BIOSYNTHESIS AND FUNCTIONAL ANALYSIS OF THE COR-LIKE METABOLITES PRODUCED BY THE COMMON SCAB PATHOGEN STREPTOMYCES SCABIES

by

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Abstract

Streptomyces scabies is a Gram-positive soil bacterium that causes common scab disease, which is identified by the round corky lesions that form on the surface of root and tuber crops such as potatoes. Virulence factors that contribute to the plant pathogenic phenotype of *S. scabies* include the phytotoxic secondary metabolite thaxtomin A and the secreted necrogenic protein Nec1. In addition, *S. scabies* produces a family of secondary metabolites called the COR-like metabolites, which are structurally similar to the COR (coronatine) phytotoxin produced by the bacterial plant pathogen *Pseudomonas syringae*. The goal of this thesis research was to characterize the biosynthesis and function of the *S. scabies* COR-like metabolites. In the first research chapter, the role of three genes, *scab79711, cfa8* and *scab79691,* in metabolite biosynthesis was elucidated by constructing gene deletion mutants in *S. scabies* and assessing the effect of each deletion on the production of the COR-like metabolites. In the second research chapter, the bioactivity of the COR-like metabolites was investigated by testing *S. scabies* culture supernatants or extracts containing the metabolites in various plant bioassays. In addition, the bioactivity of the primary COR-like metabolite, coronafacoyl-L-isoleucine, was tested alongside equimolar amounts of COR in order to compare the relative toxicity of the two metabolites. The results of this study provide important insight into the biosynthetic pathway responsible for COR-like metabolite production in *S. scabies* as well as the role of the metabolites in *S. scabies* plant pathogenicity. Future directions for this research were discussed.

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List of Symbols, Nomenclature and Abbreviations

ACP: Acyl carrier protein
amp^r: Ampicillin resistant
apra^r: Apramycin resistant
AT: Acyltransferase

BLAST: Basic Local Alignment Search Tool
bp: Base pair

cam^r: Chloramphenicol resistant
CCR: Crotonyl-CoA carboxylase/reductase
CFA: Coronafacic acid
CMA: Coronamic acid
CoA: Coenzyme A
COI1: Coronatine insensitive1
COR: Coronatine
CPC: 2-carboxy-2-cyclopentenone
CPE: 2-[1-oxo-2-cyclopenten-2-ylmethyl] butanoic acid
CYP450: Cytochrome P450

DH: Dehydratase
dNTP: Deoxyribonucleoside triphosphate
DMSO: Dimethylsulfoxide

ER: Enoylreductase

GFP: Green fluorescent protein

HPLC: High-performance liquid chromatography
hyg^r: Hygromycin B resistant

Ile: Isoleucine

JA: Jasmonic acid
JAZ: Jasmonate zim domain

kan^r: Kanamycin resistant
KR: Ketoreductase
KS: Ketosynthase

LB: Luria-Bertani medium
LC-MS: Liquid chromatography–mass spectrometry

Mb: Megabase

MEGA: Molecular Evolutionary Genetic Analysis
MeOH: Methanol

NA: Nutrient agar
NAD: Nicotinamide adenine dinucleotide
NADPH: Reduced nicotinamide adenine dinucleotide phosphate
NEB: New England Biolabs
NorVal: Norvaline
NRPS: Non-ribosome peptide synthetase
nt: Nucleotide

OBA: Oat bran agar
oriT: Origin of transfer from RK2
Oxr: Oxidoreductase

PAMP: Pathogen-associated molecular pattern
PAI: Pathogenicity island
PAS: Period circadian, aryl hydrocarbon receptor nuclear translocator and single-minded protein
PCR: Polymerase chain reaction
Pfam: protein families
PKS: Polyketide synthase
PTFE: Polytetrafluoroethylene
pv: Pathovar

rpm: Revolutions per minute
RT-PCR: Reverse transcription polymerase chain reaction

SA: Salicylic acid
SCF: Skp1-Cullin1-F-box
Sdr: Short chain dehydrogenase/reductase
SDS: Sodium dodecyl sulfate
SFMA: Soy flour mannitol agar
SFMB: Soy flour mannitol broth
SOB: Super optimal broth
SOC: Super optimal broth with catabolite repression

TBE: Tris-Borate-EDTA
tet^r: Tetracycline resistant
$thio^r$: Thiostrepton resistant
TSB: Trypticase Soy Broth

CHAPTER 1: Introduction and Overview

1.1 General features of *Streptomyces*

Streptomyces spp. are Gram-positive filamentous Actinobacteria that are abundant in heterogeneous terrestrial soil environments and can also be found in aquatic marine environments (Garrity et al. 2007; Ward and Bora 2006). Members of this genus are primarily saprophytic and can decompose complex organic compounds like starch, lignocellulose and chitin in soil, and as such they play a critical role in carbon recycling in the environment (Strap and Crawford 2006). *Streptomyces* spp. have a single, large linear chromosome that is generally 8 – 10 Mb (Megabase) in size and has a high G+C content (≥ 70%). Many species also harbour large linear and/or circular plasmids (Ventura et al. 2007). The genomes of several *Streptomyces* spp. have been sequenced (http://strepdb.streptomyces.org.uk), and among the features shared by the different genomes are the abundance of genes involved in regulation, secretion, morphological differentiation and secondary metabolism (Zhou et al. 2012). Furthermore, *Streptomyces* genomes are rich in gene duplication and lateral gene transfer events, which most likely have contributed to genomic diversification within the genus (Zhou et al. 2012). It is thought that the flexible genetic strategy of the *Streptomyces* has allowed for a more complex life cycle and for the ability to adapt to complex and variable soil environments (Bentley et al. 2002; Chen et al. 2002; Hopwood and Kieser 1993).

1.2 The *Streptomyces* life cycle

A distinguishing characteristic of the *Streptomyces* is the ability to undergo morphological differentiation as part of their life cycle. At the beginning of the life cycle (Fig. 1.1), germination of a single spore takes place under favorable environmental conditions, and the resulting filamentous cells grow by apical extension and branching to form a network of hyphae called the substrate mycelium (Elliot et al. 2008; McCormick and Flärdh 2012). When facing nutrient limitation or environmental stress, the organism begins to form structures called aerial hyphae that grow away from the colony surface, and this process is fueled by nutrients released from the autolysis of the substrate mycelium (Elliot et al. 2008). Intriguingly, the formation of aerial hyphae coincides with the production of secondary metabolites such as antibiotics, which may protect the lysing colony from invading foreign microorganisms (McCormick and Flärdh 2012). The developmental process then continues with the septation of the aerial hyphae and the formation of chains of unigenomic spores (Chater 1993), which accumulate a gray polyketide pigment that turns the aerial mycelium from white to gray (Davis and Chater 1992; Kelemen et al. 1998). The mature spores are resistant to environmental stresses such as desiccation conditions, and also are responsible for the dispersal of the non-mobile *Streptomyces* bacteria (McCormick and Flärdh 2012).

1.3 Secondary metabolism in the genus *Streptomyces*

The ability to produce a great number of secondary metabolites is the best known feature of the *Streptomyces.* Secondary metabolites are chemically diverse compounds that are usually small (MW<3000Da) and exhibit a wide range of biological activities (Berdy 2005). In contrast to primary metabolism, which is indispensable for microbial

growth, secondary metabolism is thought to have evolved to provide a selective advantage to the producing organism (Berdy 2005; O'Brien and Wright 2011). The roles proposed for *Streptomyces* secondary metabolites in nature include warfare agents for competing with other microorganisms in nutrient-poor environments, signalling molecules for intra- and inter-generic communication with other microorganisms, and regulators of symbiotic relationships between *Streptomyces* spp. and eukaryotic hosts such as plants and animals (Berdy 2005; O'Brien and Wright 2011).

To date *Streptomyces* spp. produce ~8000 bioactive secondary metabolites that have been widely used in human and/or veterinary medicine as anti-bacterial, anti-fungal, anti-parasitic, anti-viral, anti-tumor, and immuno-suppressive compounds, and also in agriculture as herbicides, insecticides, and biofertilizers for promoting plant growth (Berdy 2005; Korn-Wendisch et al. 1992; Sadeghi et al. 2012). *Streptomyces* spp. use multimodular enzymatic assembly lines to generate important families of secondary metabolites including polyketides, nonribosomal peptides and hybrid PKS/NRPS (Polyketide synthase/Non-ribosome peptide synthetase)-derived compounds (Walsh 2004; Wenzel and Müller 2005). Remarkably, the genes encoding secondary metabolite systhesis are mostly located at the unstable terminal region of the chromosome (Pang et al. 2004), where abundant transposable elements reside (Chen et al. 2002; Leblond et al. 1996). This dynamic feature may be related to the spread of antibiotic resistance among microbes since resistance genes are usually found clustered together with the corresponding secondary metabolite biosynthetic genes (Chen et al. 2002; Mazel and Davies 1999).

1.4 Plant pathogenicity in the genus *Streptomyces*

Over 580 species of *Streptomyces* have been identified so far (Garrity et al. 2007), of which only a very small number have the ability to infect living plant tissue and cause plant diseases (Bignell et al. 2010a). Three of the best studied plant-pathogenic species are *Streptomyces scabies, Streptomyces acidiscabies* and *Streptomyces turgidiscabies* (Loria et al. 2006), which cause scab disease of potato (Fig. 1.2). The main symptom associated with this disease is the formation of round-shaped corky-like lesions on the tuber surface. The lesions can be superficial, erumpent (raised) or they can extend deep into the tuber tissue (Dees and Wanner 2012). The oldest and most widely distributed pathogen, *S. scabies,* is ubiquitous in well-drained soils where root and tuber crops are typically grown, and it exhibits optimum growth at 30°C and a pH of 5.2 - 7, conditions that are associated with increased scab severity in the field (Loria et al. 1997). Studies have shown that *S. scabies* primarily penetrates the potato tuber at the immature lenticels, and rapid expansion of the tuber is required for pathogen infection and lesion expansion (Loria et al. 1997, 2006, 2008). As *S. scabies* is neither tissue nor host specific, it can also cause scab disease symptoms on other economically important root crops such as radish, carrot, beet and turnip (Dees and Wanner 2012). It has been also reported that *S. scabies* causes "pod wart" on peanuts in South Africa (De Klerk et al. 1997). Even seedlings of model plants such as *Arabidopsis thaliana* and *Nicotiana tabacum* (tobacco) can be infected by *S. scabies*, though such infections result in root stunting, swelling, necrosis and seedling death rather than scab lesion formation (Loria et al. 2006). Potato scab disease is the most important disease caused by *S. scabies* and is a worldwide problem. In Canada, the disease was estimated to cause losses of $15.3-17.3 million dollars to potato

growers in 2002 (Hill and Lazarovits 2005). In the USA, potato scab has been rated among the top five diseases affecting production of seed potatoes (Slack 1991), and in Tasmania, Australia, the disease has been reported to cause losses of up to 4% of the total industry value (Wilson 2004). The scab lesions affect the quality and market value of potato crops, and there is also evidence that infection by scab-causing pathogens can decrease the total crop yield and increase the proportion of smaller tubers in the yield (Hiltunen et al. 2005).

1.5 Control strategies for potato scab disease

Traditional ways to manage scab disease include soil irrigation during tuber growth since high moisture levels have been shown to decrease the severity of disease symptoms (Lapwood and Hering 1970). However, this strategy often fails (Dees and Wanner 2012), most likely because high soil moisture levels need to be maintained for extended periods of time, and this is impractical for many growers (Loria et al. 1997). Furthermore, maintaining high soil moisture levels can also promote the development of other undesired potato diseases (Loria et al. 1997). Reduction of soil pH (< 5.2) is another strategy that has been commonly used since *S. scabies* does not grow well under acidic conditions. This strategy also has limited success since low pH soils are unfavorable for the growth of many crops (Loria et al. 1997), and emerging pathogenic species such as *S. acidiscabies* and *S. turgidiscabies* are able to tolerate lower pH conditions than *S. scabies* (Lambert and Loria 1989a; Lindholm et al. 1997). Other chemical control methods such as soil fumigation and foliar sprays are costly and are not environmentally friendly, and

they can affect tuber size and weight (Dees and Wanner 2012). Crop rotation generally produces inconsistent results, most likely because plant pathogenic *Streptomyces* spp. are able to survive in soils as saprophytes, and they can also infect many different types of crops (Loria et al. 2006). Biological control of scab disease is considered a promising alternative to the traditional methods. Microorganisms such as non-pathogenic *Streptomyces* spp., *Pseudomonas* spp., *Bacillus* spp. and different fungal species have been reported to inhibit pathogenic *Streptomyces* spp. under controlled conditions (Beauséjour et al. 2003; Liu et al. 1995; Lorang et al. 1995; St-Onge et al. 2011; Tagawa et al. 2010). Also, the use of bacteriophages as biocontrol agents for scab disease has been studied (Goyer 2005; McKenna et al. 2001). However, more research needs to be done to determine the effectiveness of biological control in the field (Dees and Wanner 2012). The use of disease-resistant potato cultivars is considered the most desirable and reliable strategy for controlling scab disease; however, the genetic and physiological mechanisms of resistance and susceptibility are poorly understood, and true scab-resistant varieties of potato have yet been found (Dees and Wanner 2012). Overall, the lack of understanding of both the pathogen and host resistance mechanisms has hindered the development of effective control strategies for scab disease.

1.6 Virulence factors produced by scab-causing *Streptomyces* species

The successful infection of a plant host is an intricate process that requires the pathogen to detect the presence of a susceptible host, to penetrate and grow within the host tissues, and to avoid the host defense mechanisms (Chisholm et al. 2006). Plant pathogenic *Streptomyces* species are distinguished from their non-pathogenic relatives in

the ability to produce virulence determinants that participate in one or more steps in the infection process. Modern genetic tools have provided the opportunity to further characterize a number of known or predicted virulence factors in order to better understand their role in *Streptomyces* plant pathogenicity.

The primary virulence factor produced by *S. scabies* and other scab-causing pathogens is a family of phytotoxic secondary metabolites called the thaxtomins, of which thaxtomin A is the predominant member produced by these organisms (King and Calhoun 2009). Thaxtomin A is a nitrated 5, 2-diketopiperazine non-ribosomal peptide synthesized from L-phenylalanine and 4-nitro-L-tryptophan (Healy et al. 2000; Johnson et al. 2009; King and Calhoun 2009). Thaxtomin A primarily functions as a cellulose biosynthesis inhibitor. In *A. thaliana,* thaxtomin A has been shown to affect the expression of cell wall synthesis genes, it reduces the number of cellulose synthase complexes in the plant cell plasma membrane, and it causes ectopic lignification (Bischoff et al. 2009). Other physiological effects of thaxtomin A have been reported. For example in *Arabidopsis*, the influx of Ca^{2+} and efflux of H^{+} ions has been shown to be induced by thaxtomin A, thus eliciting an early defence response (Tegg et al. 2005; Errakhi et al. 2008; Bischoff et al. 2009).

Another virulence determinant that has been described is the Nec1 protein, which is produced by many, though not all scab-causing *Streptomyces* species (Bukhalid et al. 1998; Wanner 2006, 2009). Nec1 is a secreted protein that causes necrosis of potato tuber tissue (Loria and Bukhalid 1997) and is required for the colonization of radish seedling roots (Joshi et al. 2007). However unlike thaxtomin A, it is not essential for the pathogenic phenotype of *Streptomyces* spp. The *nec1* gene has a much lower GC content

(54%) than the average GC content of a *Streptomyces* genome, which suggests that it was acquired by horizontal gene transfer from another organism (Loria and Bukhalid 1997). In *S. turgidiscabies, nec1,* along with the thaxtomin biosynthetic genes, is present on a large mobilizable PAI (Pathogenicity island), and the transfer of this island is believed to facilitate the spread of plant pathogenicity among *Streptomyces* spp. in the environment (Kers et al. 2005; Bukhalid et al. 1998). Since there are no close homologues of Nec1 in database, and no characterized motifs are present in the protein sequence, the function of Nec1 remains elusive (Joshi et al. 2007).

The *S. scabies* genome encodes other putative virulence factors that may contribute to the plant pathogenic phenotype of this organism (Bignell et al. 2010a). For example, the *tomA* gene encodes a tomatinase enzyme that hydrolyzes α-tomatine, a phytoanticipin is an antimicrobial compound produced by tomato plants (Seikpe and Loria 2008). *tomA* is conserved in *S. scabies, S. turgidiscabies* and *S. acidiscabies* and is located together with *nec1* and the thaxtomin biosynthetic genes on the PAI in *S. turgidiscabies* (Kers et al. 2005; Wanner 2006, 2009). Although a *tomA* deletion mutant of *S. scabies* was not affected in virulence, it is possible that *tomA* contributes to the ability of *S. scabies* to suppress plant defense responses during infection as reported for other tomatinase – producing plant pathogens (Bouarb et al. 2002; Ito et al. 2004).

