substrates in *S. scabies*, of which more than one-third are associated with stress responses or virulence in other bacterial or fungal pathogens. This group of putative Tat-secreted virulence proteins includes putative lipoproteins, ABC transporters, phospholipases/phosphoesterases, beta-lactamase and proteins involved in Fe homeostasis (see Tables 3 and S1).

To confirm that Tat substrates are involved in infection, we inactivated genes encoding 14 candidate Tat substrates, from which strains inactivated for production of

seven confirmed Tat substrates showed a reduction in virulence (Fig. 6). Because the growth rate of these mutant strains was comparable to the WT (shown in Fig. S4 for the *SCAB03871*, *SCAB06471*, *SCAB10131* and *SCAB77391* knockout strains), it can be concluded that the Tat pathway secretes multiple virulence factors in *S. scabies*.

Inspection of the list of these authentic virulence proteins using bioinformatics reveals some interesting ecological and phylogenetic associations. Out of the seven Tat-secreted proteins affecting virulence, SCAB77391 and SCAB81841 are conserved in *S. turgidiscabies*, but the encoding genes are absent from the genomes of non-pathogenic streptomycetes for which genome sequence data are available at this time (data not presented). SCAB03871, SCAB06471 and SCAB10131 all contain homologues in fungal plant pathogens and are all predicted to interact with glycans (Table 3). Surprisingly, the amino acid sequences of the Tat secretion signals of these three proteins are highly conserved, possibly suggesting a common origin. SCAB03871 and its paralogue (confirmed by reciprocal BLAST analysis) SCAB10131 have glycosyl hydrolase and ricin-like domains. The closest homologues to these proteins are a few closely related saprophytic actinomycetes, where the twin arginine signal peptide is always conserved. Outside of the actinomycetes, the closest homologues of these proteins are encoded by saprophytic and pathogenic fungi. In keeping with the fact that fungi generally lack the Tat system, the fungal homologues have typical eukaryotic signal peptides, rather than the longer and less hydrophobic Tat signal peptides. This is suggestive that the gene for one of these proteins was acquired by an ancestoral actinomycete through horizontal transfer from a fungus and that a Tat-targeting sequence was acquired to allow secretion of the protein in the prokaryote. It is, however, not readily apparent why these proteins should be substrates for the Tat pathway rather than the Sec system – for example none of them are predicted to bind redox cofactors that would necessitate export in a folded conformation. Substrates for the Sec machinery are transported in an unfolded form and usually interact with cytoplasmic chaperones to maintain them in an unfolded, export competent state. It is possible that proteins coded by genes acquired by horizontal transfer are not recognized by host chaperones as they are non-native, and it therefore may be advantageous to export such proteins in a folded form.

Of the seven Tat-secreted proteins affecting virulence, SCAB06471 is homologous to an alpha-L-fucosidase from *G. zeae*, the cause of wheat head blight and *M. oryzae*, the rice blast pathogen (Oh *et al*., 2008). This enzyme hydrolyses the alpha-1,6-linked fucose joined to the reducing-end N-acetylglucosamine of carbohydrate moieties in glycoproteins. SCAB80581 encodes a protein for amine oxidase, both are homologous to proteins in the nonpathogenic *Streptomyces pristinaespiralis* and pathogenic *Mycobacterium* spp. SCAB81041, a putative ABC transporter, is a periplasmic-binding protein and homologous to proteins in *Streptomyces sviceus,* and in *Thermomicrobium roseum,* a Gram negative, obligately thermophilic bacterium.

Seven putative effector proteins chosen based on bioinformatic analysis did not have a virulence phenotype. One of these was SCAB08951, which was highly similar (Table 3) to proteins encoded by the Gram negative soil bacterium *Acidovorax delafieldii*, the Gram negative plant pathogen *Dickeya dadantii* (which both have predicted Sec signal peptides) and the plant pathogenic oomycete *Phytophthora infestans* (which has a predicted eukaryotic signal peptide). Since effector proteins commonly have a role in host specificity (Lindeberg *et al*., 2009), evaluation of these mutants on additional host plants, particularly potato, would be necessary.

Experimental procedures

Strains, plasmids, media and culture conditions

Streptomyces scabies strain 87-22 and the cognate Δ*tatC* strain (see below) were routinely grown on either IPM (which contained per litre of tap water 50 g Smash^{R} instant potato mash and 12 g agar), International *Streptomyces* Project medium 2, or International *Streptomyces* Project medium 4 (BD Biosciences, San Jose, CA) at 28°C. For proteomic analysis, strains were cultured on either IPM, SFM (Hobbs *et al*., 1989), R5 medium (Thompson *et al*., 1980) or oat bran broth medium (Goyer *et al*., 1998). For growth in liquid culture strains were cultured aerobically in TSB (Kieser *et al*., 2000). Phenotypic growth tests were carried out on DNA medium (Kieser *et al*., 2000) and halo diameters were determined using the image processing software GIMP (GNU Image Manipulation Program – http://www.gimp.org/).

To test candidate signal peptides for Tat dependence in the agarase assay, DNA encoding the signal peptides of interest were cloned in-frame (as *Nde*I-*Bam*HI/*Bgl*II fragments) with the mature agarase sequence in the integrative plasmid pTDW46H (which is identical to pTDW46 except that the apramycin resistance specified by the vector has been replaced by hygromycin resistance from pIJ10700 using the REDIRECT method (Gust *et al*., 2003) and the oligonucleotide primers hyg_fwd and hyg_rev. The oligonucleotide primer sequences used for signal peptide amplification are listed in Table S4, and the plasmids used in this study are listed in Table S5.

Agarase assays performed and quantified as described by Widdick *et al*. (2006; 2008).

Construction and complementation of the S. scabies Δ*tatC strain*

For construction of the *S. scabies* Δ*tatC* strain, SM1, an approximately 1000 bp region directly upstream of the *tatC*

gene (*SCAB73601*) was amplified by polymerase chain reaction (PCR) using the oligonucleotides utatcscabf1 and utatcscabr (see Table S4 for a list of oligonucleotide primers used in this study) and *S. scabies* chromosomal DNA as template, digested with EcoRI and HindIII and cloned into pBluescript (KS^+) that had been similarly digested. An approximately 1000 bp region downstream of *tatC* was subsequently amplified using the oligonucleotides dtatcscabf and dtatcscabr1, digested with EcoRI and XbaI and cloned into pBluescript already containing the *tatC* upstream region that had been similarly digested. The apramycin resistance cassette from pIJ773 (Gust *et al*., 2003) was amplified using oligonucleotides ecoriapraf and ecoriaprar, cleaved with EcoRI and cloned between the upstream and downstream regions of the *tatC* deletion allele assembled in pBluescript. To allow the detection of double-crossover strains by replica plating the ampicillin/carbenicillin resistance cassette of the carrier plasmid was replaced with a kanamycin resistance cassette, which, unlike bla, is suitable for selection in *Streptomyces*. This was achieved using the REDIRECT method of Gust *et al*. (2003), and the kanamycin resistance cassette was amplified by PCR using the oligonucleotides KanRtrans2_fwd and KanRtrans2_rev (Table S4). The plasmid was transferred by mating into *S. scabies* 87-22 and single-crossover recombinants were selected for on MS medium containing apramycin and kanamycin (Gust *et al*., 2003). Double-crossover recombinants were subsequently selected by several rounds of growth on non-selective media followed by selection for colonies that were apramycin-resistant and kanamycin-sensitive. Loss of the *tatC* gene in strain SM1 was subsequently confirmed by PCR and by Southern blot analysis.

To test for complementation of the *S. scabies* Δ*tatC* strain, a synthetic construct covering the *S. scabies tatAC* genes (covering from 260 bp upstream of *tatA* to 60 bp downstream of *tatC*) in pUC57 was purchased from GenScript, NJ, USA. The *tatAC*-containing region was excised by digestion with XbaI and cloned into similarly digested pSET-SOR-hyg (Sean O'Rourke, unpublished) to give plasmid pTDW185, which integrates site specifically into the *Streptomyces* chromosome.

Deletion of genes encoding putative Tat substrates

Deletion mutants for 14 putative Tat-secreted virulence proteins (Tables 3 and S1) were created using the PCR-based ReDirect gene disruption protocol (Gust *et al*., 2003). In this case, a cosmid library of *S. scabies* strain 87-22 containing a kanamycin resistance marker (*neo*) was used. Cosmid clones containing the target genes were individually introduced into *E. coli* BW25113 carrying an arabinose-inducible λRed-expressing plasmid pKD46 (AmpR) (Datsenko and Wanner, 2000). A gene replacement cassette [*aac(3)IV* (ApraR) + *oriT*RK2] from the plasmid pIJ773 (Gust *et al*., 2003) was PCR-amplified using gene-specific redirect primers (Table S4). The resulting PCR products were transformed into the cosmid-containing *E. coli* strain and selected for apramycin resistance. The mutant cosmids were then introduced into *S. scabies* 87-22 by intergeneric conjugation from *E. coli* ET12567 (carrying the helper plasmid pUZ8002). Exconjugants were screened for apramycin resistance (100 μg ml^{-1}) and kanamycin sensitivity (50 μg ml^{-1}), indicating a double-crossover allelic exchange in *S. scabies*; deletions were confirmed by PCR analysis.

Protein methods

Growth curves for *Streptomyces* strains were monitored by assaying total protein at regular intervals. To this end, 1×10^6 spores of each strain were inoculated into 100 ml of TSB medium and the cultures were incubated with shaking at 30°C over 56 h. Every 2 h, 3×1 ml samples were withdrawn, the cells pelleted and frozen at −20°C. The frozen samples were subsequently resuspended in 1 ml 1N NaOH, 0.1% SDS and boiled for 10 min to lyse the cells. The samples were then clarified by centrifugation and 500 μl of the resulting supernatants were diluted with an equal volume of sterile distilled water, and 25 μl of each sample was used for protein determination using the Biorad DC Protein Assay Kit.

For the preparation of extracellular proteins, autoclaved 75 mm cellophane discs were placed onto solid media plates (SFM, OBM, IPM or R5), inoculated with 10^6 spores of the *S. scabies* WT and Δ*tatC* strains and incubated for 48 h at 30°C. The biomass was scraped from the cellophane discs and extracellular proteins were released following cell wall washing exactly as described by Widdick *et al*., (2006). Separation of TCA-precipitated cell wall washes of *S. scabies* extracellular proteins by two-dimensional gel electrophoresis (2D-PAGE) was performed using the immobilized pH gradients in the pH range 3–10 as described (Antelmann *et al*., 2001). For quantification of the relative protein amounts of extracellular proteins that are decreased in the Δ*tatC* mutant proteome compared with the WT, 200 μg protein were separated by 2D-PAGE and the resulting 2D gels were stained with Coomassie-Brilliant Blue as described (Antelmann *et al*., 2001).

Quantitative image analysis was performed from the coomassie-stained 2D gels using the DECODON Delta 2D software (http://www.decodon.com). The 2D gel images from WT and the Δ*tatC* mutant cell wall proteomes were aligned using a warp transformation. Before spot detection and quantification was performed, a fused 2D gel of both images was created using the 'union fusion' algorithm of Delta2D. Spot detection was performed in the fusion gel containing all spots present in both images according to the automatically suggested parameters for background subtraction, average spot size, and spot sensitivity. The resulting spot shapes were reviewed and manually edited in the fusion gel if necessary. This reviewed spot mask served as a spot detection consensus for all gel images, which was applied to both images to guide the spot detection and quantification. This enables spot quantification in all gels at the same locations resulting in 100% matching and in a reliable analysis of complete expression profiles. Normalization was performed by calculating the quantity of each single spot in percentage related to the total spot quantity per gel. Proteins showing an induction of at least twofold in the WT compared with the Δ*tatC* mutant strain are listed in Tables 1, S2 and S3.

For standard identification of the proteins from 2D gels, spot cutting, tryptic digestion of the proteins and spotting of the resulting peptides onto the MALDI-targets (Voyager DE-STR, PerSeptive Biosystems) were performed using the Ettan Spot Handling Workstation (Amersham-Biosciences,

Uppsala, Sweden) as described previously (Eymann *et al*., 2004). The MALDI-TOF-TOF measurement of spotted peptide solutions was carried out on a Proteome-Analyzer 4800 (Applied Biosystems, Foster City, CA, USA) as described previously (Eymann *et al*., 2004). The mascot search was performed against the available *S. scabies* database (http://www.sanger.ac.uk/Projects/S_scabies/).

Thaxtomin A extraction and quantification

Streptomyces scabies WT 87-22 and the Δ*tatC* mutant were grown in 6 × 5 ml of oat bran broth medium (Johnson *et al*., 2007) in 6-well plates for 7 days at 25 ± 2°C with moderate shaking (~120 r.p.m.). Mycelia were pelleted by centrifugation and discarded. Thaxtomin A was extracted from culture supernatants and was analysed by HPLC as previously described (Johnson *et al*., 2007).

Plant virulence assays

Arabidopsis thaliana (ecotype Columbia) surface-sterilized seeds were placed on MS) (Murashige and Skoog, 1962) agar medium with 2% sucrose. Plants were grown at 21 ± 2°C with a 16 h photoperiod for 7 days, then inoculated with *S. scabies* 87-22 (WT) or the cognate deletion mutants (Δ*tatC* mutant or one of the 14 strains harbouring deletions in genes encoding Tat substrates). In all cases, spore suspensions (1x10^6 cfu) were applied to seedling root tips. Disease symptoms were noted every week and images were taken 21–28 days post inoculation. Each experiment was repeated three times with five replicates of 20–25 plants.

*Root colonization by the Δ*tatC *mutant strain*

Streptomyces scabies WT 87-22 and the Δ*tatC* mutant strains each tagged with a gene-encoding EGFP were created and used for colonization studies with *A. thaliana*. Vector pIJ8641 (Sun *et al*., 1999) or pRFSRL16 (R.F. Seipke, unpublished), carrying the *egfp* gene downstream of the constitutive *ermE*p* promoter and an antibiotic-resistant marker were replicated in the methylation-deficient *E. coli* strain ET12567 prior to conjugation into *S. scabies* WT 87-22 and the Δ*tatC* mutant strain respectively. *A. thaliana* seedlings were grown on liquid MS medium with 2% sucrose for 7 days and inoculated at the root tip with a spore suspension (1 x 10^6 cfu). Laser scanning confocal microscopy was used to visualize internal and external colonization of Arabidopsis roots at 24 h intervals. The roots of harvested plants were mounted in water immediately after harvesting and observed using a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany), with a 10 × and 63 × water immersion objectives as described (Joshi *et al*., 2007a). EGFP was visualized using a 4-line argon laser with an excitation wavelength of 488 nm and an emission wavelength of 500 to 550 nm. Differential interference contrast images were collected simultaneously with the fluorescence images using the transmitted light detector and processed using Leica LAS-AF software (version 1.8.2).

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Supplementary Figure Legends

Figure S1. Thaxtomin production by *S. scabies* wild type and Δ*tatC* strains. 10^4 spores of each strain were inoculated into 5 ml oat bran broth (OBB) and were incubated for 7 days at 30°C. After incubation thaxtomin was extracted and analysed as described in Experimental procedures. Assays were repeated in triplicate; error bars represent the standard deviation of the mean.

Figure S2. Complementation of the *S. scabies* Δ*tatC* strain with a plasmid carrying *S. scabies tatAC*. Spores of the indicated strains were streaked out from a fresh solid medium culture with an inoculation loop onto YEME medium and incubated for 72 hours at 30°C.

Figure S3. The extracellular proteome of the *S. scabies* wild type (red images) in comparison with the *tatC* mutant (green images) isolated from strains grown in four different growth media. *S. scabies* strains were grown on the following media; (A) R5, (B) SFM, (C) OBM or (D) IPM and extracellular proteins isolated and subjected to 2D PAGE as described in Experimental Procedures. Proteins that are found at a reduced level in the *tatC* mutant proteome are indicated.

Figure S4. Growth of the *S. scabies* wild type and the *SCAB03871*, *SCAB06471*, *SCAB10131* and *SCAB77391* knockout strains. 100ml of TSB medium was inoculated with each strain at a concentration of 100,000 spores per ml and incubated at 30°C with shaking. At the indicated time points, 3 x 1ml samples were removed from each culture, the hyphae pelleted and total protein content was determined using the Biorad DC Protein Assay. The error bars represent the standard error of the mean of samples taken at indicated time points, where n = 3.

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SCAB number	Putative function	Export of agarase by signal peptide?	Comments
SCAB00601	Hypothetical protein	Yes	Predicted by TATFIND 1.4 and TatP
SCAB00631	probable glycosyl hydrolase alpha-L-fucosidase precursor, putative	Not tested	Predicted to have a Tat signal peptide by both TATFIND 1.4 and TatP if the start codon is re-assigned
SCAB03671	probable glycosyl hydrolase alpha-L-fucosidase precursor, putative	Not tested	Predicted to have a Tat signal peptide by both TATFIND 1.4 and TatP if the start codon is re-assigned
SCAB03851	hypothetical protein, putative exported fucosidase	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB03871	glycosyl hydrolase domain followed by ricin domain	Yes	Predicted by TATFIND 1.4 and TatP
SCAB04451	carbon monoxide dehydrogenase (small chain), CoxS	Not tested	Only predicted by TATFIND 1.4. This is a complex cofactor-containing protein and is therefore almost certainly a Tat substrate
SCAB04591	putative hydrolase protein	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB04961	endo-xylanase	Yes	Predicted by TATFIND 1.4 and TatP
SCAB05861	phospholipase/Carboxylesterase	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB06441	putative muramoyl-pentapeptide carboxypeptidase	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB06471	putative alpha-L-fucosidase	Yes	Predicted by TATFIND 1.4 and TatP
SCAB08041	Amidohydrolase with WD40 domain repeats	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB08301	putative secreted protease	No	Predicted to have a Tat signal peptide by both TATFIND 1.4 and TatP if the start codon is re-assigned
SCAB08341	putative secreted protein	Not tested	Predicted by TATFIND 1.4 and TatP. Close homologue of SCO0736 which is a confirmed Tat substrate from *S. coelicolor* (Widdick *et al*., 2006)
SCAB08221	Putative phosphoesterase	Yes	Only predicted by TatP. Fails TATFIND 1.4 due to 'disallowed' Val in the +1 position
SCAB08951	ABC-type $Fe3^{+}$ transport system, periplasmic component	Yes	Predicted by TATFIND 1.4 and TatP
SCAB09291	putative secreted protein	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB09381	hypothetical protein	Yes	Predicted by TATFIND 1.4 and TatP
SCAB09591	Alpha-L-fucosidase	Yes	Predicted by TATFIND 1.4 and TatP
SCAB10131	glycosyl hydrolase domain followed by ricin domain	Yes	Predicted by TATFIND 1.4 and TatP

SCAB11581	Oligosaccharide deacetylase	Not tested	Only predicted by TatP. Fails TATFIND 1.4 due to 'disallowed' His in the +1 position
SCAB12481	ABC transporter substrate-binding protein	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB13491	beta-1,3-glucanase B	Not tested	Only predicted by TatP. Fails TATFIND 1.4 due to 'disallowed' Ile in the +1 position. The twin arg motif is absolutely conserved in all bacterial homologues.
SCAB13541	copper oxidase	Not tested	Only predicted by TatP. This is a predicted multicopper oxidase and they are frequently Tat substrates (e.g. Stanley *et al*., 2000)
SCAB13731	phospholipase C	Yes	Predicted to have a Tat signal peptide by both TATFIND 1.4 and TatP if the start codon is re-assigned. Close homologue of SCO6691 which is a confirmed Tat substrate from *S. coelicolor* (Widdick *et al*., 2006)
SCAB15521	sugar ABC transporter solute-binding protein	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB15571	putative secreted phosphoesterase	Yes	Predicted by TATFIND 1.4 and TatP
SCAB15581	Possible histidinol phosphatase (PHP family)	Yes	This has a long predicted signal peptide n-region. Fails TATFIND 1.4 and TatP unless truncated where it passes both
SCAB15711	putative endo-1,3-beta-glucanase	Yes	Predicted to have a Tat signal peptide by both TATFIND 1.4 and TatP if the start codon is re-assigned
SCAB16521	arabinofuranosidase	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB16551	Endo-Beta-1,4-Mannanase	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB16651	Putative L-xylulose-5-phosphate 3-epimerase	Yes	Predicted by TATFIND 1.4 and TatP Close homologue of SCO6580 which is a confirmed Tat substrate from *S. coelicolor* (Widdick *et al*., 2006)
SCAB16751	secreted solute binding protein	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB16951	hypothetical protein	Yes	Predicted by TATFIND 1.4 and TatP
SCAB18501	α-N-acetyl glucosaminidase	Yes	Predicted by TATFIND 1.4 only.
SCAB18791	Hypothetical secreted protein	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB19441	ABC transporter sugar-binding protein	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB19551	rhamnogalacturonase B precursor	Yes	Predicted by TATFIND 1.4 and TatP
SCAB20641	hypothetical protein	Not tested	Predicted by TATFIND 1.4 and TatP Close homologue of SCO6052 which is a confirmed Tat substrate from *S. coelicolor* (Widdick *et al*., 2006)
SCAB20821	lipoprotein	Not tested	Predicted to have a Tat signal peptide by both TATFIND 1.4 and TatP if the start codon is re-assigned
SCAB21061	ABC transporter solute-binding protein	Not tested	Only passes TatFind 1.4.

			However closest homologues have very good Tat motifs.
SCAB25391	hypothetical protein	Not tested	Predicted to have a Tat signal peptide by both TATFIND 1.4 and TatP if the start codon is re-assigned
SCAB25591	endo-1,4-beta-glucanase	Yes	Predicted by TATFIND 1.4 and TatP
SCAB25621	lipoprotein	Yes	Predicted by TATFIND 1.4 and TatP
SCAB25761	ABC transporter polyamine-binding lipoprotein	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB26961	phosphoesterase	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB27311	N-acetylmuramoyl-L-alanine amidase	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB31461	ABC transporter protein BldKB	No[a]	Predicted by TATFIND 1.4 and TatP
SCAB34181	putative bilirubin oxidase	No	Predicted by TATFIND 1.4 and TatP
SCAB35531	D-alanyl-D-alanine carboxypeptidase	Not tested	Predicted to have a Tat signal peptide by both TATFIND 1.4 and TatP if the start codon is re-assigned
SCAB37061	putative secreted protein	Yes	Predicted to have a Tat signal peptide by both TATFIND 1.4 and TatP if the start codon is re-assigned
SCAB38621	secreted hydrolase	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB38671	putative polysaccharide deacetylase	Not tested	Predicted to have a Tat signal peptide by both TATFIND 1.4 and TatP if the start codon is re-assigned
SCAB38731	beta-lactamase	Yes	Predicted to have a Tat signal peptide by both TATFIND 1.4 and TatP if the start codon is re-assigned
SCAB40131	hypothetical protein	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB42231	amidohydrolase 2	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB44071	hypothetical protein	Not tested	Predicted to have a Tat signal peptide by both TATFIND 1.4 and TatP if the start codon is re-assigned
SCAB44201	beta-galactosidase	Not tested	Predicted by TATFIND 1.4 and TatP Close homologue of SCO5667 which is a confirmed Tat substrate from *S. coelicolor* (Widdick *et al*., 2006)
SCAB44821	Putative phosphatase	Not tested	This has a very long predicted signal peptide n-region. Fails TATFIND 1.4 and TatP unless truncated where it passes both
SCAB44981	protocatechuate dioxygenase	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB45451	Putative glycosyl hydrolase	Not tested	Only predicted by TatP. Fails TATFIND 1.4 due to ‘disallowed’ Phe in the +1 position. Twin arginine motif completely conserved across bacterial homologues
SCAB46581	Dyp-type peroxidise	Not tested	Not predicted by either programme. Passes TatP if the signal peptide n-region is truncated. Fails TATFIND 1.4 due to ‘disallowed’ leu in the +1 position. Close homologue of SCAB66251.

			Dyp-peroxidases are frequently Tat substrates (e.g. Sturm *et al*., 2006, Jongbloed *et al*., 2004)
SCAB46711	protocatechuate dioxygenase	Yes	Predicted by TATFIND 1.4 only
SCAB47131	secreted peptidase containing M23 peptidase domain	No	Predicted by TATFIND 1.4 and TatP
SCAB48941	ATP-dependent nuclease subunit B-like	Yes	Predicted by TATFIND 1.4 and TatP
SCAB57161	secreted endo-beta-1,6-galactanase	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB57301	No homologues in database	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB57331	No homologues in database	Yes	Predicted to have a Tat signal peptide by both TATFIND 1.4 and TatP if the start codon is re-assigned
SCAB57461	ABC transporter substrate-binding protein	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB57661	putative multiple sugar ABC transporter solute-binding protein	Yes	Predicted to have a Tat signal peptide by both TATFIND 1.4 and TatP if the start codon is re-assigned
SCAB57891	PhoD family phosphatase	Not tested	Passes TATFIND 1.4 with the start codon as annotated and additionally also passes TatP if truncated. PhoD family proteins are frequently Tat substrates (e.g. Jongbloed *et al*., 2000; Widdick *et al*., 2006;2008)
SCAB57981	ferrichrome ABC transporter substrate-binding protein	Not tested	Predicted by TATFIND 1.4 only. Close homologue of SCO2780 which is a confirmed Tat substrate from *S. coelicolor* (Widdick *et al*., 2006)
SCAB58271	beta-N-acetylhexosaminidase	Not tested	Predicted by TATFIND 1.4 only. Close homologue of SCO2758 which is a confirmed Tat substrate from *S. coelicolor* (Widdick *et al*., 2006)
SCAB58511	Hypothetical protein	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB58531	Hypothetical protein	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB59231	tyrosinase co-factor	Not tested	Predicted by TATFIND 1.4 and TatP Tyrosinases shown to be exported Tat-dependently (e.g. Schaerlaekens *et al*., 2001).
SCAB59671	cyclic 3',5'-adenosine monophosphate phosphodiesterase	Yes	Predicted by TATFIND 1.4 and TatP
SCAB61981	metal transport system ABC transporter substrate-binding protein	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB62211	hypothetical protein	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB63071	putative gluconolactonase	Yes	If the sequence is truncated at the N-terminus then the truncated protein passes both TATFIND 1.4 and TatP
SCAB63391	3-ketosteroid-delta-1-dehydrogenase	Not tested	Predicted to have a Tat signal peptide by both TATFIND 1.4 and TatP if the start codon is re-assigned