1.7 The *S. scabies* COR-like metabolites

The focus of this thesis is a new virulence-associated locus that was discovered in the genome sequence of *S. scabies* 87-22 and is called CFA (Coronafacic acid)-like

biosynthetic gene cluster (Bignell et al. 2010b). The CFA-like biosynthetic gene cluster is composed of at least 15 genes, of which nine are homologous to genes from the CFA biosynthetic gene cluster found in the Gram-negative plant pathogenic bacterium *Pseudomonas syringae* (Fig. 1.3). In *P. syringae,* CFA (Fig. 1.4A) is a polyketide secondary metabolite that is linked to CMA (Coronamic acid) to form COR (Fig. 1.4B), which is a nonhost-specific phytotoxin that contributes to the plant pathogenic phenotype of the organism (Bender et al. 1999a, b). Although COR is the predominant coronafacoyl compound produced by *P. syringae,* other minor COR-like metabolites can also be made in which CFA is linked to amino acids such as L-Ile (Isoleucine) (Fig. 1.4c), L-*allo*-Ile (Fig. 1.4d), L-Val (Fig. 1.4e) and L-norVal (Norvaline) (Fig. 1.4f) (Bender et al. 1999a,b). It has been predicted that *S. scabies* also produces COR-like metabolites since the CFA-like biosynthetic gene cluster contains all of the genes needed for CFA production whereas the CMA biosynthetic genes are absent (Bignell et al. 2010a, b, 2014). Recent studies conducted in the Bignell laboratory have confirmed that at least three different COR-like metabolites are produced by *S. scabies,* the predominant of which is CFA-L-Ile (Fyans et al. 2014). Promoter reporter studies using GFP (Green fluorescent protein) have shown that the *S. scabies* CFA-like biosynthetic cluster is expressed when the pathogen is colonizing the seedling roots of both *N. tabacum* and *A. thaliana* (Bignell et al. 2010a, b), and deletion of *cfa6* from the gene cluster provided further evidence that the COR-like metabolites contribute to seedling root symptom development in *N. tabacum* (Bignell et al. 2010a, b). It is predicted that the metabolites may also be important for potato scab disease development; however, as other scab-causing *Streptomyces* spp. do not appear to produce the metabolites (Bignell et al. 2010b), it is likely that they are not required for the

disease to occur.

1.8 Biosynthesis of COR and COR- like molecules in *P. syringae*

The predicted COR biosynthetic pathway in *P. syringae* is demonstrated in Figure 1.5. The biosynthesis of CFA is thought to begin with the decarboxylation of α-ketoglutarate followed by the formation of succinic semialdehylde-CoA (Coenzyme A). This may involve either of the ligase-encoding genes *cfl* or *cfa5* found within the CFA biosynthetic gene cluster, or it may involve other genes located outside of the cluster (Rangaswamy et al. 1998a, b). Succinic semialdehylde-CoA then may serve as the starter unit for type II polyketide synthesis involving Cfa1 (ACP: acyl carrier protein), Cfa3 (KS) and Cfa2 (DH: dehydratase). Malonyl-CoA is predicted to serve as the extender unit that is linked to the –SH group of Cfa1, and chain elongation by Cfa3 may be followed by ring formation by Cfa4, a predicted cyclase, to produce the enzyme-bound intermediate 2-carboxy-3-hydroxycyclopentanone (Rangaswamy et al. 1998b). Cfa2 may then catalyze the dehydration of 2-carboxy-3-hydroxycyclopentanone to produce enzyme-bound CPC (2-carboxy-2-cyclopentenone), which may in turn serve as a starter unit for type I polyketide synthesis by the modular PKS encoded by *cfa6* and *cfa7*. The CoA ester of CPC is predicted to be loaded onto Cfa6, which possesses a loading module AT0 (Acyltransferase)-ACP0 and an extension module KS1 (Ketosynthase)-AT1-DH1-ER1 (Enoylreductase)-KR1 (Ketoreductase)-ACP1. The Cfa6 extension module would allow for CPC to be extended by a butyrate unit followed by complete reduction of the β-keto ester to give enzyme-bound CPE (2-[1-oxo-2-cyclopenten-2-ylmethyl] butanoic acid)

(Fig. 1.5). Then, CPE is predicted to be directly transferred to Cfa7, which possesses the second extension module (KS2-AT2-DH2-KR2-ACP2) that would allow for extension of CPE by malonate followed by reduction and dehydration of the β-keto ester to give CFA. Cfa7 also possesses a TE domain that presumably allows for release of CFA from the PKS. Finally, the *cfl* gene encodes the coronafacate ligase that is predicted to link the free CFA to CMA via amide bond formation to produce COR (Fig. 1.5; Bender et al. 1993; Liyanage et al. 1995). CMA is an ethylcyclopropyl amino acid derived from L-Ile, and the genes involved in its biosynthesis (*cmaABCDELT*) form a cluster that is separate from the CFA biosynthetic gene cluster (Brooks et al. 2005; Mitchell et al. 1994; Rangaswamy et al. 1998). Though CMA is the preferred substrate for ligation to CFA, the Cfl enzyme is believed to be able to utilize other amino acid substrates in order to form the minor COR-like molecules CFA-L-Ile, CFA-L-*allo*-Ile, CFA-L-Val, CFA-L-norVal (Fig. 1.4), CFA-L-Ser and CFA-L-Thr (Mitchell et al. 1986; Mitchell and Ford 1998; Mitchell and Frey 1986; Mitchell and Young 1985).

It has previously been noted that the production of COR in *P. syringae* is regulated by temperature since maximum metabolite production occurred when the organism was cultured at 18°C, while very little production occurred at 28-30°C (Palmer and Bender 1993; Ullrich et al. 1995). A chromosomal locus controlling CFA and CMA production has been identified and consists of three genes, designated *corP, corS* and *corR,* encoding a modified two-component regulatory system. CorP and CorR show significant similarity to response regulatory proteins, and CorS is related to sensor histidine protein kinases (Ullrich et al. 1995). CorR contains a helix-turn-helix DNA

binding domain and has been shown to function as a positive activator of *cfa* and *cma* gene expression by binding to promoter regions within the CFA and CMA biosynthetic gene clusters (Penaloza-Vazquez and Bender 1998; Sreedharan et al. 2006; Wang et al. 1999). The DNA binding activity of CorR is regulated by CorS, which has been shown to phosphorylate CorR *in vitro* (Rangaswamy and Bender 2000). Although CorP does not harbour any typical DNA binding motifs, it does contain a highly conserved phosphate receiving domain (Ullrich et al. 1995), and it has been proposed that CorP may function to modulate CorR and/or CorS activity (Smirnova et al. 2002). Furthermore, all three regulatory proteins are believed to be responsible for the thermoregulation of COR (Ullrich et al. 1995).

1.9 Biosynthesis of the COR- like metabolites in *S. scabies*

In *S. scabies,* the CFA-like biosynthetic gene cluster contains homologues of the *cfa1-7* genes (Fig. 1.3), which as discussed in section 1.8, are thought to be involved in synthesis of the CFA backbone in *P. syringae*. In addition, a homologue of the *cfl* gene, which in *P. syringae* encodes the coronafacate ligase enzyme required for ligation of CFA to CMA or other amino acids, is also present in the CFA-like gene cluster (Fig. 1.3). Interestingly, the Cfa7 extension module in *S. scabies* has been predicted to contain an ER domain that is absent from the Cfa7 homologue in *P. syringae* (Bignell et al. 2010b). This domain was predicted to be active based on the presence of the conserved NADPH (Reduced Nicotinamide Adenine Dinucleotide Phosphate) binding motif [LXHX(G/A)XGGVG] that is characteristic of ER domains (Donadio and Katz 1992), and it was hypothesized that the C=C double bond that is present in the CFA backbone in

P. syringae would be reduced in the metabolite produced by *S. scabies* (Bignell et al. 2010b). In addition, the *S. scabies* CFA-like biosynthetic gene cluster contains six genes that have no homologues in the *P. syringae* CFA biosynthetic gene cluster (Fig. 1.3), and four of these genes are predicted to encode enzymes that may play a role in metabolite biosynthesis. Together, these observations led to the proposal that *S. scabies* may produce novel COR-like metabolites (Bignell et al. 2010b). However, as mentioned in section 1.7, it is now known that the primary *S. scabies* COR-like metabolite is CFA-L-Ile (Fyans et al. 2014), a metabolite that is also produced by *P. syringae* in minor amounts.

Recent research in the Bignell laboratory has demonstrated that at least two of the novel genes in the CFA-like gene cluster are involved COR-like metabolite biosynthesis in *S. scabies*. Deletion of *scab79681* (*oxr:* encoding oxidoreductase) and *scab79721* (*sdr:* encoding short chain dehydrogenase/reductase), which encode a predicted oxidoreductase and a short chain dehydrogenase, respectively, resulted in a significant decrease in production of CFA-L-Ile. Production in each mutant was restored by genetic complementation with the corresponding gene (Altowairish 2014). Based on these results, a hypothetical biosynthetic pathway for CFA-L-Ile biosynthesis was proposed in which production of CFA in *S. scabies* requires not only the *cfa1-7* genes, but also the *oxr* and *sdr* genes (Altowairish 2014). In addition, it was proposed that CFA biosynthesis might also involve the *scab79691* gene, which encodes a predicted CYP450 (Cytochrome P450) monooxygenase (Altowairish 2014; Bignell et al. 2010b) and is the focus of Chapter 2 in this thesis. *scab79711* is another gene that is present in the *S. scabies* CFA-like gene cluster but not in the *P. syringae* CFA gene cluster (Fig. 1.3). Preliminary bioinformatics analysis of the gene product suggested that it may work together with the *cfa8* gene

product to produce the ethylmalonyl-CoA extender unit that is required for CFA biosynthesis (Bignell et al. 2010b). Gene *cfa8* is conserved in both *S. scabies* and *P. syringae* and encodes a predicted CCR (Crotonyl-CoA carboxylase/reductase) enzyme. A more thorough discussion of *scab79711* and *cfa8* is provided in Chapter 2.

Gene *scab79591/cfaR* (Fig. 1.3) encodes a member of the PAS (Period circadian, aryl hydrocarbon receptor nuclear translocator and single-minded protein) - LuxR family of transcriptional regulators that are found only in actinomycetes (Bignell et al. 2014a). The C-terminal LuxR domain is thought to function as a DNA binding domain for transcription activation, while the N-terminal PAS motif may control the DNA binding activity of the protein in response to environmental stimuli (Hefti 2004; Taylor and Zhulin 1999; Subramoni 2009). RT-PCR (Reverse transcription polymerase chain reaction) analysis of *scab79591/cfaR* deletion and overexpression strains indicated that the expression of the CFA-like biosynthetic genes is positively activated by Scab79591/CfaR (Bignell et al. 2010b). Furthermore, *scab79591/cfaR* was shown to be co-transcribed with *scab79581,* which encodes a ThiF-family protein of unknown function (Bignell et al. 2010b).

1.10 Biological activities of COR and COR-like molecules

COR has been shown to function as an important virulence determinant in different pathovars (pv) of *P. syringae* (Bender et al. 1999a, b). It allows the pathogen to penetrate and colonize the plant host, it facilitates the suppression of plant defense responses, and it contributes to disease symptom development (Xin and He 2013). As discussed in Chapter 3, the primary symptom induced by COR is leaf chlorosis on diverse

species of plants, although other biological effects have also been attributed to this phytotoxin (Durbin 1991; Ferguson and Mitchell 1985; Kenyon and Tuner, 1992; Bent et al. 1992; Zare et al. 2013; Lee et al. 2013). It has been shown that COR can function as a molecular mimic of the L-Ile conjugate of the plant defense and wound response signaling molecule JA (Jasmonic acid) (Katsir et al. 2008a, b; Melotto et al. 2008), and as such it plays an important role in allowing *P. syringae* to overcome plant defense responses during host colonization (Feys et al. 1994; Thilmony et al. 2006; Uppalapati et al. 2005; Zhao et al. 2003). There is the evidence that COR-like metabolites such as CFA-L-Val exhibit similar biological activities as COR, though they are not as toxic in their activity (Bender et al. 1999a; Mitchell 1991; Uppalapati et al. 2005). Work from the Bignell lab has shown that the *S. scabies* COR-like metabolites are able to induce potato tissue hypertrophy in a similar manner as COR (Altowairish 2014; Fyans et al. 2014). However, a thorough examination of other potential biological activities of the *S. scabies* COR-like metabolites, and in particular CFA-L-Ile, has not been performed.

1.11 Thesis objectives

This study has two main objectives and is divided into two separate chapters. The first objective was to characterize the role of three genes, *scab79711, cfa8* and *scab79691* (Fig. 1.3), in the biosynthesis of the CFA-L-Ile COR-like metabolite produced by *S. scabies*. As discussed in Chapter 2, deletion mutants were constructed for each gene in *S. scabies*, and the effect of each mutation on CFA-L-Ile production was assessed using HPLC (High-performance liquid chromatography) and bioassays. The second objective of this study was to characterize the biological activities of the *S. scabies* COR-like

metabolites using different plant hosts. As described in Chapter 3, culture supernatants and extracts from COR-like metabolite producing and nonproducing strains of *S. scabies* were used in different plant bioassays in order to determine whether the metabolites exhibit the same biological activities described for COR. Furthermore, the relative toxicity of pure COR and CFA-L-Ile were compared in two different bioassays. Together, these chapters provide important insights into the biosynthesis and function of the virulence-associated COR-like metabolites, which are produced by the most important and widely-distributed scab-causing pathogen.

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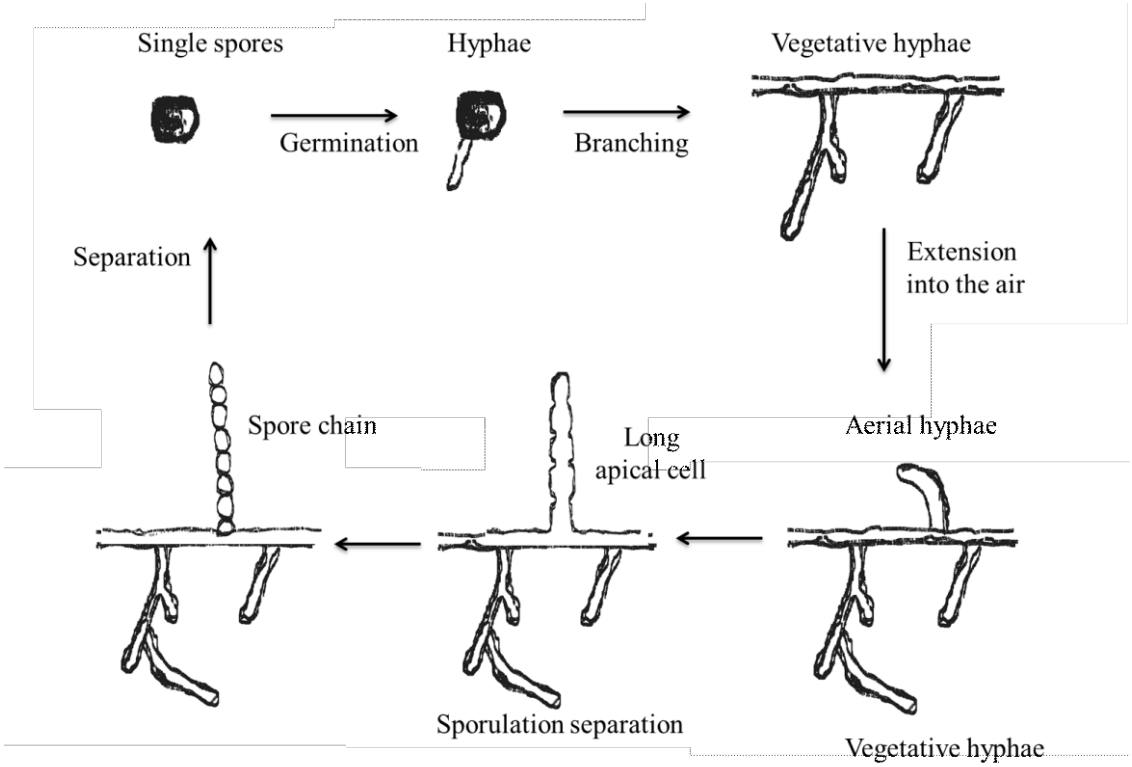


Figure 1.1: The life cycle of *Streptomyces* spp. Adapted from Elliot et al. (2008) and Flärdh and Buttner (2009).

Figure 1.2: Potato tuber showing the characteristic erumpent (raised) lesions that form as a result of infection by scab-causing *Streptomyces* spp. Image courtesy of J. Fyans.