SCAB63891	sugar ABC transporter sugar-binding protein	No	Predicted by TATFIND 1.4 and TatP
SCAB66251	iron-dependent peroxidase	Yes	Predicted by TATFIND 1.4 and TatP
SCAB66141	alkaline phosphatase	Not tested	Predicted by TATFIND 1.4 only. However This is a PhoD homologue related to SCO2286 which is a confirmed Tat substrate from *S. coelicolor* (Widdick *et al*., 2006)
SCAB68191	phospholipase D precursor	Yes	Predicted to have a Tat signal peptide by both TATFIND 1.4 and TatP if the start codon is re-assigned
SCAB68841	2',3'-cyclic-nucleotide 2'-phosphodiesterase	Yes	Predicted by TATFIND 1.4 and TatP
SCAB70561	secreted pectinesterase	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB70571	secreted pectinesterase	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB70581	Hydrolase of the alpha/beta superfamily	No	Predicted by TATFIND 1.4 and TatP
SCAB71861	Rec8/ScpA/Scc1-like protein (kleisin family)	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB71931	iron sulphur protein	Not tested	Predicted by TATFIND 1.4 and TatP Predicted iron sulphur protein so should be Tat-dependent
SCAB72801	hypothetical conserved protein	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB73161	Gluconolactonase	Yes	Predicted by TATFIND 1.4 and TatP
SCAB73521	peptidyl-prolyl cis-trans isomerase	Not tested	Predicted by TATFIND 1.4 only. Close homologue of SCO1639 which is a confirmed Tat substrate from *S. coelicolor* (Widdick *et al*., 2006)
SCAB74351	glycerophosphoryl diester phosphodiesterase	Yes	Predicted by TATFIND 1.4 and TatP
SCAB74641	lipoprotein	Yes	Predicted by TATFIND 1.4 and TatP
SCAB74681	hypothetical protein	Yes	Predicted by TATFIND 1.4 and TatP
SCAB75721	A sub-family of peptidase family C39.	Yes	Predicted by TATFIND 1.4 and TatP
SCAB76661[b]	Rare lipoprotein A	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB77391	Conserved hypothetical protein containing a galactose-binding domain	Yes	Predicted by TATFIND 1.4 and TatP
SCAB77401	Putative glycosyl hydrolase	Yes	Predicted by TATFIND 1.4 and TatP
SCAB77471	ABC transporter solute-binding protein	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB77511	Beta-galactosidase	Yes	Predicted to have a Tat signal peptide by both TATFIND 1.4 and TatP if the start codon is re-assigned
SCAB77971	alkaline phosphatase	No	Predicted by TATFIND 1.4 and TatP PhoD family protein which to date always shown to be Tat-

			dependent (e.g. Jongbloed *et al*., 2000; Widdick *et al*., 2006;2008)
SCAB78741	putative secreted protein	Yes	Predicted by TATFIND 1.4 and TatP
SCAB78851	coagulation factor 5/8 type-like	No	Predicted by TATFIND 1.4 and TatP
SCAB79011	lipase/acylhydrolase, putative	Yes	Predicted by TATFIND 1.4 and TatP
SCAB79151	putative oxygen-dependent FAD-linked oxidoreductase	Not tested	Predicted by TatP only. However this is a predicted FAD binding protein and these are usually Tat-dependent (see Berks *et al*., 2000)
SCAB79571	large secreted protein	Yes	Predicted by TATFIND 1.4 and TatP
SCAB79831	putative amidase	Not tested	Predicted by TATFIND 1.4 only. Homologue of SCO1172 which is a confirmed Tat substrate from *S. coelicolor* (Widdick *et al*., 2006)
SCAB80421	hypothetical protein	Not tested	Predicted to have a Tat signal peptide by both TATFIND 1.4 and TatP if the start codon is re-assigned This protein is predicted to have a C-terminal transmembrane helix and to be a sortase substrate
SCAB80581	Putative FAD-containing amine oxidase	Yes	Predicted by TATFIND 1.4 and TatP)
SCAB81041	putative spermidine/putrescine transporter peptide-binding protein	Yes	Only predicted by TatP. Fails TATFIND 1.4 due to ‘disallowed’ Leu in the +1 position
SCAB81841	alpha-L-rhamnosidase	Yes	Predicted by TATFIND 1.4 and TatP
SCAB81901	peptide ABC transporter peptide-binding protein	Yes	Predicted by TATFIND 1.4 and TatP
SCAB83011	hydrolase	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB84971	amidase	No	Predicted to have a Tat signal peptide by both TATFIND 1.4 and TatP if the start codon is re-assigned
SCAB85691	MelC	Not tested	Predicted by TatP only. Fails TATFIND 1.4 due to ‘disallowed’ His in the +1 position. This is MelC1 which is a known Tat substrate in *Streptomyces* (e.g. Schaerlaekens *et al*., 2001)
SCAB86391	CMP/dCMP deaminase, zinc-binding	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB86651	ABC-type Fe3+ transport system, periplasmic component	Not tested	Only passes TatP Almost identical to SCAB8951 but fails TATFIND 1.4 due to ‘disallowed’ His in the +1 position.
SCAB86701	rhamnosidase	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB88291	isoquinoline 1-oxidoreductase, beta subunit	Not tested	Only passes TatFind 1.4. However this protein binds a complex cofactor and is therefore most likely to be a Tat substrate

SCAB88501	rhamnogalacturonase B precursor	Yes	Predicted by TATFIND 1.4 and TatP
SCAB89551	4-methyl-5(B-hydroxyethyl)-thiazole monophosphate biosynthesis enzyme	Not tested	Predicted by TATFIND 1.4 and TatP
SCAB89661	putative factor C protein precursor	Yes	Predicted by TATFIND 1.4 and TatP
SCAB89701	secreted subtilisin-like protease	Not tested	Predicted to have a Tat signal peptide by both TATFIND 1.4 and TatP if the start codon is re-assigned

Table S1. Curated list of probable *S. scabies* Tat substrate proteins.

[a]The *S. coelicolor* homologue of this protein has a KR motif in its signal peptide and was also not able to mediate the Tat-dependent export of agarase (Widdick *et al*., 2006).

[b]The C-terminal part of this ORF is closely related to SCAB90061. SCAB90061 has an alternative start site 21 codons upstream that would give a signal peptide that is recognised by TATFIND 1.4 (but not TatP) as a candidate Tat targeting sequence.

Protein	R5	SFM	OBM	IPM
SCAB1191				7.8
SCAB4761	2.8			
SCAB5351	3.5			
SCAB7651				4.5
SCAB8221	8.9	10.5	11.6	6.1
SCAB8871			3.0	2.8
SCAB8951	5.0			
SCAB13321	2.5			
SCAB13551		12.0		
SCAB14911		3.5		
SCAB15571	4.3		5.0	3.0
SCAB15581	4.4	2.8	3.3	1.8
SCAB16431			3.2	
SCAB16551		4.2	3.1	
SCAB16651		4.4	8.2	4.7
SCAB16711	3.2	3.2	2.2	8.3
SCAB16721	3.1	10.7	8.1	6.5
SCAB17001		5.0		4.8
SCAB17551			1.7	
SCAB17571				4.2
SCAB18081		2.9		5.1
SCAB18501	5.6	2.5	5.2	2.5
SCAB18661		3.8		
SCAB19481				3.1
SCAB19491		2.0		
SCAB19941		1.5		
SCAB20121		2.3		
SCAB21191		1.8		2.3
SCAB24731			3.7	
SCAB24891		1.9		
SCAB25251			1.4	
SCAB25591	2.1	2.5	3.0	1.5
SCAB26011			2.6	
SCAB26841		1.6		
SCAB27771			5.2	
SCAB27931		1.4		
SCAB31531	2.1			2.6
SCAB31831				
SCAB33981		2.2		
SCAB36371				2.9
SCAB36931	5.5			
SCAB37201			2.3	
SCAB37611	2.3	6.9	1.9	3
SCAB38731			9.6	
SCAB39491	3.0	4.2		
SCAB39811				
SCAB41181		5.8		
SCAB41891	6.2			
SCAB43901				4.4
SCAB44001		4.2		
SCAB44161	2.6	7.5		
SCAB44541			5.1	5.9

SCAB44691		3.3	3.7	9.4
SCAB44821	6.6		4.1	2.9
SCAB45141				
SCAB45751	2.2			
SCAB47131			4.7	4.6
SCAB47841	7.2			
SCAB49491		2.4		3.5
SCAB50441				
SCAB51341			2.0	
SCAB51541		2.4		
SCAB54151			6.3	
SCAB55881				4.5
SCAB56441				2.5
SCAB57721			3.1	
SCAB57891	6.9		10.2	
SCAB58251		3.6		
SCAB58791	2.6			
SCAB59311	2.7			
SCAB59651		2.5		
SCAB59671	3.8			
SCAB59871		1.9		
SCAB62141	2.2			
SCAB63891		4.6		3.5
SCAB64081		2.1		3.8
SCAB64141		3.8		
SCAB64311		1.5		
SCAB66031		1.7		
SCAB67061		2.2		
SCAB68191	4.0	7.9	4.5	2.2
SCAB68841	3.0	4.5	2.8	2.9
SCAB68931		2		
SCAB69391	1.9			
SCAB69691				2.4
SCAB71391			3.0	
SCAB72441		4.4		2.4
SCAB72781				4.1
SCAB74081	4.2		5.3	4.5
SCAB74351	4.1	3.8	3.5	4.3
SCAB75721	2.1	3.2	6.1	3.8
SCAB77971	4.6	2.0	7.5	5.7
SCAB78431		2.8		
SCAB78761			4.1	
SCAB80071		1.5		
SCAB81841			2.0	
SCAB82451		1.6		
SCAB84861	2.6			
SCAB84971				6.7
SCAB85291				4.2
SCAB85681	2.5			
SCAB90091		3.1	3.0	3.5
SCAB90101		2.2		

Table S2. Comparison of the levels of protein found in each identified spot in Figures 2 and

S3 between the wild type and the *tatC* strain. The values are shown as ratios of the wild type versus *tat* mutant proteome for the growth media in which differences were detected. Ratios were calculated as described under Experimental Procedures.

Protein	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %	Total Ion Score	Total Ion Score C.I. %	Sequence coverage %
R5 Medium								
SCAB08221	72974.0234	7.83	24	162	100			47
SCAB44821	82428.1797	6.24	19	169	100			32
SCAB18501	89998.9766	5.83	16	73	100			29
SCAB59311	69194.2578	5.12	11	259	100	179	100	23
SCAB31531	74942.25	5.66	13	375	100	242	100	29
SCAB84861	84856.8281	8.05	14	201	100	131	100	28
SCAB59671	63338.2695	9.44	9	183	100	102	100	26
SCAB68841	71085.7891	6.68	12	121	100			28
SCAB77971	66756.7266	6.36	10	182	100	110	100	47
SCAB45751	101017.453	5.16	18	168	100			29
SCAB58791	53555.7188	5.5	19	393	100	234	100	50
SCAB41891	51329.0586	5.14	15	313	100	204	100	36
SCAB74081	55273.2695	6.33	8	182	100	128	100	22
SCAB74351	58092.25	5.22	8	92	100			24
SCAB44161	54932.9102	8.8	7	80	100			23
SCAB08951	50535.1719	8.18	8	79	100			30
SCAB36931	29270.8008	9.21	6	310	100	270	100	25
SCAB69391	39393.3789	5.29	12	371	100	211	100	50
SCAB47841	34552.7813	5.26	3	186	100	156	100	16
SCAB04761	20155.25	6.82	5	270	100	212	100	55
SCAB85681	40010.2617	5.5	6	54	96			19
SCAB62141	105436.75	5.14	13	185	100	85	100	24
SCAB13321	78327.2734	6.99	25	208	100			44
SCAB67061	57206.6016	5.64	18	401	100	221	100	42
SCAB67061	57206.6016	5.64	17	488	100	313	100	40
SCAB05261	64547.4609	5.1	9	332	100	265	100	25
SCAB46771	40838.4219	5.5	10	96	100			34
SCAB08801	20882.0293	6.39	5	53	96			25
SCAB72231	36120.7305	8.74	5	57	98			25
SCAB05281	44120.9883	5.15	7	63	100			31
SCAB07651	86636.0938	4.95	11	78	100			24

SCAB82671	36158.5195	4.16	7	79	100			31
SCAB81711	43087.1016	4.43	5	53	95			28
SCAB69921	80542.8906	5.08	14	245	100	152	100	31
SCAB03481	94864.6563	5.35	6	54	96			13
SCAB27611	63872.0391	4.94	13	217	100	116	100	37
SCAB59851	25073.0996	5.18	8	90	100			58
SCAB50161	48344.5313	5.22	9	109	100	17	12.386	31
SCAB42951	47713.6406	5.21	13	132	100			35
SCAB21101	102149.453	4.86	23	218	100			37
SCAB09771	138883.422	4.77	26	176	100			34
SCAB17551	23850.6504	4.74	9	91	100			49
SCAB25251	91589.3516	4.83	12	100	100			22
SCAB59611	100899.367	4.74	30	213	100			51
SCAB45651	28642.8398	4.72	7	65	100			25
SCAB66881	58180.5781	5.09	14	90	100			41
SCAB28241	56870.6406	5.19	18	249	100	69	100	55
SCAB67071	57411.2891	5.56	13	94	100			38
SCAB36201	49497.6406	5.01	19	188	100			60
SCAB25691	54188.5313	5.3	18	390	100	227	100	42
OBM and IPM media								
SCAB44541	93324.5234	7.66	27	372	100	181	100	45
SCAB44691	69131.7969	6.19	11	102	100			28
SCAB18501	89998.9766	5.83	12	69	100			24
SCAB44821	82428.1797	6.24	14	129	100			25
SCAB15571	72794.1484	5.85	11	252	100	179	100	18
SCAB68841	71085.7891	6.68	10	97	100			26
SCAB08221	72974.0234	7.83	15	113	100			37
SCAB25591	85807.4297	6.49	11	322	100	236	100	25
SCAB81841	115046.367	5.78	18	242	100	69	100	28
SCAB90091	107645.391	5.13	10	355	100	289	100	16
SCAB25251	91589.3516	4.83	7	54	96			10
SCAB15581	66970.9922	6.19	11	81	100			24
SCAB77971	66756.7266	6.36	15	100	100			31
SCAB24731	63567.4805	5.48	10	78	100			27

SCAB57721	60078.1797	5.58	15	197	100	93	100	40
SCAB47131	54055.0586	6.33	5	335	100	292	100	15
SCAB16711	59341.4102	5.14	6	62	99			12
SCAB57891	66033.0781	6.56	10	95	100			28
SCAB75721	55552.0703	6.22	12	83	100			38
SCAB78761	38410.9219	6.53	13	234	100	122	100	57
SCAB74351	58092.25	5.22	15	412	100	289	100	30
SCAB51341	45383.6484	5.6	8	76	100	8	0	29
SCAB08871	85964.2969	5.82	10	400	100	351	100	24
SCAB74081	55273.2695	6.33	9	250	100	176	100	25
SCAB26011	35574.9609	5.21	10	124	100			51
SCAB48241	62474.1094	4.88	11	82	100			31
SCAB38731	36770.9414	5.9	11	88	100			41
SCAB16431	50414.4805	5.02	9	312	100	241	100	32
SCAB16651	38215.2383	6.68	14	173	100			52
SCAB54151	26727.5391	4.79	5	201	100	151	100	24
SCAB27771	27721.3105	5.5	10	195	100	109	100	40
SCAB71391	26617.3691	5.22	7	259	100	193	100	44
SCAB37091	49618.0703	4.99	5	230	100	184	100	14
SCAB17551	23850.6504	4.74	9	91	100			49
SCAB37201	18857.3691	4.5	2	125	100	112	100	8
SCAB18081	72218.0625	5.3	14	127	100			30
SCAB05351	44984.1719	5.22	11	383	100	259	100	41
SCAB37611	42775.4883	4.99	10	404	100	307	100	36
SCAB85511	40909.3516	5.3	14	116	100			48
SCAB42951	47713.6406	5.21	11	115	100			34
SCAB52001	28249	5.85	12	263	100	141	100	46
SCAB72231	36120.7305	8.74	5	47	83			24
SCAB70681	44602.6797	5.32	9	254	100	176	100	29
SCAB66581	52602.4102	5.56	12	252	100	177	100	33
SCAB19491	54803.3281	5.53	13	363	100	228	100	49
SCAB39351	48298.0781	6.47	11	339	100	261	100	48
SCAB05851	51738.3281	5.18	6	60	99	19	47.042	22
SCAB70551	54497.9414	5.74	11	363	100	266	100	39
SCAB51091	64295.4414	9.1	12	265	100	140	100	35
SCAB13321	78327.2734	6.99	28	440	100	226	100	48

SCAB17001	65309.3711	5.25	14	98	100			37
SCAB28921	68208.4766	5.82	7	205	100	173	100	23
SCAB11431	192271.156	5.62	32	287	100	114	100	27
SCAB66701	204863.266	5.82	13	191	100	133	100	11
SCAB69691	113066.438	5.24	19	108	100			34
SCAB78431	65019.6602	8.52	16	371	100	242	100	38
SCAB28271	25764.4902	5.18	8	94	100			47
SCAB70591	46888.6484	6.14	13	181	100	85	100	42
SCAB72441	54640.0117	5.32	14	355	100	249	100	33
SCAB50261	28124.6699	4.52	9	289	100	223	100	52
SCAB56441	90484.7578	5	12	78	100			18
SCAB43901	68345.25	5.18	10	90	100			21
SCAB17571	61847.6289	8.62	19	377	100	219	100	43
SCAB31831	53965.1289	5.33	18	529	100	381	100	47
SCAB36371	58752.3086	5.11	15	568	100	463	100	32
SCAB37611	42775.4883	4.99	12	534	100	446	100	39
SCAB39491	100650.844	5.64	20	579	100	460	100	25
SCAB39811	49101.25	5.96	21	358	100	168	100	58
SCAB50441	62626.3711	4.8	11	229	100	170	100	26
SCAB55881	48744.5117	5.7	25	574	100	318	100	59
SCAB69691	113066.438	5.24	26	275	100	89	100	38
SCAB72781	105623.672	5.84	12	408	100	364	100	18
SFM medium								
SCAB90091	107645.391	5.13	10	213	100	181	100	15
SCAB59651	124069.922	5.09	13	252	100	180	100	19
SCAB08871	85964.2969	5.82	13	113	100			27
SCAB13551	97463.9297	5.88	19	362	100	218	100	34
SCAB44691	69131.7969	6.19	17	286	100	168	100	42
SCAB68841	71085.7891	6.68	15	470	100	332	100	37
SCAB68191	65376.5781	5.98	12	127	100	42	99.757	27
SCAB80071	58535.2188	6.29	16	267	100	149	100	44
SCAB78431	65019.6602	8.52	13	167	100	96	100	31
SCAB58251	61939.6992	5.83	14	332	100	211	100	33
SCAB15581	66970.9922	6.19	12	267	100	172	100	27

SCAB77971	66756.7266	6.36	11	151	100	97	100	22
SCAB64081	52636.5117	6.85	18	274	100	91	100	35
SCAB44161	54932.9102	8.8	15	396	100	255	100	44
SCAB20121	88098	5.15	17	144	100	41	99.822	29
SCAB49491	70707.8281	5.73	17	360	100	217	100	39
SCAB17001	65309.3711	5.25	12	328	100	254	100	35
SCAB90101	67403.8828	5.02	12	188	100	92	100	20
SCAB16721	95516.4375	4.9	15	477	100	345	100	23
SCAB67061	57206.6016	5.64	17	464	100	335	100	40
SCAB19491	54803.3281	5.53	12	315	100	203	100	46
SCAB68931	52951.3594	4.86	9	96	100			28
SCAB18081	72218.0625	5.3	13	204	100	115	100	29
SCAB75721	55552.0703	6.22	11	91	100	40	99.768	33
SCAB66031	61684.0898	5.84	15	237	100	154	100	45
SCAB19941	52603.9883	6.67	12	205	100	93	100	30
SCAB82451	53325.2188	5.86	15	322	100	176	100	46
SCAB74351	58092.25	5.22	16	146	100			32
SCAB27931	60719.8008	6.65	5	428	100	379	100	11
SCAB63891	48016.0703	4.91	14	314	100	184	100	50
SCAB05351	44984.1719	5.22	13	242	100	101	100	43
SCAB44001	69019.1172	6.98	7	50	90			13
SCAB72441	54640.0117	5.32	11	115	100			25
SCAB16651	38215.2383	6.68	15	173	100			52
SCAB33981	38920.1914	7.56	10	377	100	275	100	46
SCAB18661	33857.3516	5.51	6	47	80			27
SCAB16711	59341.4102	5.14	8	222	100	152	100	14
SCAB24891	35650.5508	5.11	7	220	100	174	100	29
SCAB51541	26117.6309	4.55	5	52	94			41
SCAB04761	20155.25	6.82	5	61	99			55
SCAB13321	78327.2734	6.99	28	195	100			44
SCAB13321	78327.2734	6.99	22	166	100			40
SCAB68841	71085.7891	6.68	8	277	100	223	100	20
SCAB87641	69881.7188	5.68	23	528	100	334	100	54
SCAB25181	69658.2188	5.64	22	317	100	87	100	51
SCAB27411	69808.8594	5.57	16	316	100	170	100	36
SCAB45751	101017.453	5.16	21	422	100	248	100	35

SCAB09771	138883.422	4.77	31	375	100	180	100	40
SCAB25251	91589.3516	4.83	8	76	100			15
SCAB77921	70485.8906	4.82	21	157	100			55
SCAB50441	62626.3711	4.8	21	459	100	238	100	56
SCAB36291	62594.3281	4.84	15	361	100	223	100	44
SCAB54441	51003.6016	4.48	15	318	100	180	100	47
SCAB35741	62689.6211	5.38	18	508	100	288	100	45
SCAB36211	60311.6992	5.52	10	416	100	288	100	31
SCAB83281	60270.9805	5.68	17	469	100	265	100	50
SCAB83281	60270.9805	5.68	22	582	100	339	100	53
SCAB55951	56093.6992	5.74	15	356	100	239	100	33
SCAB67061	57206.6016	5.64	16	309	100	183	100	38
SCAB67061	57206.6016	5.64	12	429	100	331	100	31
SCAB74471	48802.2813	5.72	9	340	100	244	100	28
SCAB57661	63455.3086	5.8	13	82	100			28
SCAB58791	53555.7188	5.5	11	91	100			32
SCAB28481	48873.9609	5.48	13	316	100	192	100	40
SCAB71831	47309.3086	5.41	10	391	100	292	100	33
SCAB36201	49497.6406	5.01	18	176	100			57
SCAB05151	34772.3906	5.61	13	363	100	241	100	43
SCAB37251	40177.1914	4.08	8	68	100			28
SCAB82671	36158.5195	4.16	7	458	100	394	100	31
SCAB73291	61810.4219	4.81	10	175	100	103	100	23
SCAB28761	57851.8711	4.73	18	318	100	178	100	45

Table S3. Identification of proteins by MALDI-ToF mass spectrometry from excised protein spots indicated in Figures 2 and S3. In each case spots were excised and identified from the 2D gels of the wild type cell wall proteome. PI is the isoelectric point of the protein, estimated from the amino acid sequence. Peptide count is the total number of peptides from the indicated protein. Protein score is the number of identified peptides that match the protein (peptide count) and in the neighbouring column this is shown as a percentage of all the peptides identified from that spot. The total ion score is a measure of how well the observed MS/MS spectra matches to the stated peptides, and in the neighbouring column this is indicated the % match of the sequenced peptides (ion by MS/MS) to the identified peptides. The final column gives the percentage coverage of the protein from the mass spectrometry analysis of the tryptic peptides.

Oligonucleotide	Sequence
pSM1_fwd	GCGCGCCATATGAACCACACCCCCGGTCTCCC
pSM1_rev	GCGCGCGGATCCCACCGCCGCGGAAGCCGTC
pSM2_fwd	GCGCGCCATATGCGAGGTTCCCCCACCGACCG
pSM2_rev	GCGCGCGGATCCGTACTCGGCCGCCGCGGTGC
pSM3_fwd	GCGCGCCATATGATGACCAGGACTCCACGCGC
pSM3_rev	GCGCGCGGATCCGCCGGCCGCGGCCGCACTGG
pSM4_fwd	GCGCGCCATATGACGATGACCGCCGACGACTC
pSM4_rev	GCGCGCGGATCCCGGGCGGGCCGCGGCGACCG
pSM5_fwd	GCGCGCCATATGTCCCCTCGCTTCACTCCCTC
pSM5_rev	GCGCGCGGATCCCTCCTCCGCCCGCGCCACCC
pSM6_fwd	GCGCGCCATATGCCTGAAGTCAACCGGCGGCG
pSM6_rev	GCGCGCGGATCCGGGCAGTGCCGCGGCCCGC
pSM7_fwd	GCGCGCCATATGAGCCGGCACATCACAAAGTC
pSM7_*Bgl*II_rev	GCGCGCAGATCTGATGGCCGCTTGGGCGGAAG
pSM8_fwd	GCGCGCCATATGCGTATGTCGCAATCGATCAG
pSM8_rev	GCGCGCGGATCCCGAGCCGGCCATGGCGCCCC
pSM9_fwd	GCGCGCCATATGAAGAGACGCACGCTGCTCGG
pSM9_rev	GCGCGCGGATCCGTCGGCGGCGCTGGCGGTCG
pSM10_fwd	GCGCGCCATATGTCGAGTTCGATCAGCAGACG
pSM10_rev	GCGCGCGGATCCGGGCGCGGCCTGTGCCGTGC
pSM11_fwd	GCGCGCCATATGCCGATGCCTCTGCTCGTGCC
pSM11_*Bgl*II_rev	GCGCGCGGATCCGTGGGCGGCGGACGGGCCG
pSM12_fwd	GCGCGCCATATGAGTGAACCCTCCGGCATACC
pSM12_rev	GCGCGCGGATCCGGCGGCGCCCTGCGCGGTCG
pSM13_fwd	GCGCGCCATATGTCGATCTCCCGCAGGAACTTC
pSM13_rev	GCGCGCGGATCCCGTGGCGGCGGAGCCGGAGG
pSM14_fwd	GCGCGCCATATGACGGGAAACCACACACGCAG
pSM14_rev	GCGCGCGGATCCCTCGTCCGCGAACGCCGAGC
pSM15_fwd	GCGCGCCATATGGCACCCCCGCAACCCCCGCAC
pSM15_rev	GCGCGCGGATCCGGAGGCGGCGGAGGCGGTCG
pSM17_fwd	GCGCGCCATATGCACGACGAACGTTTCACCGG
pSM17_rev	GCGCGCGGATCCGCCACCGGCCCCGGCGTCCG
pSM18_fwd	GCGCGCCATATGGGCGGCACCACGCGCAGAGG
pSM18_rev	GCGCGCGGATCCGTCCCGGGCGGAGGCCCGCC
pSM19_fwd	GCGCGCCATATGCGCACACCTCGCCACATGTC
pSM19_rev	GCGCGCGGATCCTGTTCCGGCGGTGCGTACGG
pSM20_fwd	GCGCGCCATATGTGGCTGTGCACCTTGGCCGG