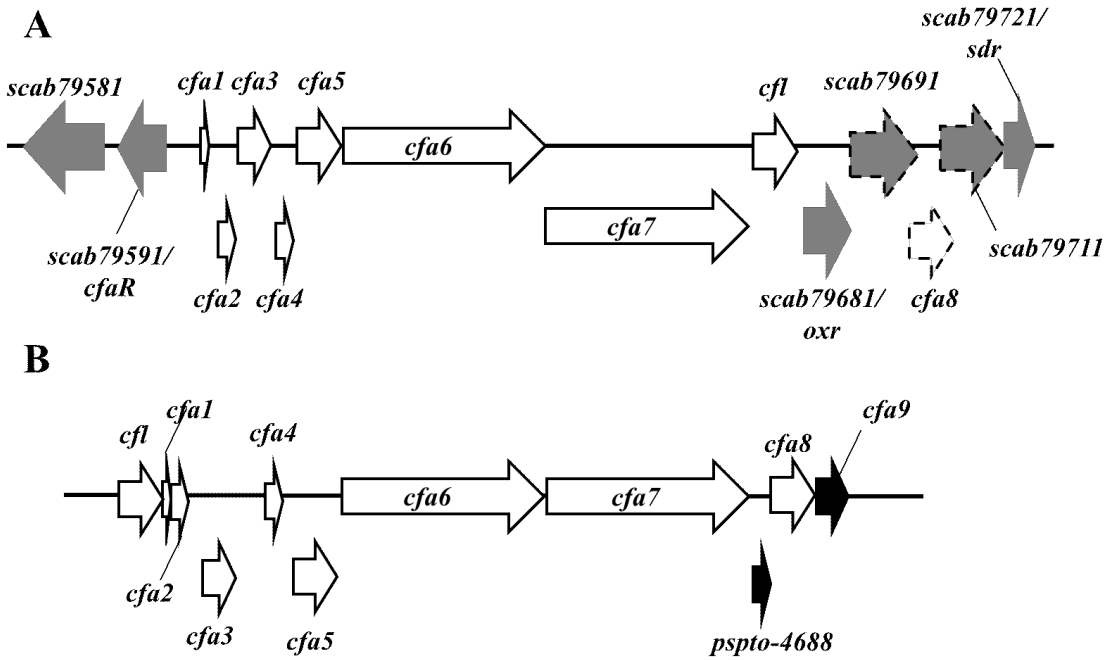


Figure 1.3: Genetic organization of the CFA-like biosynthetic gene cluster from *S. scabies* and the CFA biosynthetic gene cluster from *Pseudomonas syringae.* White arrows indicate genes encoding homologous proteins in *S. scabies* 87-22 (A) and *P. syringae* pv *tomato* DC3000 (B). Gray arrows indicate genes that are unique to the *S. scabies* cluster, and black arrows indicate genes that are unique to the *P. syringae* cluster. The *S. scabies* genes that are the focus of Chapter 2 are outlined with dash lines.

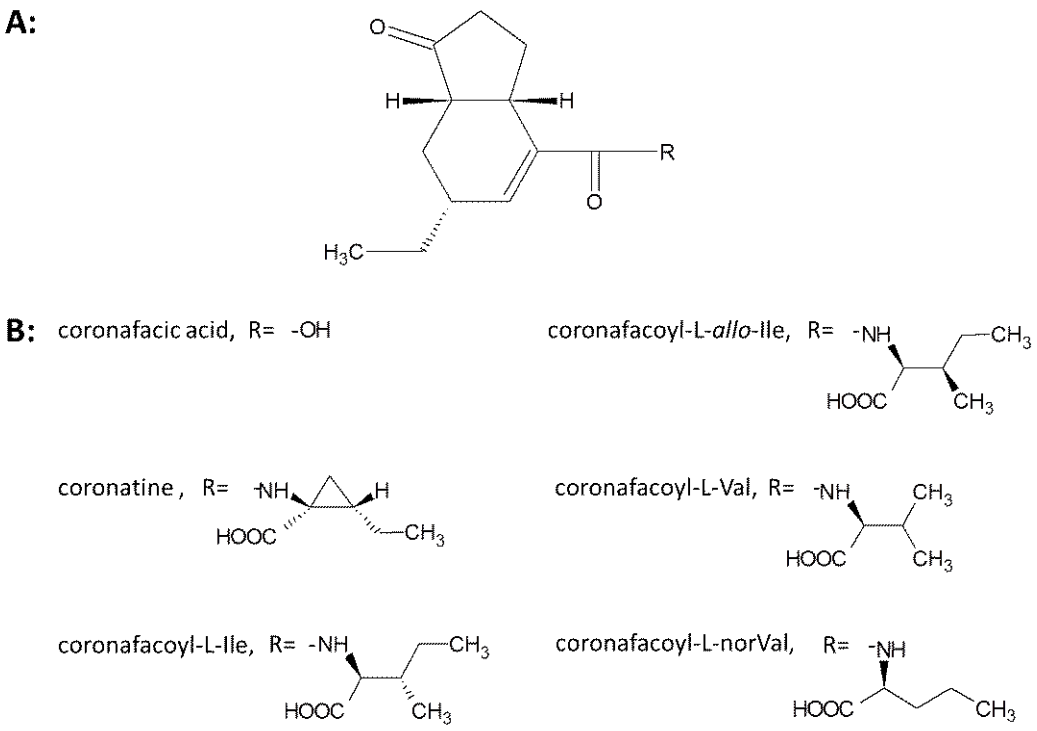
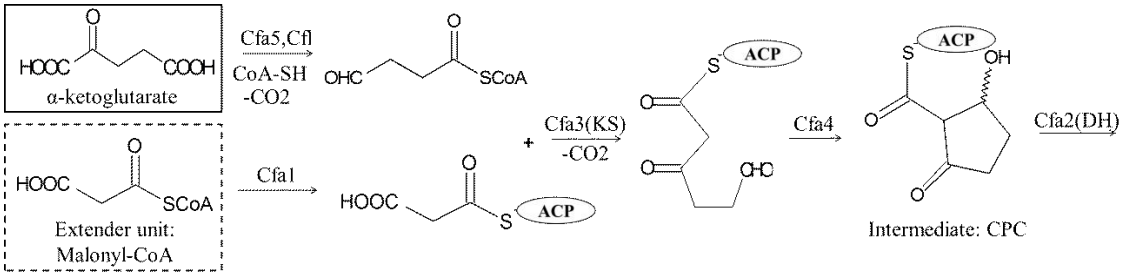


Figure 1.4: Structures of coronafacoyl compounds produced by *P. syringae*. A: core structure of the coronafacoyl compounds, B: structure of coronafacic acid (CFA), coronatine (COR), coronafacoyl-L-Ile, coronafacoyl-L-*allo*-Ile, coronafacoyl-L-Val and coronafacoyl-L-norVal.


α-ketoglutarate
Cfa5,Cfl
CoA-SH
-CO2
Extender unit:
Malonyl-CoA
Cfa1
Cfa3(KS)
-CO2
Cfa4
Cfa2(DH)
Intermediate: CPC

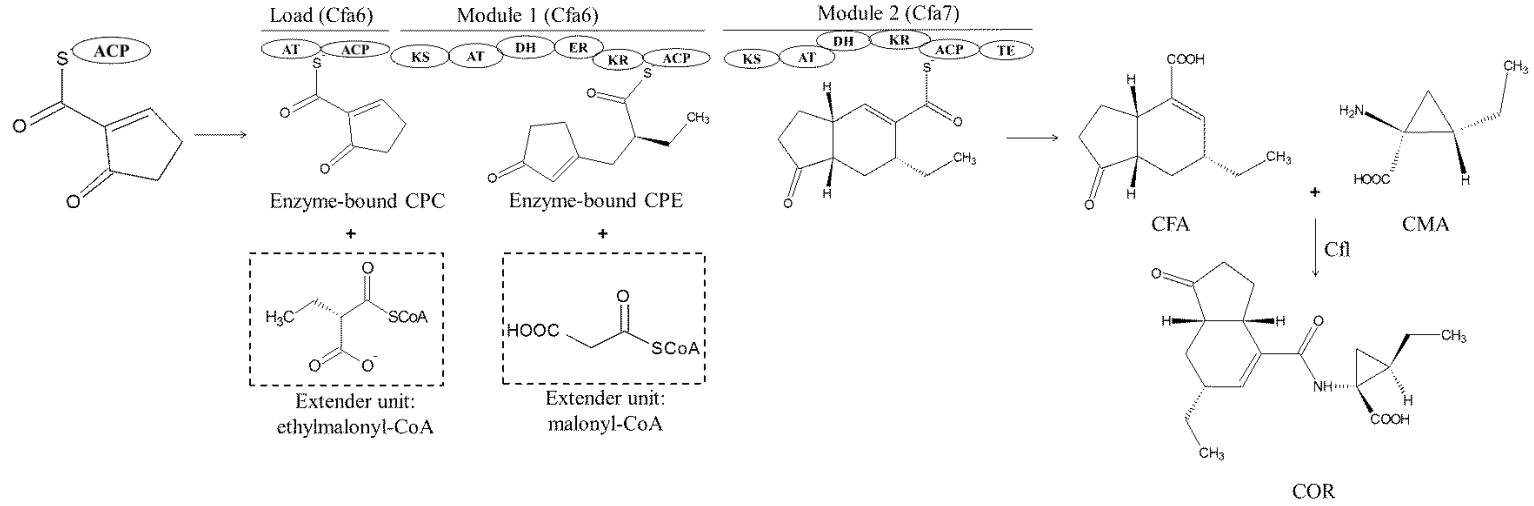

Load (Cfa6)
Module 1 (Cfa6)
Module 2 (Cfa7)
Enzyme-bound CPC
Enzyme-bound CPE
Extender unit:
ethylmalonyl-CoA
Extender unit:
malonyl-CoA
CFA
CMA
Cfl
COR

Figure 1.5: The hypothetical biosynthetic pathway for COR production in *P. syringae*. The black solid box indicates the starting unit in the biosynthetic pathway, while black dash boxes indicate extender units.

Co-Authorship Statement

Chapter 2 is a draft of a manuscript that will be submitted for publication. The basic concept of the study was designed by D. Bignell, and the experimental methodology was designed by D. Bignell and Y. Li. Y. Li performed all of the described experimental work except for the HPLC and LC-MS analyses, which were conducted by J. Fyans. The manuscript was written by Y. Li with editorial assistance provided by D. Bignell.

Parts of Chapter 3 were included in a manuscript that was recently accepted for publication [Fyans JK, Altowairish MS, Li Y and Bignell DR. Characterization of the coronatine-like phytotoxins produced by the common scab pathogen *Streptomyces scabies*. *Molecular Plant-Microbe Interactions*. In Press]. Y. Li designed and conducted the potato tuber disk assays using pure COR and CFA-L-Ile as well as the radish seedling bioassays. Y. Li also wrote the associated parts of the manuscript with editorial assistance provided by D. Bignell.

The remainder of Chapter 3 is a draft of a manuscript that will be submitted for publication in the future. The basic concept of the project was designed by D. Bignell, and the experimental methodology was designed by D. Bignell and Y. Li. Y. Li performed all of the described experimental work, and the manuscript was written by Y. Li with editorial assistance provided by D. Bignell.

CHAPTER 2: Characterizing the Role of *scab79691, scab79711* and *cfa8* in the Biosynthesis of the *Streptomyces scabies* COR-like Metabolites

2.1 Introduction

The CFA-like biosynthetic gene cluster from *S. scabies* 87-22 contains homologues of the *cfl* and *cfa1-8* genes that are present in the CFA gene cluster from *P. syringae* (Fig. 1.3). In *P. syringae,* the *cfa*1-7 and *cfl* genes are proposed to be directly involved in the biosynthesis of COR and COR-like molecules, while the *cfa8* gene encodes a protein with significant similarity to CCR enzymes involved in ethylmalonyl-CoA biosynthesis (Bender et al. 1999). CCR enzymes are well conserved in bacteria and are believed to act as both reductases and carboxylases (Erb et al. 2007). In primary metabolism, CCRs have been proposed to reduce crotonyl-CoA to butyryl-CoA (Fig. 2.1), which serves as a starter unit for fatty acid biosynthesis (Wilson and Moore 2012). As carboxylases, CCR enzymes commence the reaction by taking the hydride from NADPH and then reducing crotonyl-CoA to ethylmalonyl-CoA in the presence of CO_2 (Fig. 2.1). The resulting ethylmalonyl-CoA subsequently enters central metabolism or is passed to polyketide synthases involved in secondary metabolism (Erb et al. 2007; Wilson and Moore 2012). Although there are several pathways known for synthesizing ethylmalonyl-CoA (Fig. 2.1), CCR-dependent pathways seem to be the main supplier of this extender unit for polyketide synthesis (Wilson and Moore 2012). This was demonstrated in *P. syringae* as deletion of *cfa8* resulted in complete loss of CFA and COR production (Rangaswamy et al. 1998).

In *S. scabies*, the CFA-like biosynthetic gene cluster also contains six genes that

have no homologues in the *P. syringae* CFA gene cluster. Among these genes is *scab79711,* which was previously proposed to be involved in the biosynthesis of ethylmalonyl-CoA together with the *cfa8* gene (Bignell et al. 2010). The product of the *scab79711* gene shows similarity to 3-hydroxybutyryl-CoA dehydrogenases that catalyze the reduction of acetoacetyl-CoA to 3-hydroxybutyryl-CoA, a precursor of crotonyl-CoA (Fig. 2.1). Crotonyl-CoA, in turn, can be reduced to ethylmalonyl-CoA by the action of CCR (e.g. Cfa8) as described above. Genes that are homologous to *scab79711* and *cfa8* can be found in other polyketide biosynthetic gene clusters, including those for concanamycin A, elaiophylin and indanomycin (Chan et al. 2009; Li et al. 2009). It is likely that such genes are required to ensure a sufficient supply of ethylmalonyl-CoA for polyketide biosynthesis during secondary metabolism.

Another unique gene within the CFA-like biosynthetic gene cluster, *scab79691*, was previously predicted to encode a putative CYP450 monooxygenase that may function in the oxidative modification of the COR-like metabolite backbone at or near the end of the biosynthetic pathway (Bignell et al. 2010). CYP450s belong to a superfamily of heme-containing proteins that are characterized by an absorption maximum wavelength of 450nm (O'Keefe and Harder 1991). They catalyze the monooxygenation of a broad range of substrates including cholesterol, lipids, steroid hormones, xenobiotics drugs and toxic chemicals among all five kingdoms of life (Hasemann et al. 1995). Bacterial CYP450 superfamilies have been intensively studied and are designated CYP101 to CYP184 (Nelson et al. 1996). For their monooxygenase activity, one atom from O_2 is reduced to water while the other oxygen atom is inserted into the substrate typically as a hydroxyl group. NADPH and ferredoxin/ferredoxin reductase are usually the electron

donors needed to provide the reducing equivalents (Takemori et al. 1993). CYP450 monooxygenases have been found to be highly abundant in the genus *Streptomyces* where some may be involved in detoxifying molecules from other living organism and decaying organic material in soil, and some are associated with the biosynthesis of secondary metabolites (Lamb et al. 2002). In polyketide biosynthetic pathways within *Streptomyces* spp., CYP450s are typically involved in the post-PKS tailoring of the metabolite during the later stages of the pathway (Zhao and Waterman 2007). In the case of antibiotic secondary metabolites, CYP450s often enhance the bioactivity of the molecule through the addition one or more hydroxyl groups (Lamb et al. 2006).

In this study, the role of the *cfa8, scab79711* and *scab79691* genes in the COR-like metabolite biosynthetic pathway was elucidated. Deletion mutants of *S. scabies* were constructed for each gene, and the effect of each mutation on CFA-L-Ile biosynthesis was assessed using HPLC and bioassays. The results show that *scab79711* and *cfa8* are dispensable for CFA-L-Ile biosynthesis in *S. scabies,* though *cfa8* is required for optimum production of the metabolite. On the other hand, *scab79691* was found to be essential for metabolite biosynthesis, and the implications of this finding are discussed.

2.2 Materials and Methods

2.2.1 Bacterial strains, culturing conditions and maintenance

2.2.1.1 *Escherichia coli* strains

E. coli strains used in this study are listed in Table 2.1. Strains were routinely grown at 37°C unless otherwise indicated. Liquid cultures were grown with shaking (200

– 250 rpm: revolutions per minute) in 5 – 50 mL of Difco™ LB (Luria-Bertani medium) Lennox broth (BP1427-2; Fisher Scientific, Waltham, MA), low salt LB broth (1% w/v tryptone; 0.5% w/v yeast extract; 0.25% w/v NaCl), SOB (Super optimal broth) (Sambrook and Russell 2001) or SOC (Super optimal broth with catabolite repression) medium (B9020S; New England Biolabs, Whitby, ON), while solid cultures were grown on LB Lennox (or low salt LB) medium containing 1.5% w/v agar (105791A; NEOGEN, Michigan, US). When necessary, the solid or liquid growth media were supplemented with ampicillin (100 µg/mL final concentration; 0339-25G; Amresco, Solon, OH), kanamycin (50 µg/mL final concentration; 420311; Calbiochem, San Diego, CA), hygromycin B (100 µg/mL final concentration; 400051; Calbiochem) or chloramphenicol (25 µg/mL final concentration; AC227920250; Acros Organic, New Jersey, USA). *E. coli* strains were maintained at 4°C for short-term storage or at - 80°C as glycerol stocks for long-term storage. Glycerol stocks were prepared by growing the strains overnight in 2 – 5 mL of LB or low salt LB liquid medium (with or without antibiotics) and then pelleting the cells by centrifugation (13,000g) for 5 min. The resulting cell pellets were resuspended in 0.5 – 1 mL of 20% v/v glycerol and were frozen at - 80°C.