pSM20_*Bgl*II_rev	GCGCGCAGATCTCGAGAGGGCCAGCGCCGGCAC
pSM21_fwd	GCGCGCCATATGACCAGTCGCAGATCTCACTC
pSM21b_fwd	GCGCGCCATATGGGTTCGGCCGCGCCGAGCCG
pSM21_rev	GCGCGCGGATCCCTCCGCCGCGAGGGCCGGCAG
pSM22_fwd	GCGCGCCATATGGACCCCAAGGAGACATCAATG
pSM22_rev	GCGCGCGGATCCCGACTGGGCCTCGGCCGTCG
pSM23_fwd	GCGCGCCATATGCACCGCCGTACCTTCCTCGC
pSM23_rev	GCGCGCGGATCCGGTCGACGACGATGACGGCG
pSM24_fwd	GCGCGCCATATGACCGCACGACGCGTCGACCG
pSM24_rev	GCGCGCGGATCCGAGGTCCGCGCCCGCCCAGAG
pSM25_fwd	GCGCGCCATATGACGCCGAACTCCGCCCCCTC
pSM25_rev	GCGCGCGGATCCCGAGCCGCCGCAGGCGGAC
pSM26_AtoG_fwd	GCGCGCCATATGATGGTCACGTATGTGAATGG
pSM26_rev	GCGCGCGGATCCGGAGGCGGCGTGCGCGGCGG
pSM27_fwd	GCGCGCCATATGACCGAGCGTCAGAACGAGCC
pSM27_rev	GCGCGCGGATCCCGTCGCGGCCGCGGCTCTCAC
pSM29_fwd	GCGCGCCATATGCCCATGACACAAAGACGTCG
pSM29b_fwd	GCGCGCCATATGCGTCCGCAGCCCTGTC
pSM29_rev	GCGCGCGGATCCGTCGACGGCCGACGCCGGAAAG
pSM31_fwd	GCGCGCCATATGTCGGACGGGGTTCGGGAGAG
pSM31_rev	GCGCGCGGATCCTTCGGTCGCCATCGCCGCCTG
pSM32_fwd	GCGCGCCATATGTCGCGGCCCGGCATGTCCCG
pSM32_rev	GCGCGCGGATCCGGAGCGGGCGGCCGGCTCGTC
pSM33_fwd	GCGCGCCATATGGCCGAATCCCCCACCCCGTC
pSM32_fwd	GCGCGCCATATGTCGCGGCCCGGCATGTCCCG
pSM33_rev	GCGCGCGGATCCGCCGAAGGCCGCGGCGCGGG
pSM34_fwd	GCGCGCCATATGAGGGAGCACGTGGACCGTCG
pSM34_rev	GCGCGCGGATCCCAGGTCGGCGCCGGGTGGCG
pSM35_fwd	GCGCGCCATATGAAGGAGCACACCTTGGACGC
pSM35b_fwd	GCGCGCCATATGAAGGAGCACACCTTGGA
pSM35c_fwd	GCGCGCCATATGGACGCGTCAGCCTCGGC
pSM35_rev	GCGCGCGGATCCGGACTGCGCGAACGCCGGCAG
pSM37_fwd	GCGCGCCATATGCCGGCCGGTGAACCGGCGGG
pSM37_rev	GCGCGCGGATCCCGGGGCGGCACCGGCGGCGC
pSM38_fwd	GCGCGCCATATGCAGCAGAACAGGAACGTTGAG
pSM38_rev	GCGCGCGGATCCTCCGTCCCCCGAGCCGCTGC
pSM39_fwd	GCGCGCCATATGTCCCCGATCGACCGCAGGTC
pSM39_*Bgl*II_rev	GCGCGCAGATCTCGGATCCGCCTTCGCCGCCTC

pSM40_fwd	GCGCGCCATATGCCCAACGCACCCAGAAGACG
pSM40_rev	GCGCGCGGATCCGAAGGCGGCGTCCGCGGCCG
pSM41_fwd	GCGCGCCATATGTCCGAGTCACAGCCCGCCAC
pSM41_rev	GCGCGCGGATCCGGTCAGGGCGTCCGCGGTCG
pSM42_fwd	GCGCGCCATATGAGTTCTGACATGACAGGTTTG
pSM42_*Bgl*II_rev	GCGCGCAGATCTCACAGCGGCGGACGCGGCAC
pSM43_fwd	GCGCGCCATATGGTCCTCACCACCCGTCGCAG
pSM43_rev	GCGCGCGGATCCTTCGCCGGCCGACGCGCTCG
pSM44_fwd	GCGCGCCATATGTCCGAAGTGACACGCAGACG
pSM44_rev	GCGCGCGGATCCGTCGGCGGCCCGGACTTCCG
pSM45_fwd	GCGCGCCATATGAACGGAACCACCATGTCCGC
pSM45_rev	GCGCGCGGATCCTCCCGCCGCGTCGGCGGTGG
pSM47TtoC_fwd	GCGCGCCATATGGACCCCGGACGCACACGCAG
pSM47_rev	GCGCGCGGATCCGCCCGCCGCACGCGCGGTCG
pSM49_fwd	GCGCGCCATATGCACCCGTACGACGACAACAG
pSM49_rev	GCGCGCGGATCCACCGGCCGCGCCCGCCGAGG
pSM50_fwd	GCGCGCCATATGTCGATGACCCTCACCCCCCAG
pSM50_rev	GCGCGCGGATCCGTCCGCCGAGAAGGCCATGG
pSM51_fwd	GCGCGCCATATGAGACGAAGAGCGAGATCGATC
pSM51_rev	GCGCGCGGATCCCTCGGCAGCCTGGGCCAGCG
pSM52_fwd	GCGCGCCATATGACCGAGGAGGTGCCCATGTAC
pSM52_rev	GCGCGCGGATCCGGGCGCCTGGCCCGCGTGGG
pSM53_fwd	GCGCGCCATATGCCCCTGAACCGCCGGAAGTTTC
pSM53_rev	GCGCGCGGATCCCTCCGCCGCCTGCGCGCTCG
pSM55_fwd	GCGCGCCATATGACCAGTCGCAGATCTCACTC
pSM55_*Bgl*II_rev	GCGCGCAGATCTCGAGAGGGCCAGCGCCGGCAC
pSM56_fwd	GCGCGCCATATGCGTCACACGCGCCGCACAGG
pSM56_rev	GCGCGCGGATCCGCTGTCGGCGGACGCCGTCG
pSM58_fwd	GCGCGCCATATGAGATCCACCCCCCACAGACG
pSM58_rev	GCGCGCGGATCCACCGCGTGCCATCGCCGGGG
pSM59_fwd	GCGCGCCATATGCAGAGGAACCGACGACTGCC
pSM59_rev	GCGCGCGGATCCGCGGTCCGCCTCATCGGAC
pSM62_fwd	GCGCGCCATATGACGACACCTACTTCCAGGAG
pSM62_rev	GCGCGCGGATCCGCTGGAGGCCGAGGCGGCCC
pSM63_fwd	GCGCGCCATATGGGGACGCAGGAGTCGCGGC
pSM63_rev	GCGCGCGGATCCCTCGGCGGCCGTGGCCGTGC
pSM65_fwd	GCGCGCCATATGTGCACCTCGCACGAACC
pSM65_rev	GCGCGCGGATCCGGGCGCCGCCTGTGCGGTGG

pSM67_fwd	GCGCGCCATATGCGCACCCCACAGCCGAG
pSM67_rev	GCGCGCGGATCCGGTGTGAGCCTGGGCTGTCG
pSM68_fwd	GCGCGCCATATGTCAGAGCGCGACCCTCG
pSM68_rev	GCGCGCGGATCCCTGTGCGCCGGGCGCCGCGG
pSM69_fwd	GCGCGCCATATGCCGCGCATACGCTCAG
pSM69_rev	GCGCGCGGATCCGGTGGCGGCATGGGCGGC
pSM70_fwd	GCGCGCCATATGATGATCATGCGCACTCG
pSM70_rev	GCGCGCGGATCCGTTCTGGCCGCAGGCGGTC
pSM71_fwd	GCGCGCCATATGACGAGCCTTTCCGAGCC
pSM71_rev	GCGCGCGGATCCTTCCGGTGCTGGTGCGCGAC
pSM72_fwd	GCGCGCCATATGGCGCAGGACTTCTCACG
pSM72_rev	GCGCGCGGATCCGACCGCGGCCATGCCCACCG
pSM73a_fwd	GCGCGCCATATGTCGCGCCGGGGCCTGC
pSM73b_fwd	GCGCGCCATATGTCCGACCCGTCCGGCC
pSM73_rev	GCGCGCGGATCCGGGGCCCGCCGCGGCCGGTG
pSM74_fwd	GCGCGCCATATGACTCCGTTCTACGCGCG
pSM74_rev	GCGCGCGGATCCGGGCTGGGCGGTGGCGCCCG
pSM75_fwd	GCGCGCCATATGGAATCCGGAGCCCCCAC
pSM75_rev	GCGCGCGGATCCGGGCGCCGCCCCGGCTATG
pSM76_fwd	GCGCGCCATATGGACCTGACGCGACGTC
pSM76_rev	GCGCGCGGATCCGGTGATGGCGCCGGCCCGG
pSM77_fwd	GCGCGCCATATGCACCTCACACCTTCCGG
pSM77_rev	GCGCGCGGATCCGGTCTTCGCGTCACCGGCC
pSM78_fwd	GCGCGCCATATGGTCGGCAGTTTCAGCAG
pSM78_rev	GCGCGCGGATCCGGTGGCGGCCCGGGCGGTG
pTDW168_fwd	GGCGCGCATATGATGATCATGCGCACTCG
pTDW168_rev	GGCGGATCCGTCGTCGCTGCCACCCTCGCTG
pTDW169_fwd	GGCGCGCATATGCGGACCGTAGGAACCACAGG
pTDW169_rev	GGCGGATCCGGGGGCCGCGGCATACGCCGAC
pTDW170_fwd	GGCGCGCATATGGTCGGCAGTTTCAGCAGG
pTDW170_rev	GGCGGATCCGGAGCCGGCCGCCGACGCGGTG
pTDW171_fwd	GGCGCGCATATGAGCCGCATCTCCCAGCCCGA
pTDW171_rev	GGCGGATCCGCCAGCGGCGGCCTGGGCGGGG
pTDW172_fwd	GGCGCGCATATGTCAGAGCGCGACCCTCGCA
pTDW172_rev	GGCGGATCCGCCGGGCGCCGCGGTGGCCGCAG
pTDW173_fwd	GGCGCGCATATGCCGTCATCCGCCGGGCAC
pTDW173_rev	GGCGCGCGGATCCCGCGGTGGCGGCCTTGGCCTC
pTDW174_fwd	GGCGCGCATATGCAGAGGAACCGACGACTGC

pTDW174_rev	GGCGCGGGATCCCGCCTCATCGGACACGGCCGGT
pTDW175_fwd	GGCGCGCATATGCCGCTCGCCCGTCGTGC
pTDW175_rev	GGCGCGGGATCCTGTGTCGTCCGAGGGGATGG
pTDW176_fwd	GGCGCGCATATGACTCCGTTCTACGCGCGTC
pTDW176_rev	GGCGCGGGATCCCGCGGGCTGGGCGGTGGC
pTDW177_fwd	GGCGCGCATATGACATCCTTACTGACTTC
pTDW177_rev	GGCGCGGGATCCGGGGGCCGCCGCCGAAGC
pTDW178_fwd	GGCGCGCATATGACGACACCTACTTCCAGG
pTDW178_rev	GGCGCGGGATCCGTGGCTGGAGGCCGAGGC
pTDW181_fwd	GGCGCGCATATGAAAGACGCCCGGATAGAC
pTDW181_rev	GGCGCGGGATCCGCCACCGCTGCCGCCGGCCGT
pTDW182_fwd	GGCGCGCATATGAGAACCCCCAGAAATCC
pTDW182_rev	GGCGCGGGATCCCGGGTCGGCGGCGTGGGCGG
pTDW183_fwd	GGCGCGCATATGAAGAAACGCACAGCTGC
pTDW183_rev	GGCGCGGGATCCCGGGGCGGCGGCGGAAGCG
pTDW184_fwd	GGCGCGCATATGAGGCCGACCAGAAGGGC
pTDW184_rev	GGCGCGGGATCCGCCGTCCGCGGCGCTTGCCAC
utatcscabf1	CGCGCGAAGCTTAGCAGTGTCCGGACGGGCAG
utatcscabr	CGCGCGGAATTCCCCACGTCCTCATCTCGTGC
dtatcscabf	CGCGCGGAATTCGACTCGCACGTGAGTGAGGG
dtatcscabr1	CGCGCGTCTAGACGGCGAACTCGCCGACGATC
ecoriapraf	CGCGCGGAATTCATTCCGGGGATCCGTCGACC
ecoriaprar	CGCGCGGAATTCTGTAGGCTGGAGCTGCTTC
hyg_fwd	CTATGATCGACTGATGTCATCAGCGGTGGAGTGCAATGTCGTGACA CAAGAATCCCTGTTACTTC
hyg_rev	CAGCGCGACCTTGCCCCTCCAACGTCATCTCGTTCTCCGCTCAGG CGCCGGG
KanRtrans2_fwd	TGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGATTGAACAA GATGGATTGC
KanRtrans2_rev	AATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTATCAGAAGAA CTCGTCAAGAAG
MJ384_RD-fwd-SCAB77391	GAATCCTCATGGGCAGCCTCAAGTAAAGGATCAGCCGTGATTCCG GGGATCCGTCGACC
MJ385_RD-rev-SCAB77391	GGGGAACCGGGCGTCGGCCCGGCTCCCCCGGTGCGGTCATGTAG GCTGGAGCTGCTTC
MJ394_RD-fwd-SCAB80581	TCGTGCGCCGGTACTTACCGACGGTAAGGGGTAGCGATGATTCCG GGGATCCGTCGACC
MJ395_RD-rev-SCAB80581	GCGCACTGCCCTGAACGGGCCGCGGGGGTGCGGTCGTCATGTAG GCTGGAGCTGCTTC
MJ396_RD-fwd-SCAB81041	TCACCTCCCGCGGCCGGCACCACCAGGAGGCACACCATGATTCCG GGGATCCGTCGACC