2.2.1.2 *Streptomyces scabies* strains

S. scabies strains used in this study are listed in Table 2.1. Strains were routinely grown at 28°C unless otherwise indicated. Liquid cultures were typically grown with shaking (200 rpm) in TSB (Trypticase Soy Broth) (DF0370173; BD Biosciences, Mississauga, ON) medium in 50 or 125 mL flasks with stainless steel springs. Plate

cultures were grown on SFMA (Soy flour mannitol agar) (Kieser et al. 2000), OBA (Oat bran agar) (Johnson et al. 2007) or Difco NA (Nutrient agar) (DF0003178, BD Biosciences) containing 1.5% w/v agar. When necessary, the growth media were supplemented with hygromycin B (50 µg/mL final concentration), apramycin (50 µg/mL final concentration), nalidixic acid (50 µg/mL final concentration; BP908-25; Thermo Fisher Scientific), kanamycin (50 µg/mL final concentration), or thiostrepton (25 µg/mL final concentration; T8902-1G; Sigma-Aldrich, Oakville, ON). *S. scabies* strains were maintained at 4°C for short-term storage or at - 80°C as spore or mycelial stocks for long-term storage. Spore stocks were prepared by scraping gray spores from a 7-10 day old OBA plate and then transferring the spores to a sterile 1.5 mL microcentrifuge tube containing 1 mL of 40% v/v glycerol. The contents were mixing thoroughly and the tubes were placed into the - 80°C freezer. Mycelial stocks were prepared by inoculating spores into 25 mL of TSB and then growing for 48 – 72 hrs or until the culture was dense. Next, 950 µL of the TSB culture was transferred into sterile 1.5 mL microcentrifuge tubes containing 50 µL of 100% v/v DMSO. After mixing the contents, the tubes were frozen at - 80°C. For production of the COR-like metabolites, a single DMSO stock tube for each strain was thawed on ice, inoculated into 9 mL of TSB, and incubated for 24 – 48 hrs until the culture was dense. Then, the seed culture was subcultured (1% v/v) into 5 or 50 mL of SFMB (Soy flour mannitol broth) (Kieser et al. 2000) in 6-well tissue culture plates (353046; BD Falcon) or in 2 × 125 mL spring flasks, respectively, and were incubated for 7 days at 25 or 28°C with shaking (125 rpm for 6-well plates, 200 rpm for spring flasks).

2.2.2 DNA manipulations

Standard molecular biology procedures were implemented for all DNA manipulations performed in this study (Sambrook and Russell 2001). Restriction digestions were performed using enzymes purchased from New England Biolabs according to the manufacturer's instructions. DNA was routinely analyzed by agarose gel electrophoresis using 1% w/v agarose gels in 1× TBE (Tris-Borate-EDTA) buffer, and the size of the DNA bands was estimated using 100bp (base pair) and 1kb DNA ladders (DM001-0500 and DM010-0500; FroggaBio Inc., Toronto, ON). The EZ-Vision® In-Gel stain (N391-0.5ML; Amresco LLC, Solon, OH) or ethidium bromide was used for visualizing the DNA in the gel, and gel images were acquired using a GelDoc-It Imager (UVP, Upland, CA). Gel extraction of DNA was routinely performed using the Wizard® SV Gel and PCR (Polymerase chain reaction) Clean-Up system (A9281; Promega Corporation, Madison, WI) as per the manufacturer's directions. Quantification of DNA was performed using a P300 Nanophotometer™ (Implen Inc., Westlake Village, CA) according to the instrument instructions. Cloning of PCR products was performed using the pGEM®-T Easy vector system (PR-A1360, Promega Corporation) as per the manufacturer's protocol. Prior to cloning, the PCR products were modified using the A-tailing procedure described in the pGEM®-T Easy vector system protocol and using Taq DNA polymerase (M0273S, New England Biolabs). Cloning of other DNA fragments was performed using T4 DNA ligase (M0202S; New England Biolabs) as per the manufacturer's instructions except that the reactions were incubated at room temperature

for 4hrs. A vector: insert ratio of 1: 3 was used for all ligation reactions, and reactions were routinely transformed into NEB (New England Biolabs) 5-alpha high efficiency competent *E. coli* cells (C2987H; New England Biolabs) as per the manufacturer's directions. Plasmid and cosmid DNA was routinely isolated from overnight cultures of *E. coli* using the alkaline lysis with SDS (Sodium dodecyl sulfate) minipreparation method (Sambrook and Russell 2001). When highly pure DNA was required, the EZ-10 Spin Column Plasmid DNA kit (BS614-250Preps; Bio Basic Inc., Markham, ON) was used as described by the manufacturer. All cosmids and plasmids that were used or constructed in this study are listed in Table 2.1. Genomic DNA was prepared using the SpeedMill PLUS Beat Beater (MBI lab equipment, Montreal, CA) and the QIAamp DNA Mini Kit (51304; QIAGEN) or the One-Tube Bacterial Genomic DNA Extraction Kit (BS8413-100Preps; Bio Basic Inc.). Sequencing of DNA was performed by The Centre for Applied Genomics (Toronto, ON). Standard desalted oligonucleotide primers used for sequencing were purchased from Integrated DNA Technologies (Coralville, IA) and are listed in Table 2.2.

2.2.3 PCR

2.2.3.1 Generation of the *hyg* + *oriT* (Origin of transfer from RK2) extended resistance cassettes

PCR was performed in order to generate the *hyg* + *oriT* extended resistance cassettes that were used for constructing the Δ*scab79711,* Δ*cfa8,* and Δ*scab79691* mutant cosmids (Table 2.1) as part of the Redirect PCR targeting system (Gust et al. 2003a,b). Reactions (50μL) contained 50 – 100 ng of pIJ10700 plasmid template, 1× Taq reaction

buffer with KCl, 2 mM $MgCl_2$, 200 µM dNTPs (Deoxyribonucleoside triphosphate), 50 pmol each of the long (58-59 nt: nucleotide) forward and reverse primers, 5% v/v DMSO (Dimethylsulfoxide) and 2.5 U of Taq DNA polymerase (FEREP0402, Thermo Fisher Scientific, Waltham, MA). The cycling conditions started with an initial denaturation step of 95°C for 2 min followed by 10 cycles of denaturation at 95°C for 45 sec, primers annealing at 50°C for 45 sec, and extension at 72°C for 90 sec. Then, an additional 15 cycles was carried out with denaturation at 95°C for 45 sec, annealing at 55°C for 45 sec and extension at 72°C for 90 sec. At the end, a final extension step was performed at 72°C for 5 min. The PCR reactions described in this section were performed in 0.2 mL thin walled PCR tubes (10011-776; VWR International, Mississauga, ON) using a Mastercycler Pro thermal cycler (Eppendorf Canada, Mississauga, ON). Following PCR, the extended resistance cassettes were analyzed by agarose gel electrophoresis and were gel-purified as described in section 2.2.2.

2.2.3.2 Verification of constructed mutant cosmids and strains

PCR reactions were also performed in order to verify the constructed mutant cosmids (1770/Δ*scab79711,* 1770/Δ*cfa8* and 1770/Δ*scab79691*) and the *S. scabies* mutant strains (Δ*scab79711,* Δ*cfa8* and Δ*scab79691*). Reactions were performed in 25 µL volumes using cosmid or genomic DNA (2.5 µL) as template and using gene-specific oligonucleotide primers (25 pmol each). Reactions were performed using the same enzyme and reaction conditions as described in section 2.2.3.1, and the cycling conditions consisted of an initial denaturation step at 95°C for 1 min followed by 30 cycles of

denaturation at 95°C for 30 sec, primer annealing at 60°C for 30 sec and extension at 72°C for 1 min/kb. All reactions were performed using an Eppendorf Mastercycler Pro thermal cycler, and the resulting products were analyzed by agarose gel electrophoresis (section 2.2.2).

2.2.3.3 Amplification of the *scab79691* and *cfa8* genes

The *scab79691* and *cfa8* genes were PCR-amplified for subsequent cloning into the pGEM®-T Easy vector (Table 2.1). Amplifications were conducted in 50 µL reaction volumes with 25-50 ng of Cosmid 1770 DNA as a template, 1× high GC buffer, 10mM dNTPs, 25 pmol of forward and reverse primer, 3% v/v DMSO, and 0.5U of Phusion DNA polymerase (M0530S, New England Biolabs). The cycling conditions consisted of an initial denaturation step at 98°C for 30 sec followed by 30 cycles of denaturation at 98°C for 10 sec, primer annealing at 60°C and 72°C for *scab79691* and *cfa8,* respectively, for 45 sec and extension at 72°C for 5 min. Then, a final extension step of 72°C for 5 min was followed. The PCR reactions described in this section were all performed using a C1000™ Thermal Cycler (BIO-RAD, Mississauga, Ontario), and the resulting products were analyzed by agarose gel electrophoresis prior to cloning into pGEM®-T Easy. All oligonucleotide primers used for PCR are listed in Table 2.2.

2.2.4 Construction of the *S. scabies* gene deletion mutants

The Redirect PCR targeting system (Gust et al. 2003a,b) was used to construct mutant cosmids harbouring a deletion of the *S. scabies scab79711, cfa8* and *scab79691*

genes (Table 2.1). Each gene was replaced with an extended resistance cassette (which was PCR amplified as described in section 2.2.3.1) harbouring the *hyg* gene (conferring resistance to hygromycin B) and *oriT,* which allowed for conjugal transfer of the mutant cosmids into *S. scabies* from *E. coli.* Intergeneric conjugations were performed as described previously (Altowairish 2014), and *S. scabies* exoconjugants were selected using hygromycin B and nalidixic acid. To confirm that gene replacement had occurred in the resulting exconjugants due to a double-crossover event, the strains were cultured on NA containing kanamycin in order to identify those strains that were hygromycin resistant and kanamycin sensitive. A total of six hygromycin resistant and kanamycin sensitive isolates for each mutant were selected and numbered as Δ*scab79711* N. 5/10/11/12/14/18, Δ*cfa8* N. 2/7/10/12/17/18 and Δ*scab79691* N. 1/2/3/4/5/6. Each isolate was streaked for single colonies, and spores from a single colony were then spread over the surface of an entire OBA plate containing the appropriate antibiotics. Finally, spore and mycelial stocks of each isolate were prepared as described in section 2.2.1.2.

2.2.5 Chemical extraction of the COR-like metabolites

2.2.5.1 Small-scale extractions for HPLC analysis

S. scabies SFMB cultures grown in 6 well plates (see section 2.2.1.2) were used for small-scale extraction of the COR-like metabolites. Samples (1.5 mL) of the SFMB cultures were centrifuged at 13,300 *g* for 5 min, and 1 mL of each culture supernatant was transferred into a clean 2 mL microcentrifuge tube (87003-298, VWR). The pH of the supernatants was adjusted to ~10-11 with 1N NaOH, after which the supernatants were

extracted twice with 0.5 volumes of chloroform (319988-4L; Sigma Aldrich Canada Co., Oakville, ON) to remove non-acidic hydrophobic compounds. Next, the aqueous supernatants were adjusted to pH 1-2 with 1N HCl and were then extracted three times with 0.5 volumes of chloroform. The acidic organic extracts were pooled and dried down in a fume hood, and the resulting material was redissolved in 100 µL of 100% v/v HPLC-grade methanol (34860-4L-R; Sigma Aldrich Canada Co., Oakville, ON). The samples were filtered using a 0.2 µm pore size syringe filter (28145-491; VWR) to remove any undissolved particulates prior to HPLC analysis.

2.2.5.2 Large-scale extractions for potato bioassays

S. scabies SFMB cultures grown in 2 × 125 mL flasks (section 2.2.1.2) were used for large-scale extraction of the COR-like metabolites. Culture supernatants were harvested by centrifugation (4000 rpm for 5 min) and were extracted as described in section 2.2.5.1 except that the dried extracts were redissolved in 200 µL of 100% v/v HPLC-grade methanol.

2.2.6 HPLC analysis of COR-like metabolite production

Filtered culture extracts were transferred to flat bottom glass inserts (5181-3377; Agilent Technologies Canada Inc., Mississauga, ON), which were then placed into 2 mL glass screw cap vials (5182-0716; Agilent Technologies Canada Inc.) and were sealed with a PTFE (Polytetrafluoroethylene)/silicone rubber septum (5182-0731; Agilent Technologies Canada Inc.). Samples (10 µL) of each culture extract were analyzed using

an Agilent 1260 Infinity Quaternary LC system with a Poroshell 120 EC-C18 column (4.6 × 50 mm, 2.7 µm particle size; Agilent Technologies Inc.). The initial mobile phase concentration consisted of acetonitrile and water (30:70) with 0.1% v/v formic acid. After 1.5 min, the concentration was increased linearly to 50:50 acetonitrile:water over a time period of 2.5 min, and this concentration was held for 1 min. Next, the mobile phase concentration was returned to the initial conditions (30:70 acetonitrile:water) in 1.5 min using a linear gradient, and this concentration was held for 2 min prior to injection of the next sample. The flow rate (1 mL/min), column temperature (40°C) and detection wavelength (230 nm) were kept constant throughout the analysis. A synthetic standard of CFA-L-Ile (provided by Carol Bender) was included to assist in the identification of the primary COR-like metabolite. All data were collected and analyzed using the ChemStation software (version B.04.03, Agilent Technologies Canada Inc.), and the Student's *t*-test was used to identify significant differences in metabolite production in the mutants compared to the Δ*txtA*/pLDRB51-1 strain.

2.2.7 LC-MS (Liquid chromatography–mass spectrometry) analysis

Culture extracts (10 µL) were also analyzed by LC-MS using an Agilent 1100 series HPLC system (Agilent Technologies Inc.) interfaced to a Waters G1946A single quadrupole mass spectrometer (Waters Corporation). Separation was achieved using the Agilent Technologies ZORBAX SB-C18 column (4.6 × 150 mm, 5 µm particle size) and flow rate and column temperature as described in 2.2.6 except that the solvent gradient system was modified. The initial mobile phase concentration consisted of acetonitrile and

water (30:70) with 0.1% v/v formic acid. After 4.5 min, the concentration was increased linearly to 50:50 acetonitrile: water over a time period of 12 min, and this concentration was held for 3 min. Next, the mobile phase concentration was returned to the initial conditions (30:70 acetonitrile: water) in 4.5 min using a linear gradient, and this concentration was held for 6 min prior to injection of the next sample. Detection was by ultraviolet radiation (230 nm) and by electrospray ionization MS in negative ion mode.

2.2.8 Potato tuber slice bioassay

This was performed as described previously (Bignell et al. 2010) with some modifications. Potato tubers were peeled and the surface was sterilized by immersing in 15% v/v bleach (Chlorox) for 10 min with occasional stirring. After rinsing twice with sterile HPLC - grade water, the tubers were cut into equivalent size pieces (approximately 2.5 × 1.5 × 1 cm) using a sterile knife, and the potato pieces were placed onto pre-wetted Whatman™ filter paper in sterile Petri dishes (4-6 potato pieces per dish). Next, one 6 mm diameter sterile paper disk (2017-006; Whatman) was placed onto each potato piece, and 25 µL of filter-sterilized acidic culture extract was pipetted onto each disk. Pure COR (500 ng) (C8115-1MG; Sigma Aldrich Canada Co., Oakville, ON) was included as a positive control in this experiment while 100% v/v methanol was used as a negative control. The potato disks were incubated under high humidity for 5-7 days at ambient temperatures in the dark, and then they were assessed for the presence of tissue hypertrophy.

2.2.9 Complementation of the Δ*cfa8* and Δ*scab79691* mutants

The basic strategy that was used is outlined in Figure 2.18. The target genes (*cfa8* and *scab79691)* were PCR-amplified as described in section 2.2.3.3 using primers containing engineered NdeI and XhoI restriction sites (Table 2.2). After cloning the PCR products into pGEM®-T EASY, the resulting clones were verified by digestion with EcoRI and by sequencing. Next, the pGEM clones were digested with NdeI and XhoI to release the cloned inserts, which were then gel-purified. The *Streptomyces* expression plasmid pMSAK13, which contains the strong constitutive *Streptomyces* promoter *ermE*p* and integrates into the chromosome at the ϕBT1 site (Table 2.1), was digested with the same enzymes and was also gel-purified. Ligation reactions were set up using the digested plasmid and the *cfa8* or *scab79691* DNA fragments. Recombinant plasmids obtained were verified by PCR and sequencing, and a single positive clone was introduced into the corresponding *S. scabies* deletion mutant strain (Δ*cfa8* or Δ*scab79691*) by conjugation with *E. coli* as described previously (Altowairish 2014). As a control, the pMSAK13 plasmid without cloned insert was also introduced into each strain. After this, all the resulting strains were tested for COR-like metabolite production using HPLC as described above.

2.2.10 Bioinformatics analyses

S. scabies DNA and protein sequences were obtained from the StrepDB website (http://strepdb.streptomyces.org.uk). Amino acid sequence alignments were performed using the Geneious 6.1.2 software. Predicted protein functions were assigned based on

database similarity searches using the National Center for Biotechnology Information Protein BLAST (Basic Local Alignment Search Tool) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the StrepDB BLAST program. Protein domain analyses were performed using the Pfam (protein families) database (http://pfam.sanger.ac.uk/) (Finn et al. 2008). Phylogenetic trees were constructed using maximum likelihood method of MEGA (Molecular Evolutionary Genetic Analysis) program version 5.10 (Tamura et al. 2011). The significance of the branch order was tested using the bootstrapping method with 1000 repetitions.