MJ397_RD-rev-SCAB81041	CCGGCCACCTTCACCGACGGTGCGGCGACACCGAATTCATGTAGGCTGGAGCTGCTTC
MJ398_RD-fwd-SCAB81841	CCTCAGCCCCCGGACCCCCGACCGAAGGTATGCACCATGATTCCGGGGATCCGTCGACC
MJ399_RD-rev-SCAB81841	TGAGCGGTCGGCTCAGGGGCCCGTTCAGGGGCCTGTTCATGTAGGCTGGAGCTGCTTC
MJ408_fwd-SCAB77391	GAACACCCTCGTCCTCACCG
MJ409_rev-SCAB77391	GAGTGTCAGGGGGTGGCAGAT
MJ418_fwd-SCAB80581	ATCGGTGTGTCGCTCCTATCCTC
MJ419_rev-SCAB80581	TCAAGGACACGACACCCGAACA
MJ420_fwd-SCAB81041	ACGGACGCATCTTCTACGGCAT
MJ421_rev-SCAB81041	ATCATCAGCGTCGTGGTCTTGC
MJ422_fwd-SCAB81841	TCGGCACGGAACTCTAACCTC
MJ423_rev-SCAB81841	TCTGACACAACACCCCCGAA
MJ440_RD-fwd-SCAB03871	CCGGGCTGTCGCTCTCCCCTCACCGAAAGGTCCAGGATGATTCCGGGGATCCGTCGACC
MJ441_RD-rev-SCAB03871	AAATCGGGCAGTCCTGGGGAGCGGTTGGGGGTGGTGTCATGTAGGCTGGAGCTGCTTC
MJ442_RD-fwd-SCAB06471	CCTCCCCCTGTCTCCCCTCCGCATCAGGGACGTCCCATGATTCCGGGGATCCGTCGACC
MJ443_RD-rev-SCAB06471	GCGTACCGGCCGGCCGTCGTCCTGGGCCGGGGTGGGTCATGTAGGCTGGAGCTGCTTC
MJ446_RD-fwd-SCAB10131	CCAGCCCGTGACGCGCCACGCCCGACCGTCGGACGTGTGATTCCGGGGATCCGTCGACC
MJ447_RD-rev-SCAB10131	AGGTAATGCCCAGTTCACGGCCTTGTGCCACACGCCTCATGTAGGCTGGAGCTGCTTC
MJ448_fwd-SCAB03871	TCACCCTCGGCACATCCAAT
MJ449_rev-SCAB03871	CGAGCAGCACGGAAAGATGTC
MJ450_fwd-SCAB06471	ACCACTTTCATCCCTGCACGC
MJ451_rev-SCAB06471	ATGTCCCGTTCCTCCGCACT
MJ454_fwd-SCAB10131	TCGTCCGCAGGTCATCGTGTA
MJ455_rev-	ACAGGTAATGCCCAGTTCACGG

SCAB10131

Table S4. Oligonucleotide primers used in this study. Introduced restriction sites are shown underlined.

Plasmid	Description	Amino acid sequence tested in agarase assay
pSM1	pTDW46H with signal sequence of SCAB89661	MNHTPGLPFSRRGLIRTGAGASLAALGLAAGAQTASAAV
pSM2	pTDW46H with signal sequence of SCAB84971	MRGSPTDRRPTPRPGRARRSALAAAPAVLLLPLLGAAPPAGLPSAAPARLQGAFGTAAAEY
pSM3	pTDW46H with signal sequence of SCAB78851	MMTRTPRAHDRRRPLMVLALFLTTMGMLLSPSSSAAAAG
pSM4	pTDW46H with signal sequence of SCAB66251	MTMTADDSPEPGSAPEHPRTPSRRALIGWGGAGLALGAAAAGGAVAAARP
pSM5	pTDW46H with signal sequence of SCAB37061	MSPRFTPSTICHGAEQIQPSGRPVEGCGMSADVSRRTVLGAGAGLAVVPALPGVARAEE
pSM6	pTDW46H with signal sequence of SCAB13731	MPEVNRRRFLQVAGATTAFSALSASIQRAAALP
pSM7	pTDW46H with signal sequence of SCAB10131	MSRHITKSKQPLCRIERLTMSPSVSRRRLLQLAGATVAASATGSLVGASSAQAAI
pSM8	pTDW46H with signal sequence of SCAB09591	MRMSQSISRRRLLTGATAVVLATAATDVIAAGGAMAGS
pSM9	pTDW46H with signal sequence of SCAB09381	MKRRTLLGAALAGAVATPALGAATASAAD
pSM10	pTDW46H with signal sequence of SCAB06471	MSSSISRRRLLAGATCVAAAAVAGGVLGAGTAQAAP
pSM11	pTDW46H with signal sequence of SCAB03871	MPMPLLVPHPTAGSVSHRHSRPGCRSPLTERSRMTSPVSRRRLLQAAGASAAASAAGSLIGP SAAH
pSM12	pTDW46H with signal sequence of SCAB15711	MSEPSGIPLGRRRPLRRVFLAMAGALALATAWATTAQGAA
pSM13	pTDW46H with signal sequence of	MSISRRNFVIASSVATAGGALVLSACSSGGGGGTAGGGAASGSAAT

	SCAB31461	
pSM14	pTDW46H with signal sequence of SCAB46711	MTGNHTRRTITRRRALAVTGGTVAAGGLVATGYRSAFADE
pSM15	pTDW46H with signal sequence of SCAB73161	MAPPQPPHLPSARHLSRRTLLTTSAAGAAALLVGSSAATASAAS
pSM17	pTDW46H with signal sequence of SCAB81901	MHDERFTGLRRRGFLAAGGAVALGSLALTGCGDGGGTDAGAGG
pSM18	pTDW46H with signal sequence of SCAB80581	MGGTTRRGFLGTAAAAGAGGGIGLGVPGRASARD
pSM19	pTDW46H with signal sequence of SCAB79011	MRTPRHMSRRTLVAAGAAGLAATVVSPASATVRTAGT
pSM20	pTDW46H with signal sequence of SCAB77971	MWLCTLAGSVTGGAFMSHRPPSPLPGRRNVLRGSLAASAALALPGSVALGSVPALALS
pSM21a	pTDW46H with signal sequence of SCAB68191	MTSRRSHSSPDSPAQPMGSAAPSRRTVVKAAAAGAVLAAPLAAALPALAAE
pSM21b	pTDW46H with signal sequence of SCAB68191	MGSAAPSRRTVVKAAAAGAVLAAPLAAALPALAAE
pSM22	pTDW46H with signal sequence of SCAB57331	MDPKETSMAIALPFGRRTALAALAITAAAATTLTLPATAEAQS
pSM24	pTDW46H with signal sequence of SCAB34181	MTARRVDRRRLLKLGLLGIPATAAVAGGGLTYLWAGADL
pSM25	pTDW46H with signal sequence of SCAB25621	MTPNSAPSSPSRRSFLASTAVATAAVAGGLPLLSACGGS
pSM26	pTDW46H with signal sequence of SCAB16951	MMVTYVNGWPMTRTRRNVLGAALGGAAAATAGLPAPAAHAAS
pSM27	pTDW46H with signal sequence of	MTERQNEPERGRGGSPSRRTVLAAVGALPILTAVAPPAAGAVRAAAAT

	SCAB04961	
pSM29a	pTDW46H with signal sequence of SCAB00601	MPMTQRRRRFLPRLRATLIASAAALACMLVTLSAPFPASAVD
pSM29b	pTDW46H with signal sequence of SCAB00601	MRPQPCLTSGTTDTEGTIMPMTQRRRRFLPRLRATLIASAAALACMLVTLSAPFPASAVD
pSM31	pTDW46H with signal sequence of SCAB08301	MSDGVRESQAMRHARRRVVKRVARLTAAGGLICGTLMVAQAAMATE
pSM33	pTDW46H with signal sequence of SCAB19551	MAESPTPSEAASGPGRRAFVVGAAATAAGAALTGPLAGTARAAAFG
pSM34	pTDW46H with signal sequence of SCAB48941	MREHVDRRRRALLSVSVVAATAALGAAVFGGGPVERPAPPGADL
pSM35a	pTDW46H with signal sequence of SCAB63071	MPRTAPPVVTDRRRRAPARPAFPPRHRTNRRTNHRMKEHTLDASASASRRTLLRLATAAGLG GALAATPLPAFAQS
pSM35b	pTDW46H with signal sequence of SCAB63071	MKEHTLDASASASRRTLLRLATAAGLGGALAATPLPAFAQS
pSM35c	pTDW46H with signal sequence of SCAB63071	MDASASASRRTLLRLATAAGLGGALAATPLPAFAQS
pSM37	PTDW46H with signal sequence of SCAB70581	MPAGEPAGVPDPPGTRRSVGRRAFVAGAGAAALGTGAGSPAAGAEPPGAAGAAP
pSM38	PTDW46H with signal sequence of SCAB74641	MQQNRNVERRTVLKAAGASLAVAGLGATATACGGGSGSGDG
pSM39	PTDW46H with signal sequence of SCAB74681	MSPIDRRSLLKAAAVAGAAAQFSWALGSADAQAAPLAEAAKADP
pSM40	PTDW46H with signal sequence of SCAB77391	MPNAPRRRDVLKYAGTTGVAAAGLGLLNSPSAAAAPAADAAF
pSM41	PTDW46H with signal sequence of	MSESQPATPASPSRRRFLQITAVGTTAAATGIAAAPTADALT

	SCAB77401	
pSM42	PTDW46H with signal sequence of SCAB77511	MSSDMTGLPSRRQVLGGTAAGVLATLAGIPHAGAASAAV
pSM43	PTDW46H with signal sequence of SCAB78741	MVLTTRRRALTTLGAALAGAVTLPATSASAGE
pSM44	PTDW46H with signal sequence of SCAB79571	MSEVTRRRFLAAGAAAGAAVVPGGWTAVARAGSAAPPEVRAAD
pSM45	PTDW46H with signal sequence of SCAB88501	MNGTTMSAHGRRRPTRRDVLLRTAAGGLVLGGGAAVCGPLLSTADAAG
pSM47	PTDW46H with signal sequence of SCAB90091	MDPGRTRRTLRRALTATATALALPLSMLATGATTARAAG
pSM49	PTDW46H with signal sequence of SCAB16721	MHPYDDNRHPTGATRTLGRRGLRGPVALLSALLLAGASLSLTAPSAGAAG
pSM50	PTDW46H with signal sequence of SCAB59651	MTLTPQPGPRSGSRRVARTAVAAGLVAALSAAGPIPMAFSAD
pSM51	PTDW46H with signal sequence of SCAB69691	MRRRARSILAAGALLLGGGAGLAPLAQAAE
pSM52	PTDW46H with signal sequence of SCAB13551	MYLRGLSDAKRRAWAATLTASVVTAGLLSGPVAHAGQAP
pSM53	PTDW46H with signal sequence of SCAB68841	MPLNRRKFLKKSAVTGAGVAIAGSAMAPSAQAAE
pSM55	PTDW46H with signal sequence of SCAB77971	MRHTRRTGRTHRPRARTHPLLRTGGFDTVRMRTLPAATALAVLFSSAALTVAPTASADS
pSM56	PTDW46H with signal sequence of SCAB44691	MNKTIRRSAVFCLLLVLALLVRATWVQFYEGTALADS
pSM58	PTDW46H with signal sequence of	MQRNRRLPAALAGAALLIAGVAGPAVSDEADR

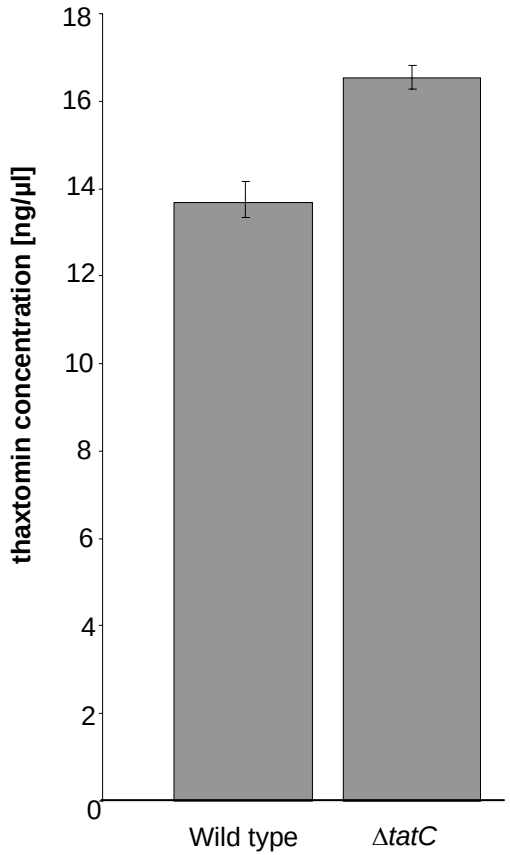
	SCAB07651	
pSM59	PTDW46H with signal sequence of SCAB16711	MSRTARLSSSLVRTAAVAGVAALTLTACGSGSGSTSTGSGSGSGSG
pSM62	PTDW46H with signal sequence of SCAB72441	MGTQESRQSQDSNEQGRGAGRRALLGAAMLGAGGAVLGLPGTATAAE
pSM63	PTDW46H with signal sequence of SCAB74351	MCTSHEPGQESARAHSGRRNFLRATALLGAAATVALPTATAQAAP
pSM65	PTDW46H with signal sequence of SCAB15571	MTLTPQPGPRSGSRRVARTAVAAGLVAALSAAGPIPMAFSAD
pSM67	PTDW46H with signal sequence of SCAB25591	MRTPQPSRRTVLAGTAAAAALTTFPAPTAQAHT
pSM68	PTDW46H with signal sequence of SCAB47131	MSERDPRNRTGRRPLLRGLAAAVLTAGLLVASTGQGHGSAAATAAPGAQ
pSM69	PTDW46H with signal sequence of SCAB59671	MPRIRSVAAAATHPDRRAFLAATGAVGLAAGAGFALRPETAAHAAT
pSM70	PTDW46H with signal sequence of SCAB63891	MMIMRTRRAALAALASTASLALTLTACGQN
pSM71	PTDW46H with signal sequence of SCAB75721	MTSLSEPSRRTVLTAAVTVAATAAAGASPARAVADTTSGRGAALARRAPAPE
pSM72	PTDW46H with signal sequence of SCAB81841	MAQDFSRRRLLQFSGIAAASVVLSAPRTLAGPGPVGMAAV
pSM73a	PTDW46H with signal sequence of SCAB15581	MSRRGLLRGAGLFGAAFAMGAAGSLTTPAAAGP
pSM73b	PTDW46H with signal sequence of SCAB15581	MSDPSGLSGTHEQQLPAWADPSVSPADLDAQGVSRRGLLRGAGLFGAAFAMGAAGSLTTPAAAGP
pSM74	PTDW46H with signal sequence of	MTPFYARRRRTTLAIATAVAAGALLTTGLTTGATAQP

	SCAB27931	
pSM75	PTDW46H with signal sequence of SCAB37611	MESGAPTMKLSVPAARRATLAAAVAVATLFTTGSIAGAAP
pSM76	PTDW46H with signal sequence of SCAB05261	MDLTRRQLLWAGGAIGAGVMLTACGGGDEKPTASATGDSAKPRKGGTLRVGALGRAGAIT
pSM77	PTDW46H with signal sequence of SCAB57661	MHLTPSGLSVPGPSRRTVLRGVGGAVALGAGVPLLSACGGSGSSAGDAKT
pSM78	PTDW46H with signal sequence of SCAB08951	MVGSFSRRRVLTTSAGAALGGLAAAGTARAAT
pTDW168	pTDW46H with signal sequence of SCAB63891	MMIMRTRRAALAALASTASLALTLTACGQNSEGGSDD
pTDW169	pTDW46H with signal sequence of SCAB38731	MRTVGTTGAHPRRRAMLGLGGLGVAGVLGSLGAGSAYAAAP
pTDW170	pTDW46H with signal sequence of SCAB8951	MGSFSRRRVLTTSAGAALGGLAAAGTARAATASAAGS
pTDW171	pTDW46H with signal sequence of SCAB16651	MSRISQPDPELTHRLSRRGMLGVAAGATVAALLGAAAPAQAAAG
pTDW172	pTDW46H with signal sequence of SCAB47131	MSERDPRNRTGRRPLLRGLAAAVLTAGLLVASTGQGHGSAAATAAPG
pTDW173	pTDW46H with signal sequence of SCAB8221	MPSSAGHHTLDRRVFLRNSLGVTAGLIAAPTVASLWQAPEAKAATA
pTDW174	pTDW46H with signal sequence of SCAB16711	MQRNRRLPAALAGAALLIAGVAGPAVSDEA
pTDW175	pTDW46H with signal sequence of SCAB18501	MPLARRALLAALAAGPAVACAPARAIPSDDT
pTDW176	pTDW46H with signal sequence of	MTPFYARRRRTTLAIATAVAAGALLTTGLTTGATAQPA

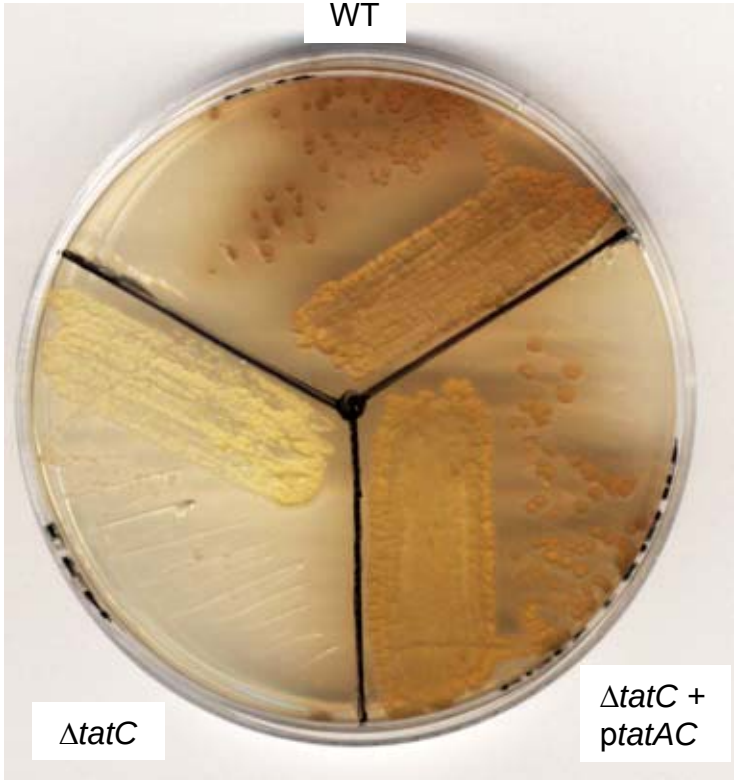
	SCAB27931	
pTDW177	pTDW46H with signal sequence of SCAB64081	MTSLLTSRRARGATICLAALLAGLCTPASASPASPSPSAASAAAP
pTDW178	pTDW46H with signal sequence of SCAB72441	MTTPTSRSGSGRRSIASLICASLAAGGLAAAGVTVLEPGAASASSH
pTDW181	pTDW46H with signal sequence of SCAB81041	MKDARIDRRLFLRGIGGVTAGVAAATTLSACGTGTSRTAGGSGG
pTDW182	pTDW46H with signal sequence of SCAB36371	MRTPRNPRRARLTPLLATLTALAAALIATAPTANAAHAADP
pTDW183	pTDW46H with signal sequence of SCAB17571	MKKRTAALCGAVAVLAGMVTAVPAGASGASAAAP
pTDW184	pTDW46H with signal sequence of SCAB78431	MRPTRRAAAFGSAGLLVTATLIAGAVAAPVASAADG
pTDW185	pSET-SOR-hyg with the *S. scabies tatAC* genes	Not applicable

Table S5. Plasmids constructed in this study

The amino acid sequence of the signal peptides fused to agarase for each of the given constructs is shown. For some signal peptides, DNA coding for two or more variants of the sequence, differing by choice of start codon, were cloned upstream of *dagA*. Two variants of the SCAB15581 signal peptide were tested, of which only the longer variant produced from plasmid pSM73b gave any measurable agarase activity. Two variants of the SCAB68191 signal peptide were tested, of which only the longer variant produced from plasmid pSM21a gave detectable agarase activity. Three possible versions of the SCAB63071 signal peptide were tested, from plasmids pSM35a, pSM36b and pSM35c, respectively. No agarase activity was detected from the longest version of the signal peptide (plasmid pSM35a). The signal peptide encoded in pSM35b gave the highest activity, whilst that from pSM35c (slightly shorter) gave lower activity. Two versions of the SCAB00601 signal peptide was tested, from which only the variant produced from plasmid pSM29b gave any activity.