2.3 Results

2.3.1 Bioinformatics analysis of Scab79711, Cfa8 and Scab79691

2.3.1.1 Scab79711 and Cfa8 homologues in the database

Protein BLAST analysis indicated that the most similar homologues of Scab79711 and Cfa8 are IdmE and IdmF, respectively, from *Streptomyces antibioticus* (Table 2.3). IdmE is a predicted hydroxybutyryl-CoA (or hydroxyacyl-CoA) dehydrogenase and IdmF is a predicted CCR, and both are responsible for supplying a sufficient pool of the extender unit ethylmalony-CoA for indamycin biosynthesis (Li et al. 2009). Pfam analysis identified two 3-hydroxyacyl-CoA dehydrogenase NAD (Nicotinamide adenine dinucleotide) binding domains and two C-terminal 3-hydroxyacyl-CoA dehydrogenase domains in Scab79711 (Table 2.3). This is consistent with the fact that the N-terminal domain of hydroxybutyryl-CoA dehydrogenases is usually responsible for the binding of NAD^+ and the C-terminal domain is involved in binding acyl-CoA substrates (Barycki et

al. 1999; Kim et al. 2014). Pfam analysis of Cfa8 suggested the presence of a conserved C-terminal NAD(P)H/NAD(P)$^+$ binding domain that binds the electron donor NAD(P)H, and an N-terminal alcohol dehydrogenase GroES-like domain that is responsible for binding a zinc cofactor atom(s) (Table 2.3; Hedlund et al. 2010; Murzin 1996). CCR enzymes are members of the zinc-dependent alcohol dehydrogenase-like medium chain dehydrogenase/reductase family (Rosas et al. 2003), which prefer NADPH as the electron donor and usually require zinc atom(s) as the cofactor (Hedlund et al. 2010). Homologues of Scab79711 and Cfa8 were identified in several *Streptomyces* spp. including the plant pathogenic species *S. turgidiscabies, S. acidiscabies* and *S. ipomoeae* (Fig. 2.2 and 2.3). Based on the phylogenetic analysis performed, the Cfa8 protein from *S. scabies* 87-22 is more closely related to CCRs from other *Streptomyces* spp. than to Cfa8 homologues from *P. syringae* pathovars (Fig. 2.3).

2.3.1.2 Scab79691 homologues in the database

Protein BLAST and phylogenetic analyses revealed that an unspecific monooxygenase from *S. ipomoeae* is the closest relative of Scab79691 (Table 2.3 and Fig. 2.4). Several known or predicted CYP450 monooxygenases from other actinomycetes were also found to be similar to Scab79691 (Fig. 2.4). In addition, Scab79691 contains a cytochrome P450 domain according to Pfam analysis (Table 2.3). Thus, Scab79691 is predicted to serve as a monooxygenase in the biosynthesis of CFA-L-Ile. CYP450 enzymes are known to catalyze a variety of different reactions including primary oxidation reactions, reductions and some non-redox P450 reactions (Guengerich and Munro 2013). Monooxygenation catalyzed by CYP450s requires the input of two protons

to insert one oxygen group into the substrate (De Mot and Parret 2002). Alignment of the Scab79691 amino acid sequence with that of other known or predicted CYP450s indicated three conserved features: (1) the consensus sequence FXXGXXXCXG, which contains the invariant residue cysteine (C) and is found in the heme-binding loop adjacent to the L-helix; (2) an EXXR motif, which stabilizes the core structure by building up an extensive network of hydrogen bonds with other amino acids and is found in the K-helix on the proximal side of heme; (3) a P450 signature consensus sequence A/GGXD/ETT, which is involved in proton transfer and is found on the distal side of the heme group (Fig. 2.5; Werck-Reichhart and Feyereisen 2000). This further supports the idea that Scab79691 functions as a CYP450 monooxygenase enzyme that catalyzes a hydroxylation reaction during COR-like metabolite biosynthesis.

2.3.2 Construction of the *S. scabies* gene deletion mutants

Using the Redirect PCR targeting system, Δ*scab79711,* Δ*cfa8* and Δ*scab79691* mutant cosmids were constructed, and each was verified by PCR (Fig. 2.6 – 2.8). The mutant cosmids were then transferred into the *S. scabies* Δ*txtA/*pRLDB51-1 strain by conjugation with *E. coli*. Strain Δ*txtA/*pRLDB51-1 was used to study the biosynthesis of the COR-like metabolites since it is able to produces high levels of the COR-like metabolites due to overexpression of the *scab79591* regulatory gene, and it is also unable to produce the dominant virulence factor thaxtomin A (Bignell et al. 2010). Exconjugants obtained from the conjugations were screened to confirm that they arose from a double crossover event, and spore and mycelial stocks were prepared for six Δ*scab79711*

isolates, six Δ*cfa8* isolates and six Δ*scab79691* isolates. All mutant isolates were confirmed by PCR (Fig 2.9 – 2.11).

2.3.3 The *S. scabies* deletion mutants differ in their ability to produce the CFA-L-Ile COR-like metabolite

To analyze the production of the COR-like metabolites by the Δ*scab79711,* Δ*cfa8* and Δ*scab79691* mutants, the strains, together with the original Δ*txtA/*pRLDB51-1 strain, were grown in SFMB, a medium which is known to support the production of the *S. scabies* COR-like metabolites (Fyans et al. 2014). The resulting culture supernatants were then extracted with chloroform under basic and acidic conditions, and the acidic organic extracts were assessed for the presence of the primary COR-like metabolite (CFA-L-Ile) using HPLC. As shown in Fig. 2.12 and 2.13, the majority of the Δ*scab79711* isolates were not significantly different in production of CFA-L-Ile from the Δ*txtA/*pRLDB51-1 strain, indicating that *scab79711* is not required for metabolite production. On the other hand, all of the Δ*cfa8* mutant isolates produced significantly less CFA-L-Ile as compared to the Δ*txtA/*pRLDB51-1 strain, though all were still able to produce the metabolite (Fig. 2.12 and 2.14). Quantitative analysis revealed that the Δ*cfa8* isolates produced 45-64% of the averaged metabolite level in the Δ*txtA/*pRLDB51-1 cultures (Fig. 2.14). In contrast, the Δ*scab79691* isolates were all found to be abolished in CFA-L-Ile production (Fig. 2.15; data not shown), and instead they accumulated a metabolite with a longer retention time. LC-MS analysis of the extracts from Δ*scab79691* N.2 and N.4 revealed that the accumulated metabolite has a mass of 307.2 (Fig. 2.16D). It is also noteworthy that the

basic extracts from the Δ*scab79691* isolates were also analyzed by HPLC, and no accumulated metabolites were found in those extracts (data not shown). Thus, *scab79691* appears to be essential for COR-like metabolite biosynthesis, whereas *cfa8* contributes to metabolite production but is not essential, and *scab79711* is not required for metabolite biosynthesis. Furthermore, the accumulated metabolite in the Δ*scab79691* mutant extracts may represent an intermediate in the COR-like metabolite biosynthetic pathway.

2.3.4 Culture extracts from the Δ*scab79691* mutant display reduced bioactivity on potato tuber tissue

The bioactivity of the mutant culture extracts was also assessed using a potato tuber bioassay, which detects the tissue hypertrophy-inducing activity of COR and COR-like molecules (Bignell et al. 2010; Bignell et al. 2014). As shown in Figure 2.17, the Δ*scab79691* mutant culture extract was found to induce only a very small amount of tissue hypertrophy, which is consistent with the inability of this mutant to produce CFA-L-Ile. The fact that some hypertrophy was observed suggests that the accumulated metabolite in the culture extract (Fig. 2.15) may exhibit some of the same bioactivity as CFA-L-Ile. On the other hand, the Δ*scab79711* and Δ*cfa8* mutant extracts exhibited a similar level of hypertrophy - inducing activity as the Δ*txtA*/pRLDB51-1 culture extract, a finding that is consistent with the HPLC results for these extracts.

2.3.5 Genetic complementation of the Δ*cfa8* and Δ*scab79691* mutants

Genetic complementation was next performed to confirm that the observed

decrease in CFA-L-Ile production in the Δ*cfa8* and Δ*scab79691* mutants is due to deletion of the corresponding gene. Given that there is only one promoter that drives expression of the entire CFA-like metabolite biosynthetic gene cluster and that this promoter is located upstream of *cfa1* (Bignell et al. 2010), it was necessary to clone *scab79691* and *cfa8* into a plasmid that harbours a promoter that would allow for expression of the genes in the corresponding mutant. As such, the *scab79691* and *cfa8* coding sequences were PCR-amplified and cloned into the pMSAK13 plasmid, which integrates into the ϕBT1 phage site in *Streptomyces* chromosomes and harbours the *ermE*p* promoter, allowing for strong constitutive expression of the cloned genes (Table 2.1; Altowairish 2014). This particular promoter was chosen as it has been successfully used in complementation studies involving other COR-like metabolite biosynthetic mutants (Altowairish 2014). The complementation plasmids were each verified by restriction digestion using the same enzymes that were used for cloning (Fig. 2.19), after which they were introduced into the corresponding mutant by conjugation with *E. coli.* As a control, the pMSAK13 without any cloned insert was also introduced into each mutant.

As shown in Figure 2.20, the ability of pMSAK13/*scab79691* to restore CFA-L-Ile production in the Δ*scab79691* (isolate N.2) mutant was variable since two of the complementation strains did not produce CFA-L-Ile (Fig. 2.20D, E) while a third strain did produce a small amount of the metabolite (Fig. 2.20F). When complementation was attempted using a second Δ*scab79691* mutant strain (isolate N.4), the pMSAK13/*scab79691* plasmid again failed to restore CFA-L-Ile production in the mutant (data not shown). Attempts to complement the Δ*cfa8* (isolate N.18) mutant were also

unsuccessful as introduction of the pMSAK13/*cfa8* plasmid severely affected the growth of the complemented strain, and production of CFA-L-Ile was determined to be much lower in the complementation strain than in the original Δ*cfa8* mutant (data not shown).

2.4 Discussion

This study focused on the role of three genes in the biosynthesis of the CFA-L-Ile COR-like metabolite in *S. scabies*. One gene, *cfa8,* is conserved in the *P. syringae* CFA biosynthetic gene cluster and was previously proposed to encode a CCR enzyme involved in the biosynthesis of the ethylmalonyl-CoA extender unit (Bignell et al. 2010). Mutation of *cfa8* in *P. syringae* resulted in loss of CFA and COR production, indicating that the gene is required for COR biosynthesis in that organism (Rangaswamy et al. 1998). The other two genes, *scab79711* and *scab79691,* are unique to *S. scabies* and were previously predicted to be involved in ethylmalonyl-CoA biosynthesis and tailoring of the COR-like metabolite backbone, respectively (Bignell et al. 2010). It was predicted that all three genes may be required for COR-like metabolite biosynthesis in *S. scabies,* and therefore gene deletion mutants were constructed in order to test this hypothesis.

2.4.1 *scab79711* and *cfa8* are dispensable for COR-like metabolite biosynthesis

Previously, it was suggested that *scab79711* encodes a 3-hydroxybutyryl-CoA dehydrogenase that functions to reduce acetoacetyl-CoA to 3-hydroxybutyryl-CoA, which is an intermediate in crotonyl-CoA biosynthesis (Fig. 2.1; Bignell et al. 2010). The bioinformatics analyses performed in this study support this idea as Scab79711 was

shown to display significant homology to other predicted 3-hydroxybutyryl-CoA dehydrogenases that are currently in the database (Table 2.3 and Fig. 2.2). Four functional domains that are responsible for catalyzing the reduction reaction are conserved in Scab79711 and in its closest homologue IdmE from *S. antibioticus*. Though it was predicted that *scab79711* may be required for supplying a sufficient amount of ethylmalonyl-CoA for CFA and COR-like metabolite biosynthesis in *S. scabies,* deletion of the gene caused only a slight reduction of CFA-L-Ile production as determined by HPLC and bioassay, indicating that Scab79711 is dispensable for COR-like metabolite biosynthesis.

Bioinformatics analysis revealed that Cfa8 is most similar to IdmF (Table 2.3), which is a predicted CCR enzyme encoded along with IdmE within the indamycin biosynthetic gene cluster in *S. antibioticus* (Li et al. 2009). Cfa8 was found to contain predicted domains that are conserved in other CCR homologues and which bind NADPH and zinc (Murzin 1996; Hedlund et al. 2010). CCR enzymes are well conserved in *Streptomyces* spp. and catalyze both reduction and carboxylation reactions involved in ethylmalonyl-CoA biosynthesis (Fig. 2.1; Erb et al. 2007; Wilson and Moore 2012). Although the BLAST results indicated that Cfa8 is most similar to IdmF from *S. antibioticus,* the phylogenetic analysis revealed that Cfa8 may form a distinct CCR lineage as compared to IdmF and other *Streptomyces* homologues (Fig. 2.3). This is probably because the amino acids conserved in Cfa8 are more distinguishable than other *Streptomyces* homologues according to the protein alignment used for the tree. As with *scab79711,* it was predicted that Cfa8 is required to supply a sufficient amount of ethylmalonyl-CoA for CFA and COR-like metabolite biosynthesis in *S. scabies*. The

results of this study show that although deletion of *cfa8* in *S. scabies* did significantly reduce the CFA-L-Ile production levels, production could still occur in the absence of the enzyme. This is in contrast to the *P. syringae cfa8* mutant, which was abolished in CFA and COR biosynthesis (Rangaswamy et al. 1998).

In *Streptomyces tsukubaensis*, which produces the macrolide secondary metabolite FK520, it has been shown that the total disruption of metabolite biosynthesis requires the deletion of two *ccr* homologues in the genome (Kosec et al. 2012). A search of the *S. scabies* 87-22 genome sequence revealed the presence of genes encoding three possible homologues of Scab79711 and one possible homologue of Cfa8 (Table 2.4). Scab17601, a predicted oxidoreductase, was found to be most closely related to Scab79711, and the same domain properties were found in this protein as in Scab79711. The homologue of Cfa8, Scab17621, is encoded within the same vicinity of Scab17601 and is 88% identical to Cfa8 at the amino acid level (Table 2.4). It is possible that the homologous proteins allow *S. scabies* to produce ethylmalonyl-CoA for CFA-L-Ile biosynthesis in the absence of Cfa8 or Scab79711, though Cfa8 is apparently needed for optimum metabolite biosynthesis.

Alternatively, or in addition, it is possible that *S. scabies* can use alternative pathways for ethylmalonyl-CoA production that are independent of CCR and/or 3-hydroxybutyryl-CoA activity. For example, in *P. syringae,* there are no homologues of Scab79711 encoded within the genome, and it was previously suggested that ethylmalonyl-CoA production in this organism occurs via the conversion of acetoacetyl-CoA to butyryl-CoA, a pathway that is dependent on CCR but not on 3-hydroxybutyryl-CoA dehydrogenase (Fig. 2.1; Rangaswamy et al. 1998). In *Streptomyces hygroscopicus*

var. ascomyceticus, it has been reported that the storage compound polyhydroxybutyrate can be metabolized by polyhydroxybutyrate depolymerase to produce hydroxybutyryl-CoA, which can then be converted to ethylmalony-CoA by the action of CCR (Wu et al. 2000). Further studies showed that this organism can also generate ethylmalonyl-CoA directly from butyryl-CoA through the β-oxidation pathway (Fig. 2.1; Wu et al. 2000). Whether such alternative pathways play a role in ethylmalonyl-CoA production in *S. scabies* is currently unknown and warrants further investigation.

Attempts to complement the Δ*cfa8* mutant phenotype were not successful due to the fact that the mutant strains containing pMSAK13/*cfa8* grew very poorly. This could be because the *ermE*p* promoter used for *cfa*8 expression is a strong, constitutive promoter, and perhaps the high level of *cfa8* expression is toxic to the cells. The use of an alternative promoter for *cfa8* expression might therefore be the answer for successful complementation of this mutant.

2.4.2 *scab79691* is required for COR-like metabolite biosynthesis

Analysis of the *scab79691* gene product revealed that it is most closely related to CYP450 monooxygenase enzymes from *Streptomyces* spp. and other actinomycetes (Table 2.3 and Fig. 2.4). The EXXR, FXXGXXXCXG and A/GGXD/ETT motifs that are characteristic of CYP450s were found to be well conserved in Scab79691 (Fig. 2.5), supporting the idea that Scab79691 is in fact a CYP450 enzyme. CYP450s are commonly associated with the biosynthesis of secondary metabolites in *Streptomyces* spp. For example, CYP450s are required for hydroxylation, epoxidation and/or carbonylation reactions involved in the biosynthesis of pharmaceutically important secondary

metabolites such as pladienolide B, oleandomycin, FD-891, filipin, albaflavenone and 7,30,40-trihydroxyisoflavonein (Urlacher and Girhard 2012). In *S. scabies* and other thaxtomin A - producing species, two CYP450s, TxtC and TxtE, are required for hydroxylation and nitration of thaxtomin A, respectively (Barry et al. 2012). HPLC analysis of the culture extracts from the Δ*scab79691* mutant isolates showed no detectable production of CFA-L-Ile, indicating that Scab79691 is essential for COR-like metabolite biosynthesis. Interestingly, a possible biosynthetic intermediate with a molecular weight of 307.2 was found to accumulate in the mutant culture extracts (Fig. 2.15 and 2.16). Given that the culture extracts were shown to exhibit some hypertrophy-inducing activity in the potato tuber bioassay (Fig. 2.17), it appears as though the putative intermediate displays some of the same bioactivity as CFA-L-Ile.

The genetic complementation of the Δ*scab79691* mutant was also unsuccessful overall, although CFA-L-Ile production did occur at low levels in one of the mutant isolates containing pMSAK13/*scab79691* (Fig. 2.20). Given that the pMSAK13 plasmid integrates into the *S. scabies* chromosome at a different location than the CFA-like biosynthetic gene cluster, it is possible that the expression of the cloned *scab79691* gene is affected by the surrounding genetic context. Alternatively, there might be an unknown problem with the pMSAK13/*scab79691* expression plasmid itself that does not allow for proper expression of the *scab79691* gene. It also cannot be completely ruled out that the deletion of *scab79691* has polar effects on the expression of downstream genes in the CFA-like biosynthetic gene cluster. Future work should therefore focus on ruling out any potential polar effects of the *scab79691* gene deletion by testing for expression of the downstream genes or by making a marker-less deletion mutant. In addition, the

construction of a new *scab79691* expression plasmid may also be useful in future complementation studies with this mutant.

2.4.3 Proposed role of Scab79691 in COR-like metabolite biosynthesis

The results of this study as well as those from a previous study show that at least three of the novel genes within the *S. scabies* CFA-like biosynthetic gene cluster are necessary for normal production of the CFA-L-Ile COR-like metabolite. Altowairish (2014) demonstrated that deletion of the *scab79681/oxr* and *scab79721/sdr* genes leads to a drastic decrease in CFA-L-Ile production, and in the case of the Δ*scab79721/sdr* mutant, three new metabolites are known to accumulate in the mutant culture extracts. Based on these findings, a hypothetical biosynthetic pathway for production of CFA-L-Ile in *S. scabies* was proposed in which Scab79681/Oxr and Scab79721/Sdr are directly involved in the biosynthesis of the CFA moiety (Fig. 2.21). Scab79681/Oxr is a putative F_{420}-dependent oxidoreductase, and it has been proposed to be the enzyme that introduces the C=C double bond that is present in the CFA molecule (Fig. 2.21; Altowairish 2014). Scab79721/Sdr is a predicted short chain dehydrogenase/reductase, and this enzyme has been proposed to catalyze the formation of the carbonyl group on the CFA backbone by reduction of a hydroxyl group. Support for the role of Scab79721/Sdr comes from the fact that two of the accumulated metabolites in the mutant culture extract have a molecular mass that is the same as the mass of (e), which is the proposed Scab79721/Sdr substrate (c) linked to isoleucine (Fig. 2.21; Altowairish 2014). Based on the results of the current study, it can now be said that the Scab79691 CYP450 is also directly involved in the

biosynthesis of the CFA moiety, and it may do so by serving as the enzyme that introduces the hydroxyl group that is subsequently reduced by Scab79721/Sdr (Fig. 2.21). The mass of the accumulated metabolite in the Δ*scab79691* mutant culture extract (307.2) is consistent with the predicted Scab79691substrate (b) linked to isoleucine, which agrees with the proposed role of Scab79691. The fact that (b) itself was not detected in the mutant culture extracts suggests that the Cfl enzyme is able to utilize CFA biosynthetic intermediates as substrates for ligation with isoleucine (Fig. 2.21), an idea that was also proposed based on the Δ*scab79721/sdr* mutant results (Altowairish 2014).

The involvement of Scab79691, together with Scab79681/Oxr and Scab79721/Sdr, in the biosynthesis of the CFA moiety of the *S. scabies* COR-like metabolites is a novel finding. Similar enzymes have not previously been implicated in CFA biosynthesis in *P. syringae,* and a search of the *P. syringae* pv *tomato* DC3000 genome sequence (http://pseudomonas-syringae.org/) revealed that there are no homologues of Scab79691 produced by this organism. It is possible that *S. scabies* uses a novel biosynthetic pathway to produce the same family of phytotoxins that is made by *P. syringae.*

2.5 References

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Table 2.1: Bacterial strains, plasmids and cosmids used in this study.

Strain or plasmid/cosmid	Genotype or comments	Antibiotic resistance	Source or reference
***Escherichia coli* strains**			
BW25113	Host for Redirect PCR targeting system	N/A	Gust et al. 2003a,b
DH5α	General cloning host	N/A	Gibco-BRL
ET12567	Non-methylating host (*dam*$^-$ *dcm*$^-$ *hsdS*$^-$)	Cam^r (Chloramphenicol resistant), Tet^r (Tetracycline resistant)	MacNeil et al. 1992
NEB 5-alpha	DH5α derivative; high efficiency competent cells used for transformation	N/A	New England Biolabs
***Streptomyces scabies* strains**			
Δ*txtA*/ pRLDB51-1	*S. scabies* strain containing a deletion of the *txtA* gene and carrying the *scab79591* overexpression plasmid pRLDB51-1 integrated at the ϕC31 *attB* site	Thio^r, Apr^r (Apramycin resistant)	Bignell et al. 2010
Δ*scab79711*	Δ*txtA*/pRLDB51-1 derivative containing a deletion of the *scab79711*gene.	Thio^r (Thiostrepton resistant), Apr^r, Hyg^r (Hygromycin B resistant)	This study
Δ*cfa8*	Δ*txtA*/pRLDB51-1 derivative containing a deletion of the *cfa8* gene.	Thio^r, Apr^r, Hyg^r	This study
Δ*scab79691*	Δ*txtA*/pRLDB51-1 derivative containing a deletion of the *scab79691* gene.	Thio^r, Apr^r, Hyg^r	This study
Plasmids or cosmids			
pIJ790	λRED recombination plasmid; expresses the λRED recombinase genes (*gam, bet* and *exo*) from the L-arabinose inducible pBAD promoter	Cam^r	Gust et al. 2003a,b
pUZ8002	Encodes the machinery for	Kan^r (Kanamycin	Kieser et al.

	the conjugal transfer of DNA from *E. coli* into *Streptomyces*	resistant)	2000
Cosmid 1770	SuperCos1 derivative containing the *S. scabies* CFA-like gene cluster	Apr^r, Kan^r	Bignell et al. 2010
1770/ Δ*scab79711*	Cosmid 1770 derivative in which the *scab79711* gene was replaced with the [*hygr+oriT*] disruption cassette	Amp^r (Ampicillin resistant), Kan^r Hyg^r	This study
1770/Δ*cfa8*	Cosmid 1770 derivative in which the *cfa8* gene was replaced with the [*hygr+oriT*] disruption cassette	Amp^r, Kan^r Hyg^r	This study
1770/ Δ*scab79691*	Cosmid 1770 derivative in which the *scab79691* gene was replaced with the [*hygr+oriT*] disruption cassette	Amp^r, Kan^r Hyg^r	This study
pMSAK13	pIJ10257 derivative containing the *neo* gene and promoter sequence cloned into the EcoRV site; integrates into the ϕBT1 *attB* site in *Streptomyces* chromosomes and carries the strong, constitutive promoter *ermE*p*	Hyg^r, Kan^r	Altowairish 2014
pGEM® - T Easy	Cloning vector for PCR products	Amp^r	Promega
pMSAK13/*cfa8*	pMSAK13 derivative containing the *cfa8* gene cloned into the NdeI and XhoI sites	Hyg^r, Kan^r	This study
pMSAK13/ *scab79691*	pMSAK13 derivative containing the *scab79691* gene cloned into the NdeI and XhoI sites	Hyg^r, Kan^r	This study

Table 2.2: Oligonucleotide primers used in this study.

Primer Name	**Sequence* (5′→3′)**	**Use**
YL1	ATGACACCGCACAAGCCCGTGGTCGGGATCGTCGGCCTCATTCCGGGGATCCGTCGACC	Redirect primer for Δ*scab79711* mutant cosmid construction
YL2	TCACTGCGATCTCAGTCCCTTCCGGTTCTTGCGTCCCAATGTAGGCTGGAGCTGCTTC	Redirect primer for Δ*scab79711* mutant cosmid construction
YL4	TCATGACGAAGGTGTCCCCTTCTCCGGCAGGGAAGCGGCTGTAGGCTGGAGCTGCTTC	Redirect primer for Δ*cfa8* mutant cosmid construction
YL5	CCGCGGGCGACCCGTCGGCGGCGCGGGGAAACCCGATGAATTCCGGGGATCCGTCGACC	Redirect primer for Δ*cfa8* mutant cosmid construction
JS3	GTGGTGCTGGGCGCGGAGTTCGTGCGGAATCCGCATGAGATTCCGGGGATCCGTCGACC	Redirect primer for Δ*scab79691* mutant cosmid construction
YL7	CGCTGTGCCGTCCGGACGGGCAGGGACCGCAGCCCGTGGTGTAGGCTGGAGCTGCTTC	Redirect primer for Δ*scab79691* mutant cosmid construction
YL12	GTGAGGCGTGGGAGGCCAAC	PCR verification of Δ*scab79711* mutant cosmid and strain (junction 1)
YL13	GCGTCAGCCGGGCAGGATAG	PCR verification of Δ*scab79711* mutant cosmid and strain (junction 1)
YL14	GACTTCGCCCGCGAACTGCT	PCR verification of Δ*scab79711* mutant cosmid and strain (junction 2)
YL15	TCCCCAGCTCCACCACGGAG	PCR verification of Δ*scab79711* mutant cosmid and strain (junction 2)
YL10	CCCCAGCATGCTCCACCACG	PCR verification of Δ*cfa8*

		mutant cosmid and strain
YL11	AGGCCGACGATCCCGACCA	PCR verification of Δ*cfa8* mutant cosmid and strain
YL8	GGGACAAGGAGTGCGGAGCC	PCR verification of Δ*scab79691* mutant cosmid and strain
YL9	CATCGGGTTTCCCCGCGCC	PCR verification of Δ*scab79691* mutant cosmid and strain
YL18	GCGC**CATATG**ATGAGTTCCACAACGAG TGA	PCR amplification of *scab79691* for construction of complementation plasmid
YL19	GCGC**CTCGAG**ACGATGTCCTTCATCGG GTT	PCR amplification of *scab79691* for construction of complementation plasmid
YL22	CCATGGTCAGGACGGTCATT	Verification of *scab79691* complementation plasmid
YL23	CGCGATGAACACGAACTCAC	Verification of *scab79691* complementation plasmid
YL20	GCGC**CATATG**ATGACCGCGGGCGACC CGTC	PCR amplification of *cfa8* for construction of complementation plasmid
YL21	GCGC**CTCGAG**AATCCGATCACCTCGTG CCC	PCR amplification of *cfa8* for construction of complementation plasmid
YL24	GGGCGACAACATCCTCATCT	Verification of *cfa8* complementation plasmid
YL25	GGAGTACACCTTGGAGAGCG	Verification of *cfa8* complementation plasmid
ermEp* For	GCGATGCTGTTGTGGGC	Sequencing of pMSKA13 clones

* Non-homologous extensions are underlined, while engineered restriction sites are indicated in bold. N/A, not applicable.

Table 2.3: Closest homologue, predicted protein domains, and predicted function of the Scab79711, Cfa8 and Scab79691 proteins encoded in the *S. scabies* CFA-like gene cluster.

Protein	**Closest homologue in database (% identity/ similarity)**	**Domains present (E-value)**	**Predicted Function**
Scab 79711	*S. antibioticus* Hydroxybutyryl-CoA dehydrogenase (69/78)	3-hydroxyacyl-CoA dehydrogenase NAD binding domain (1.8e-58); 3-hydroxyacyl-CoA dehydrogenase NAD binding domain (9.7 e-23); 3-hydroxyacyl-CoA dehydrogenase, C terminal domain (7.1e-25); 3-hydroxyacyl-CoA dehydrogenase, C terminal domain (1.3e-24)	Hydroxybutyryl-CoA dehydrogenase
Cfa8	*S. antibioticus* crotonyl-CoA reductase/carboxylase (85/93)	Zinc-binding dehydrogenase family (3.7e-41); Alcohol dehydrogenase GroES-like domain (6.6e-07)	Crotonyl-CoA reductase/carboxylase
Scab 79691	*S. ipomoeae* unspecific monooxygenase (70/81)	Cytochrome P450 domain (3.4e-22)	CYP450 monooxygenase

Table 2.4: Closest homologues of the Scab79711 and Cfa8 proteins encoded on the *S. scabies* 87-22 chromosome.

Protein	**Predicted Function**	**Homologues**	**% Identity /Similarity**	**E-value**
Cfa8	Crotonyl-CoA carboxylase/reductase	Scab17621	88/95	0.0
Scab79711	Hydroxybutyryl-CoA dehydrogenase	Scab17601	45/59	6e-158
		Scab74071	41/55	4e-57
		Scab28641	37/55	6e-52

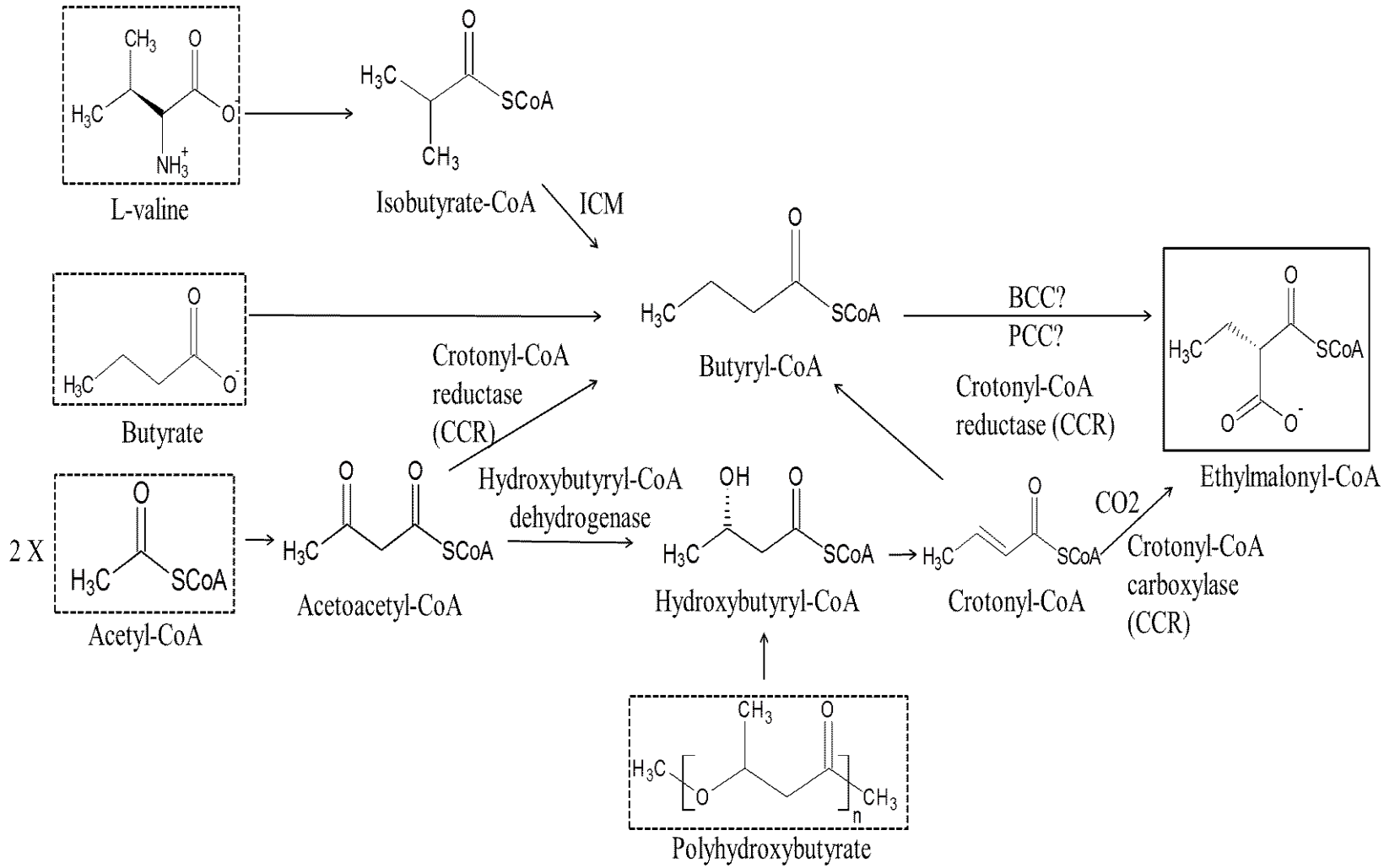
L-valine
Isobutyrate-CoA
ICM
Butyrate
Crotonyl-CoA
reductase
(CCR)
Butyryl-CoA
BCC?
PCC?
Crotonyl-CoA
reductase (CCR)
Ethylmalonyl-CoA
2 X
Acetyl-CoA
Acetoacetyl-CoA
Hydroxybutyryl-CoA
dehydrogenase
Hydroxybutyryl-CoA
Crotonyl-CoA
CO2
Crotonyl-CoA
carboxylase
(CCR)
Polyhydroxybutyrate

Figure 2.1: Metabolic pathways leading to the formation of ethylmalonyl-CoA in bacteria (Chan et al. 2009). The extender unit is in the solid box, and starting metabolites that can lead to the extender unit are in dashed boxes. CCR, crotonyl-CoA carboxylase/reductase; BCC, butyryl-CoA carboxylase; PCC, propionyl-CoA carboxylase.