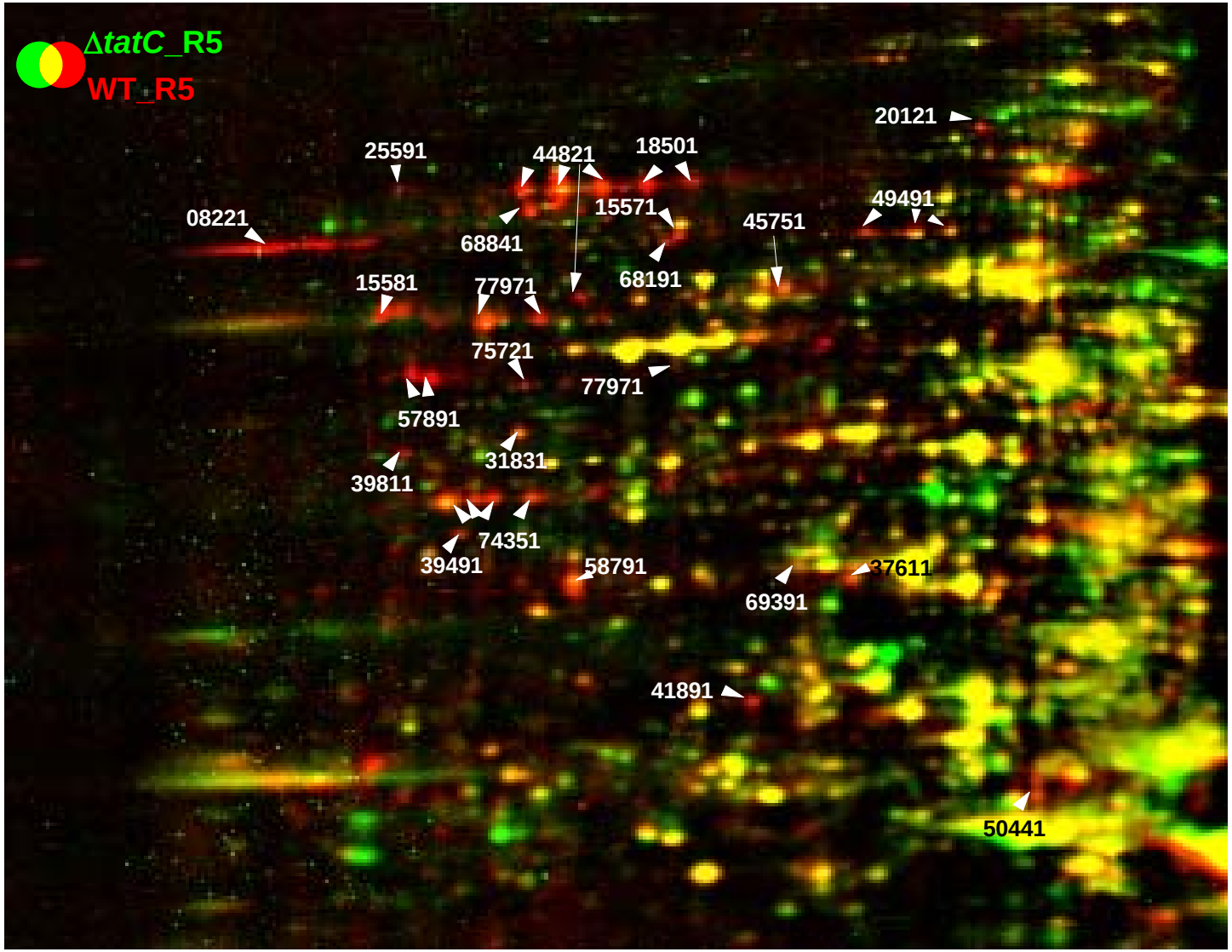


Joshi *et al*. Fig S1



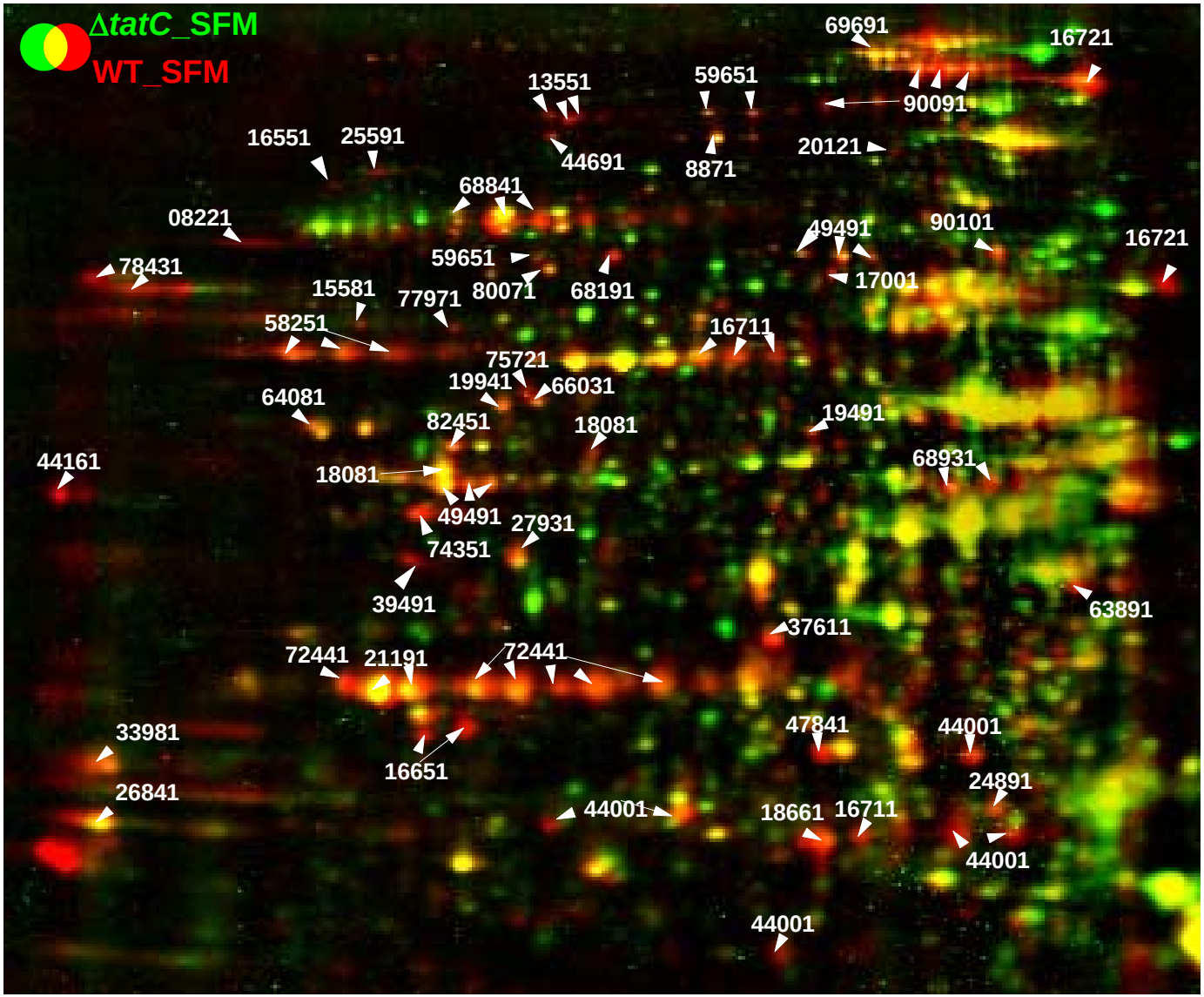
Joshi *et al*. Fig S2

A



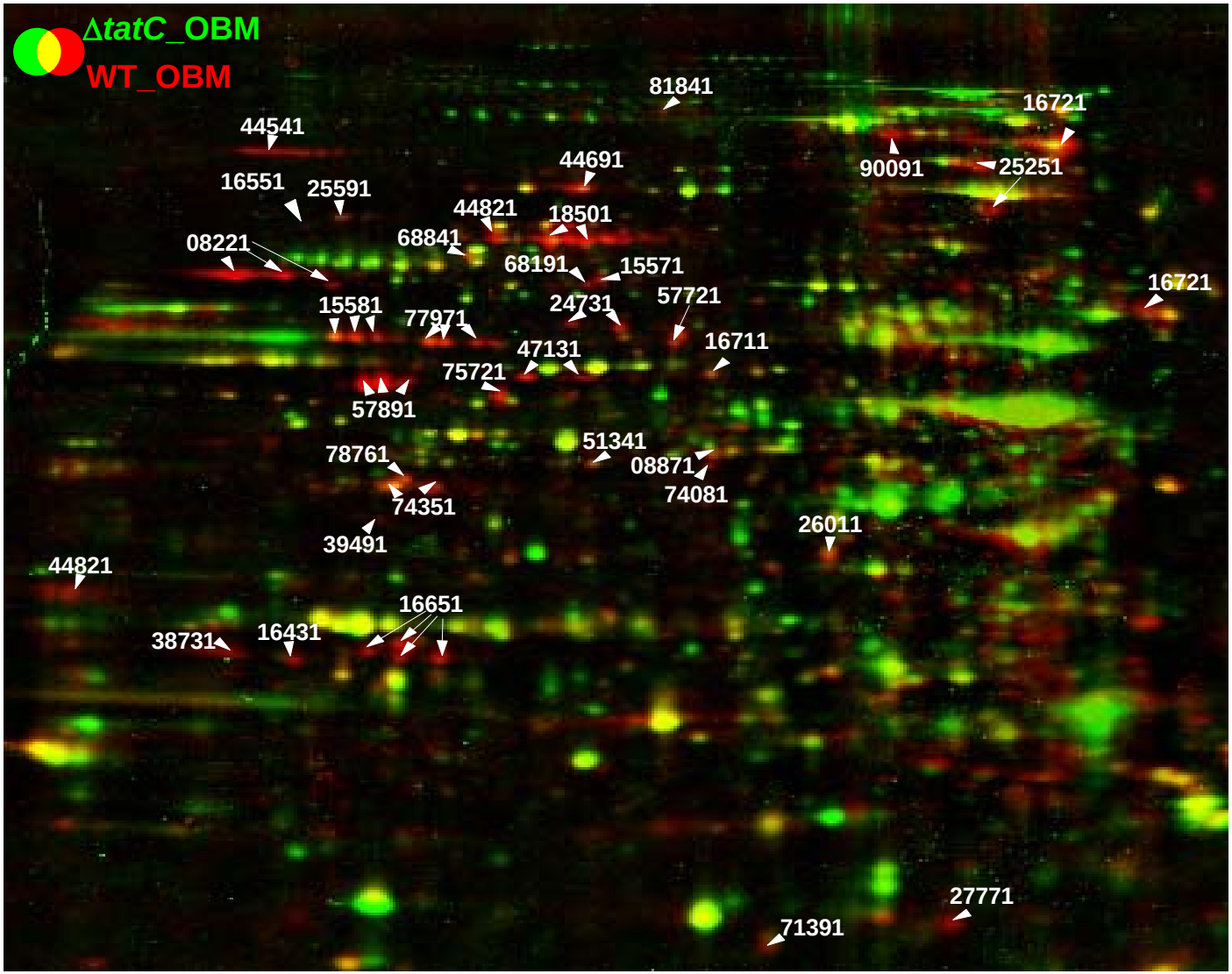
Joshi *et al*. Fig S3

B



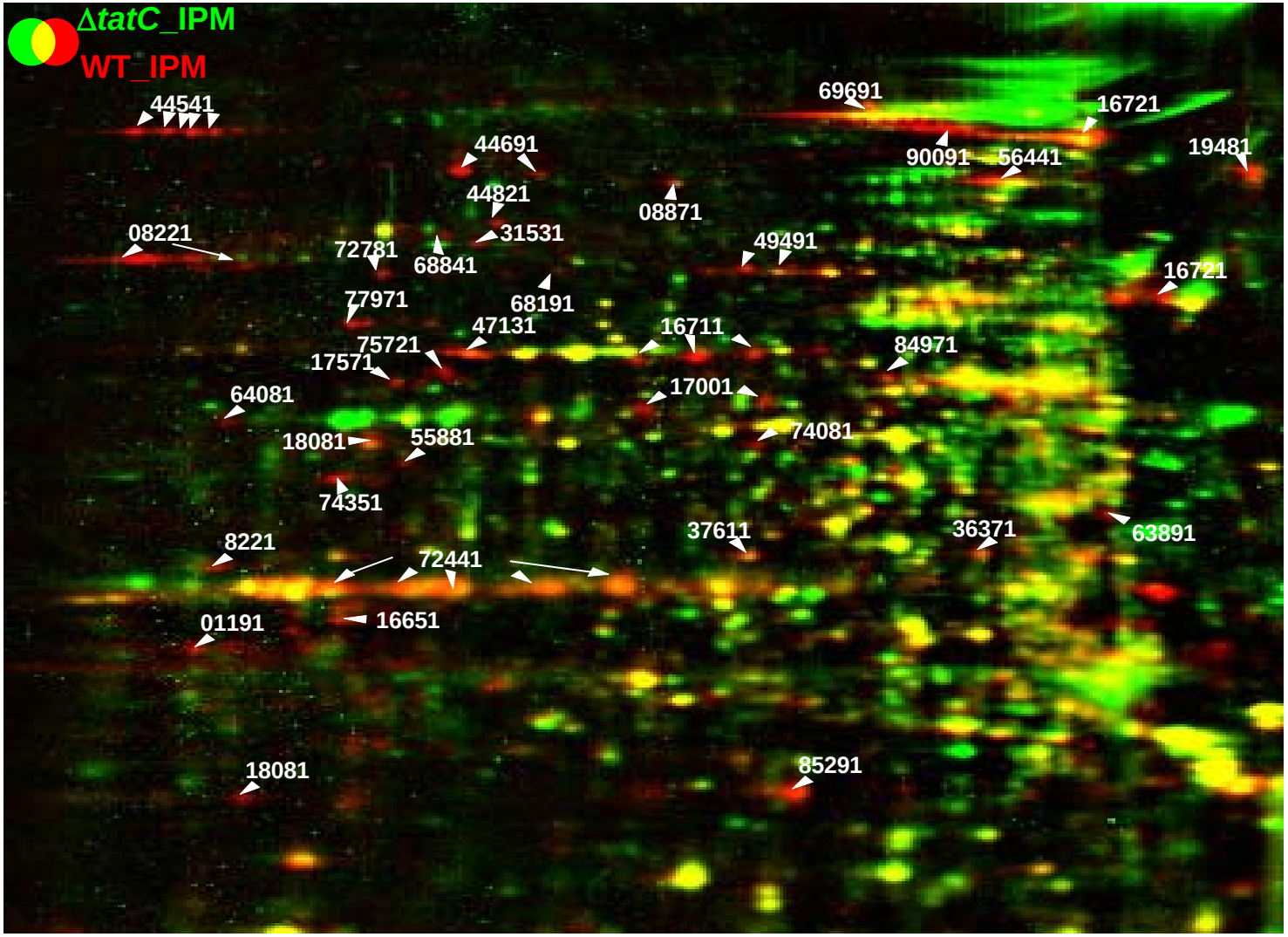
Joshi et al. Fig S3

C

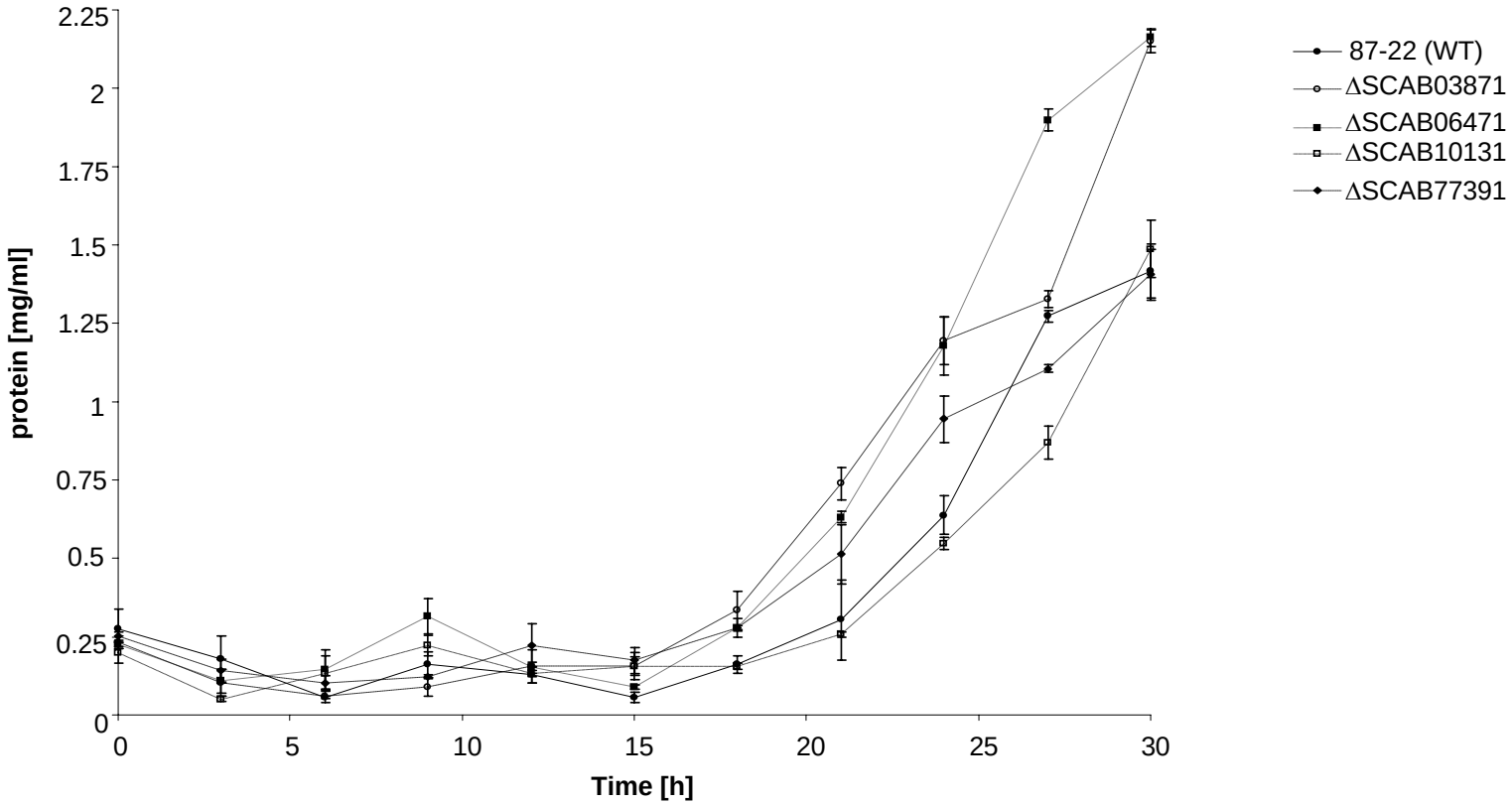


Joshi *et al*. Fig S3

D



Joshi *et al*. Fig S3



Joshi *et al*. Fig S4