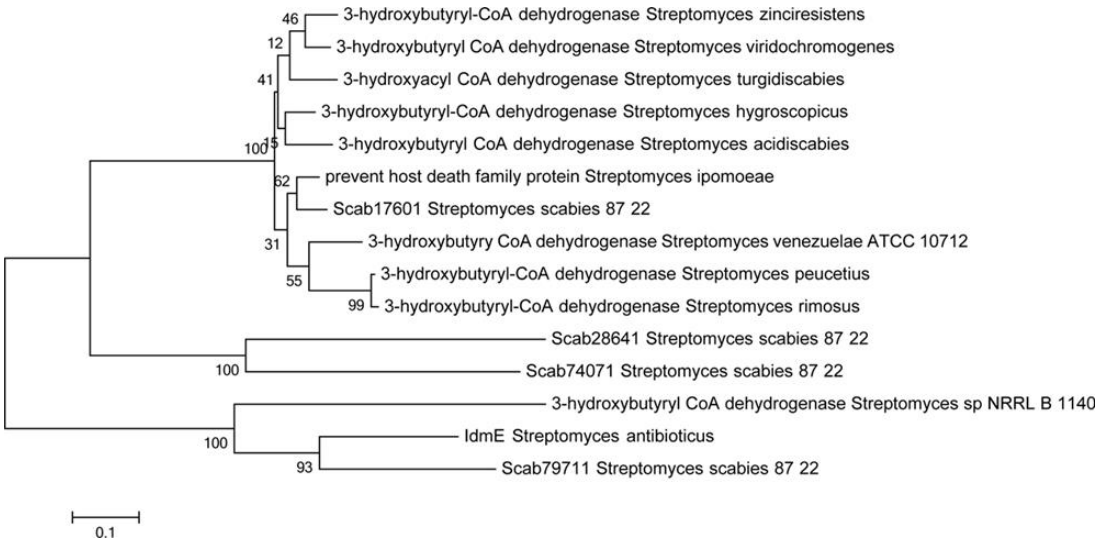

Figure 2.2: Phylogenetic relationships of hydroxybutyryl-CoA dehydrogenase homologues from *Streptomyces* spp. Bootstrap values are shown at the branch points. The scale bar indicates the number of amino acid substitutions per site. Accession numbers for each sequence are as follows: 3-hydroxybutyryl-CoA dehydrogenase *Streptomyces zinciresistens* (WP_007498531.1); 3-hydroxybutyryl-CoA dehydrogenase *Streptomyces viridochromogenes* (WP_003994089.1); 3-hydroxyacyl-CoA dehydrogenase *Streptomyces turgidiscabies* (WP_006377336.1); 3-hydroxybutyryl-CoA dehydrogenase *Streptomyces hygroscopicus* (WP_014675988.1); 3-hydroxybutyryl-CoA dehydrogenase *Streptomyces acidiscabies* (WP_010355092.1); prevent host death family protein *Streptomyces ipomoeae* (WP_009327483.1); Scab17601 *Streptomyces scabies* 87-22 (WP_012999615.1); 3-hydroxybutyryl-CoA dehydrogenase *Streptomyces peucetius* (WP_031189204.1); 3-hydroxybutyryl-CoA dehydrogenase *Streptomyces rimosus* (KEF19360.1); Scab28641 *Streptomyces scabies* 87-22 (WP_013000643.1); Scab74071 *Streptomyces scabies* 87-22 (WP_013004929.1); 3-hydroxybutyryl-CoA dehydrogenase

Streptomyces sp. NRRL B-1140 (AGZ94354.1); IdmE *Streptomyces antibioticus* (ACN69981.1); Scab79711 S*treptomyces scabies* 87-22 (WP_013005381.1).

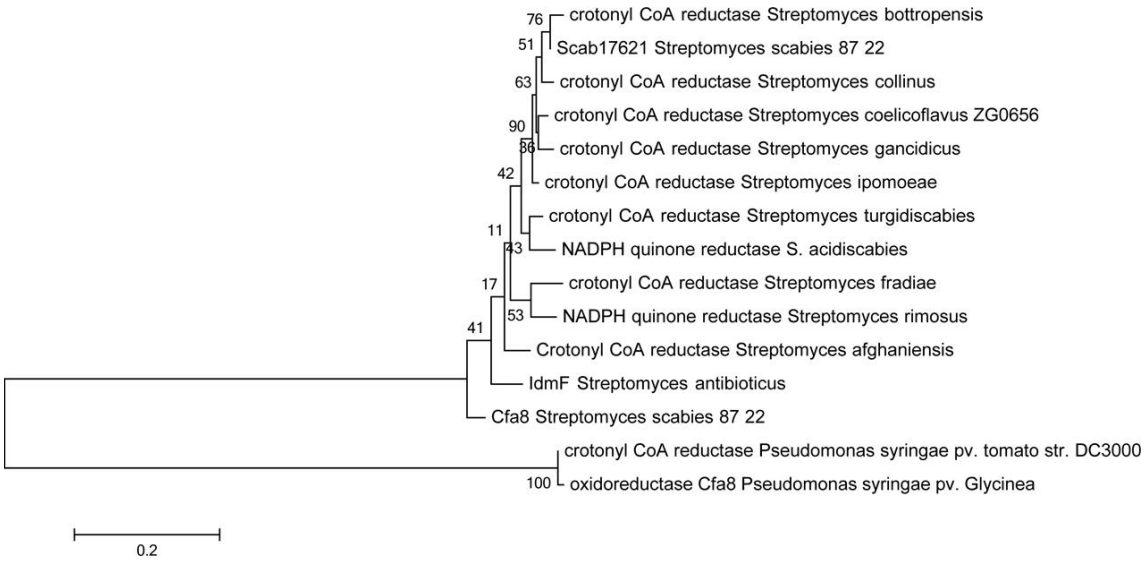

Figure 2.3 Phylogenetic relationships of CCR homologues in the database. Bootstrap values are shown at the branch points. The scale bar indicates the number of amino acid substitutions per site. Accession numbers for each sequence are as follows: Scab17621 S*treptomyces scabies* 87-22 (WP_012999617.1); crotonyl CoA reductase *Streptomyces bottropensis* (WP_005482325.1); crotonyl CoA reductase *Streptomyces collinus* (WP_020943170.1); crotonyl CoA reductase *Streptomyces coelicoflavus* ZG0656 (EHN79708.1); crotonyl CoA reductase *Streptomyces gancidicus* (EMF26267.1); crotonyl CoA reductase *Streptomyces ipomoeae* (WP_009327493.1); crotonyl CoA reductase *Streptomyces turgidiscabies* (WP_006377331.1); NADPH quinone reductase *S. acidiscabies* (WP_010355090.1); Crotonyl CoA reductase *Streptomyces afghaniensis* (WP_020277510.1); IdmF *Streptomyces antibioticus* (ACN69982.1); crotonyl CoA reductase *Streptomyces fradiae* (CAA57474.2) ; NADPH quinone reductase *Streptomyces rimosus* (WP_030642327.1) ; Cfa8 *Streptomyces scabies* 87-22 (WP_013005380.1); crotonyl CoA reductase *Pseudomonas syringae* pv. *tomato* str.

DC3000 (AAO58133.1); oxidoreductase Cfa8 *Pseudomonas syringae* pv. *glycinea* (AAC38656.1).

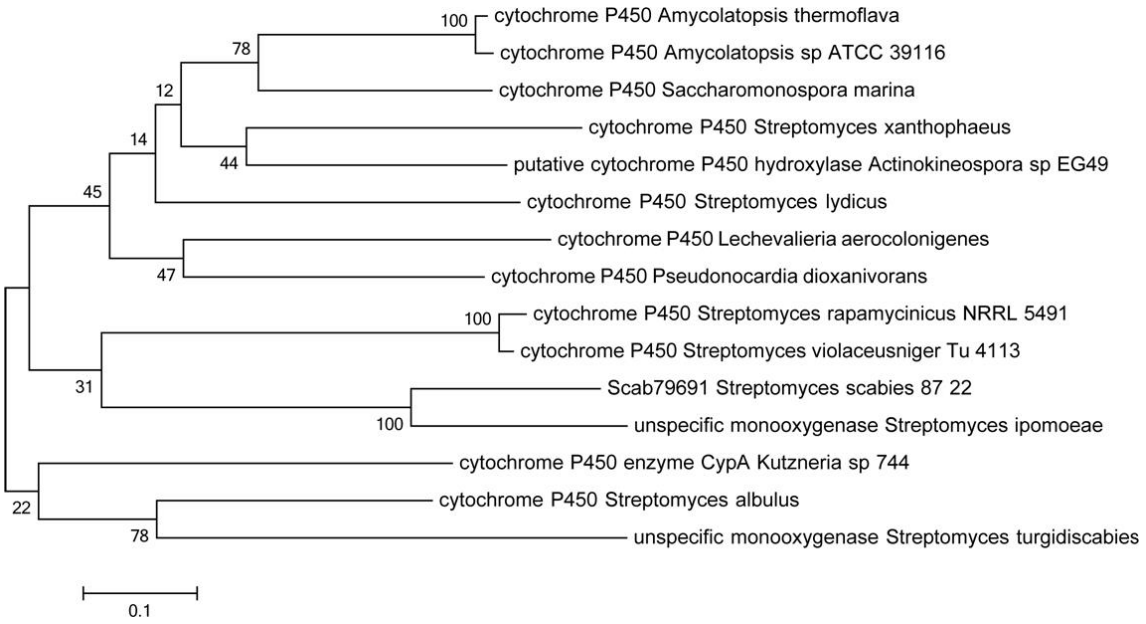

Figure 2.4: Phylogenetic relationships of CYP450 monooxygenase homologues in the database. Bootstrap values are shown at the branch points. The scale bar indicates the number of amino acid substitutions per site. Accession numbers for each sequence are as follows: cytochrome P450 *Amycolatopsis thermoflava* (WP_027934798.1); cytochrome P450 *Amycolatopsis sp.* ATCC 39116 (WP_020421854.1); cytochrome P450 *Saccharomonospora marina* (WP_009152790.1); ; cytochrome P450 *Actinokineospora sp.* EG49 (EWC63042.10); cytochrome P450 *Streptomyces lydicus* (CBA11565.1); cytochrome P450 *Lechevalieria aerocolonigenes* (WP_030471252.1); cytochrome P450 *Pseudonocardia dioxanivorans* (WP_013675876.1); cytochrome P450 *Streptomyces rapamycinicus* NRRL 5491 (WP_020870458.1); cytochrome P450 *Streptomyces violaceusniger* (WP_014058477.1); Scab79691 *Streptomyces scabies* 87-22 (WP_013005379.1); unspecific monooxygenase *Streptomyces ipomoeae* (WP_009328794.1); cytochrome P450 enzyme CypA *Kutzneria sp.* 744 (EWM16617.1); cytochrome P450 *Streptomyces albulus* (WP_016577953.1); unspecific monooxygenase

Streptomyces turgidiscabies (WP_006383617.1).

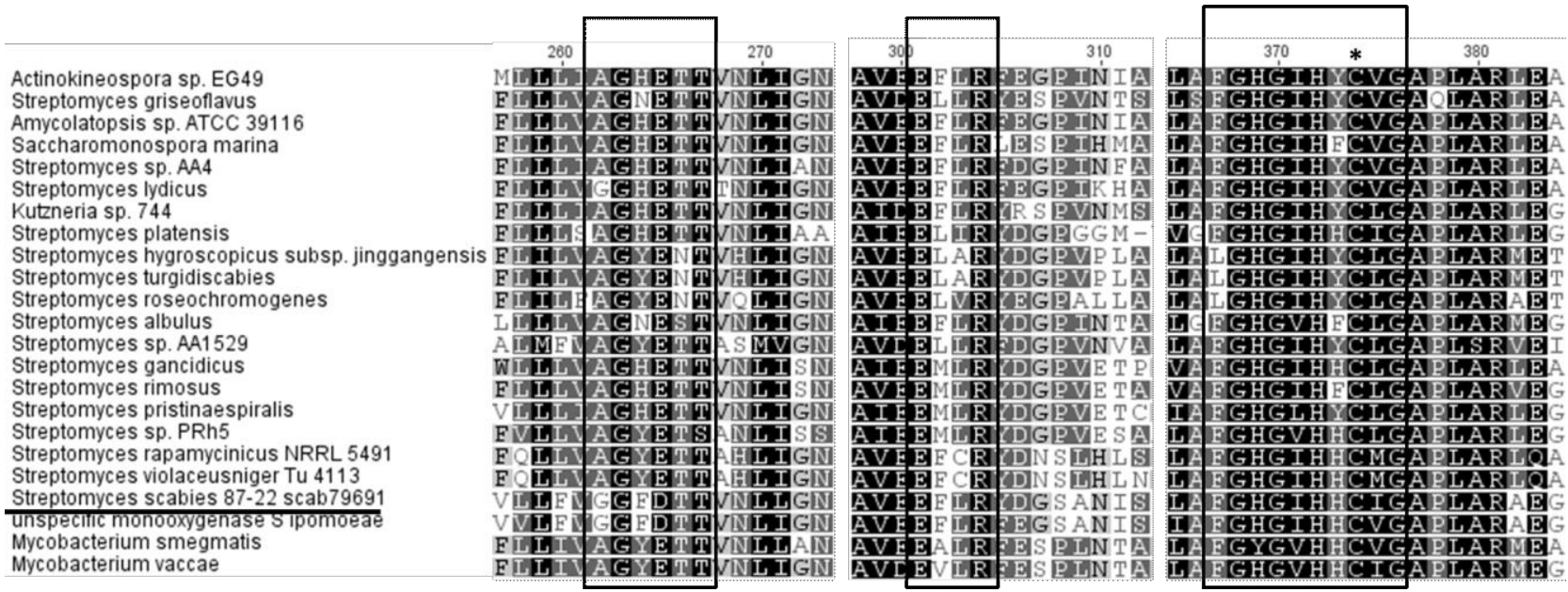
260
270
300
310
370
*
380
Actinokineospora sp. EG49
Streptomyces griseoflavus
Amycolatopsis sp. ATCC 39116
Saccharomonospora marina
Streptomyces sp. AA4
Streptomyces lydicus
Kutzneria sp. 744
Streptomyces platensis
Streptomyces hygroscopicus subsp. jinggangensis
Streptomyces turgidiscabies
Streptomyces roseochromogenes
Streptomyces albulus
Streptomyces sp. AA1529
Streptomyces gancidicus
Streptomyces rimosus
Streptomyces pristinaespiralis
Streptomyces sp. PRh5
Streptomyces rapamycinicus NRRL 5491
Streptomyces violaceusniger Tu 4113
Streptomyces scabies 87-22 scab79691
unspecific monooxygenase S ipomoeae
Mycobacterium smegmatis
Mycobacterium vaccae

Figure 2.5: Partial amino acid alignment of CYP450 monooxygenase homologues in the database. Black indicates 100% amino acid identity at a given position, while dark gray indicates 80-99% similarity, light gray indicates 60-79% similarity, and white indicates < 60% similarity. The conserved A/GGXD/ETT, EXXR and FXXGXXXCXG motifs are highlighted by the black boxes. The invariant cysteine residue found in all CYP450s is shown with the asterisk.

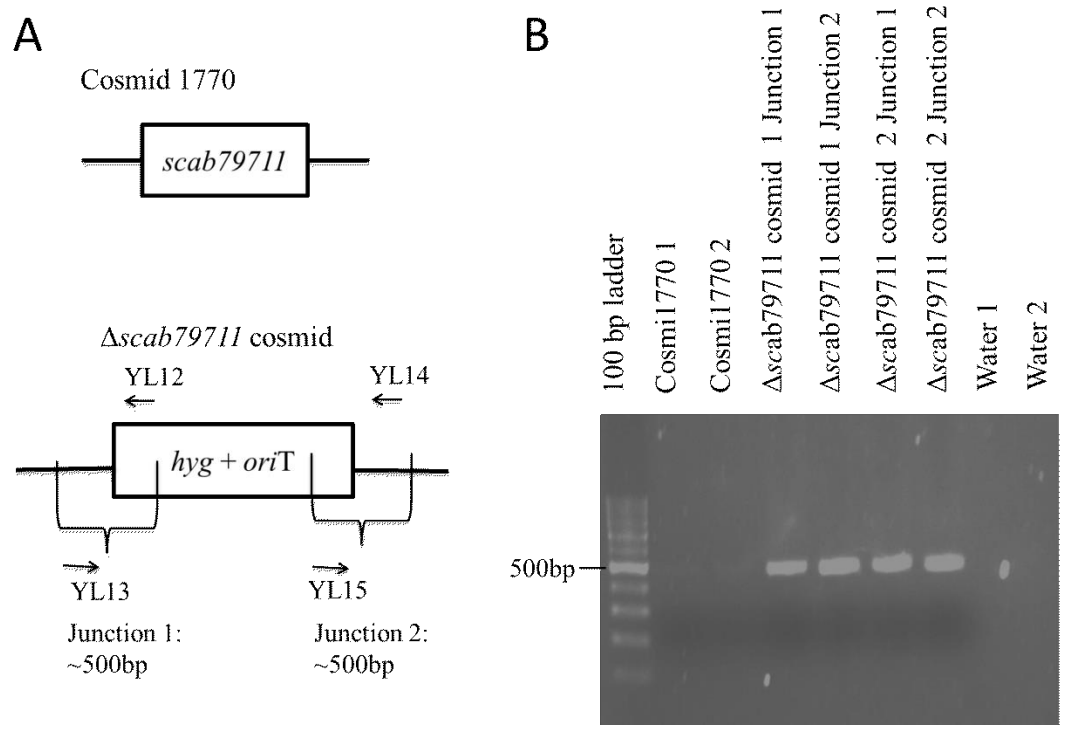

Figure 2.6: PCR verification of the Δ*scab79711* mutant cosmid DNA. (A) Strategy used to verify the mutant cosmid. Two pairs of primers (indicated by the arrows) were used to amplify the junctions of the [*hyg* + *ori*T] cassette that replaced the *scab79711* gene. Only the Δ*scab79711* cosmid template was expected to result in the correct PCR products (~500 bp in size each). (B) Agarose gel electrophoresis of the PCR products obtained. Negative control reactions were conducted using cosmid 1770 DNA as template or using water in place of template DNA. The size (bp) of each product was estimated according to the 100bp ladder used.

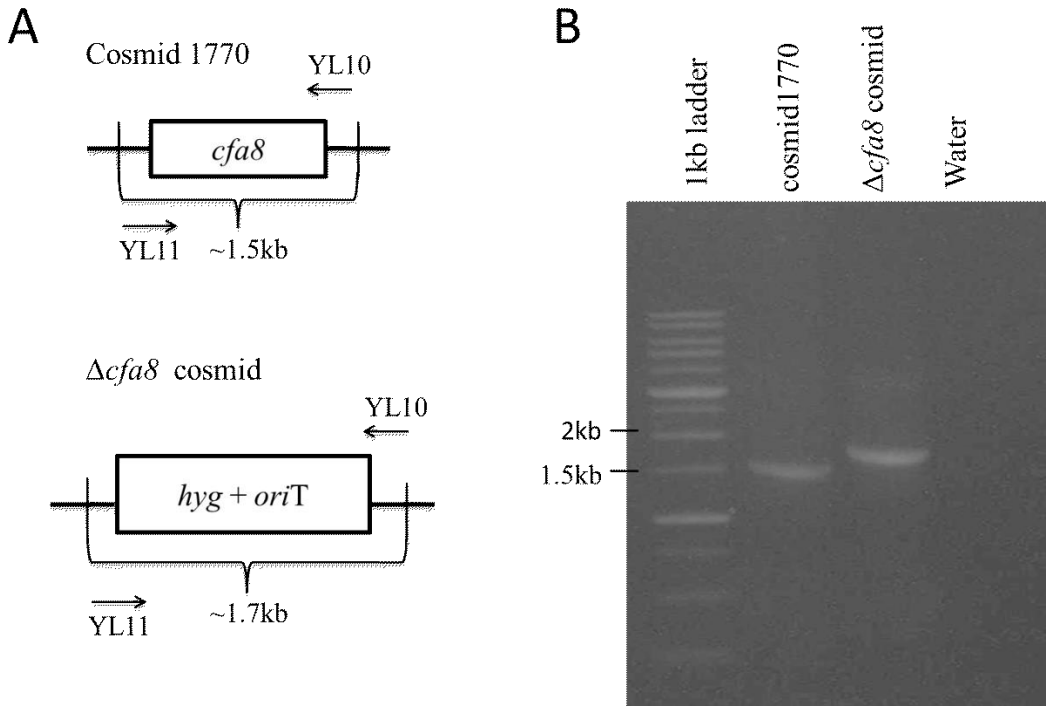

Figure 2.7: PCR verification of the Δ*cfa8* mutant cosmid DNA. (A) Strategy used to verify the mutant cosmid. The primers used for amplification are indicated by the arrows. The expected product sizes using cosmid 1770 and the Δ*cfa8* cosmid as template are indicated. (B) Agarose gel electrophoresis of the resulting PCR products. Negative control reactions were conducted using water in place of template DNA. The size (kb) of each product was estimated according to the 1kb ladder used.

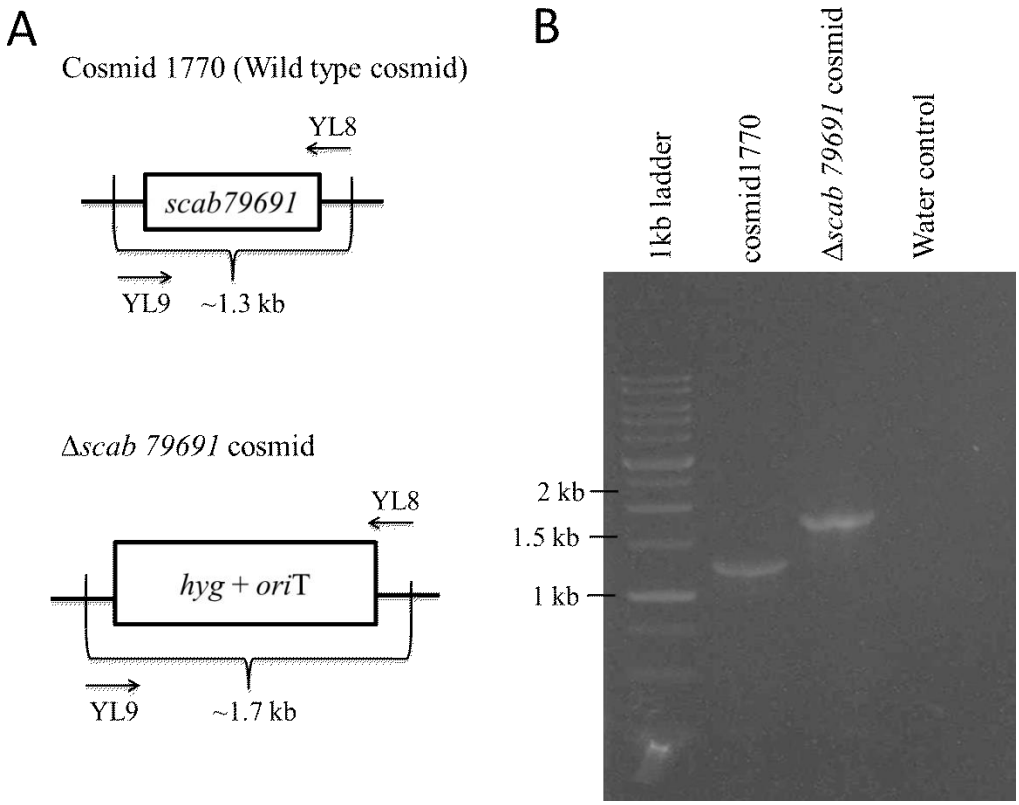

Figure 2.8: PCR verification of the Δ*scab79691* mutant cosmid DNA. (A) Strategy used to verify the mutant cosmid. The primers used for amplification are indicated by the arrows. The expected product sizes using cosmid 1770 and the Δ*scab79691* cosmid as template are indicated. (B) Agarose gel electrophoresis of the PCR products obtained. Negative control reactions were conducted using water in place of template DNA. The size (kb) of each product was estimated according to the 1kb ladder used.

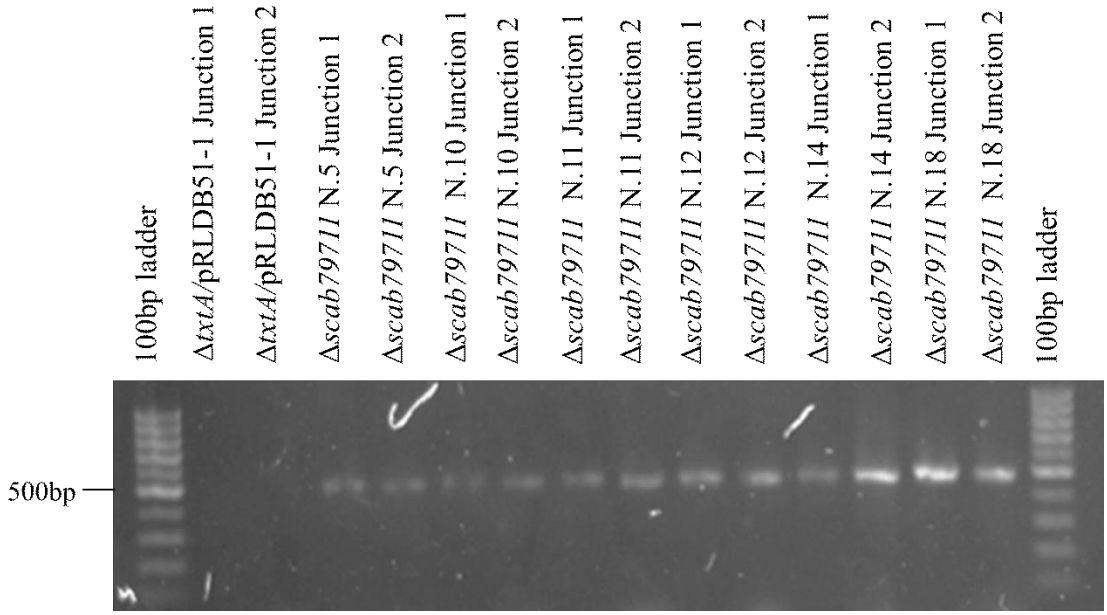

Figure 2.9: PCR verification of the six *S. scabies* Δ*scab79711* mutant isolates. Genomic DNA was harvested from each isolate and was used as a template in the PCR reactions. The primers used and the expected product sizes are described in Fig. 2.6. Negative control reactions were conducted using genomic DNA from the *S. scabies* Δ*txtA/*pRLDB51-1 strain. The size (bp) of each product was estimated according to the 100bp marker ladder that was used.

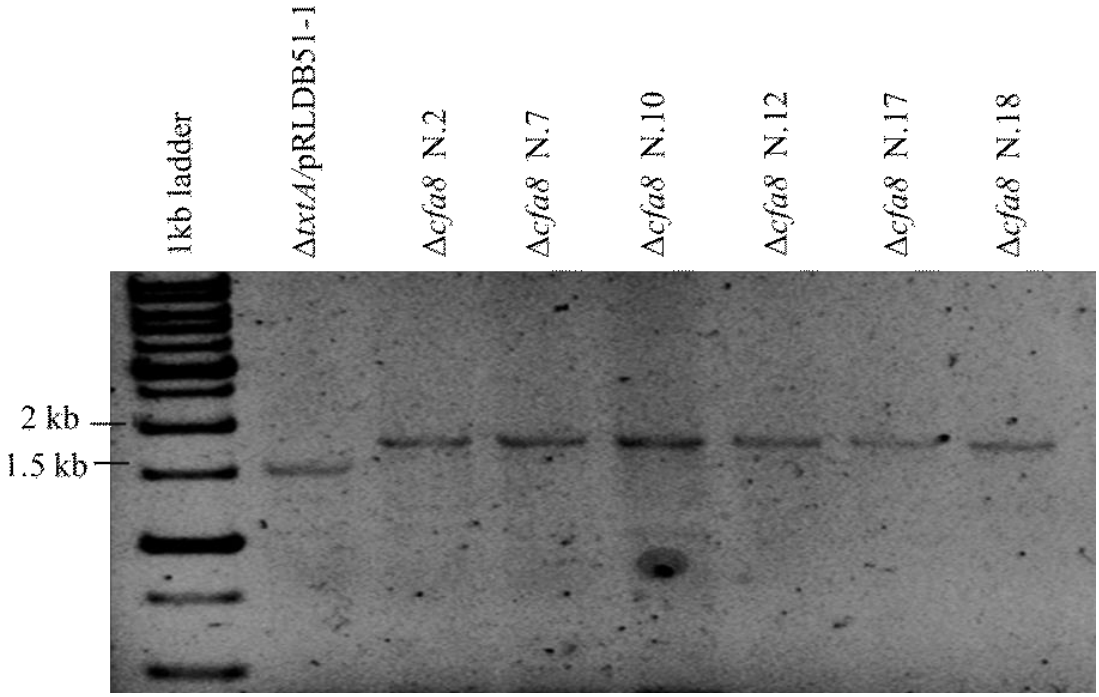

Figure 2.10: PCR verification of the six *S. scabies* Δ*cfa8* mutant isolates. Genomic DNA was harvested from each isolate and was used as a template in the PCR reactions. The primers used and the expected product sizes are described in Fig. 2.7. Genomic DNA from the *S. scabies* Δ*txtA/*pRLDB51-1 strain was included as a control. The size (kb) of each product was estimated according to the 1kb marker ladder that was used.

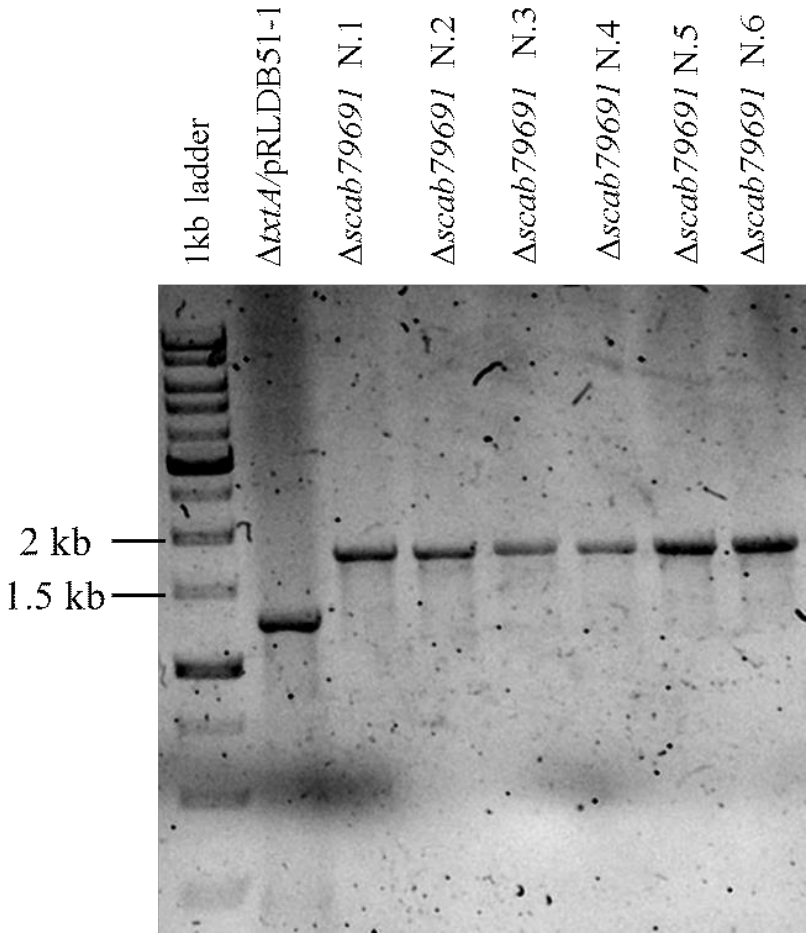

Figure 2.11: PCR verification of the six *S. scabies* Δ*scab79691* mutant isolates. Genomic DNA was harvested from each isolate and was used as a template in the PCR reactions. The primers used and the expected product sizes are described in Fig. 2.8. Genomic DNA from the *S. scabies* Δ*txtA*/pRLDB51-1 strain was included as a control. The size (kb) of each product was estimated according to the 1kb marker ladder used.

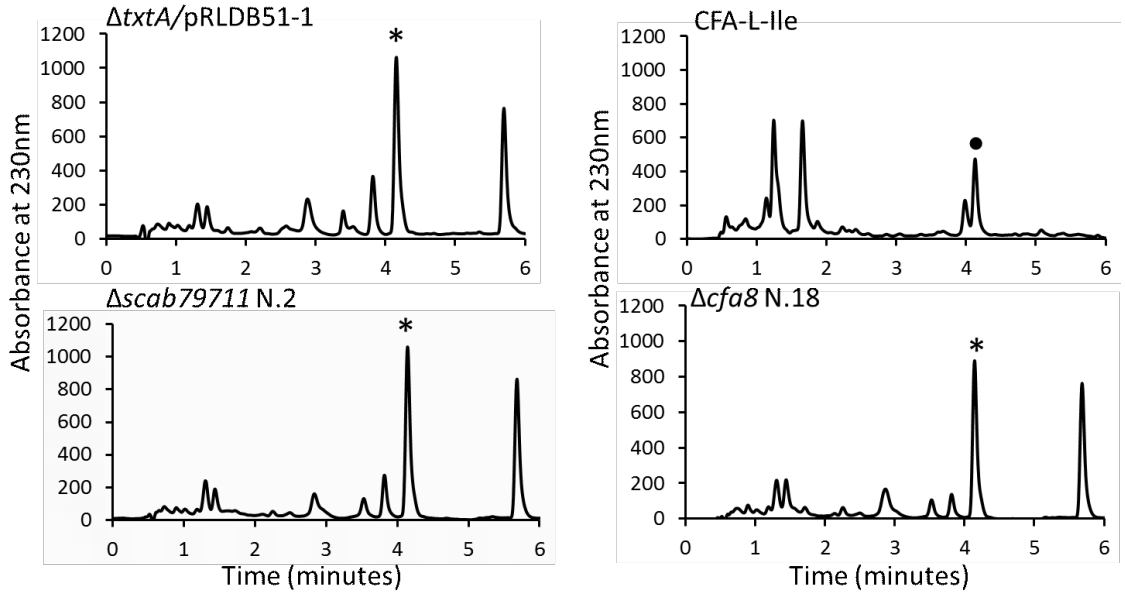

Figure 2.12: HPLC analysis of acidic culture extract from the *S. scabies* Δ*txtA/*pRLDB51-1 strain and the Δ*scab79711* (isolate N.2) and Δ*cfa8* (isolate N.18) mutants. * Indicates the peak corresponding to the primary COR-like metabolite (CFA-L-Ile) produced by *S. scabies.* The HPLC chromatogram of an authentic CFA-L-Ile standard (where ● indicates the CFA-L-Ile peak) is also shown.

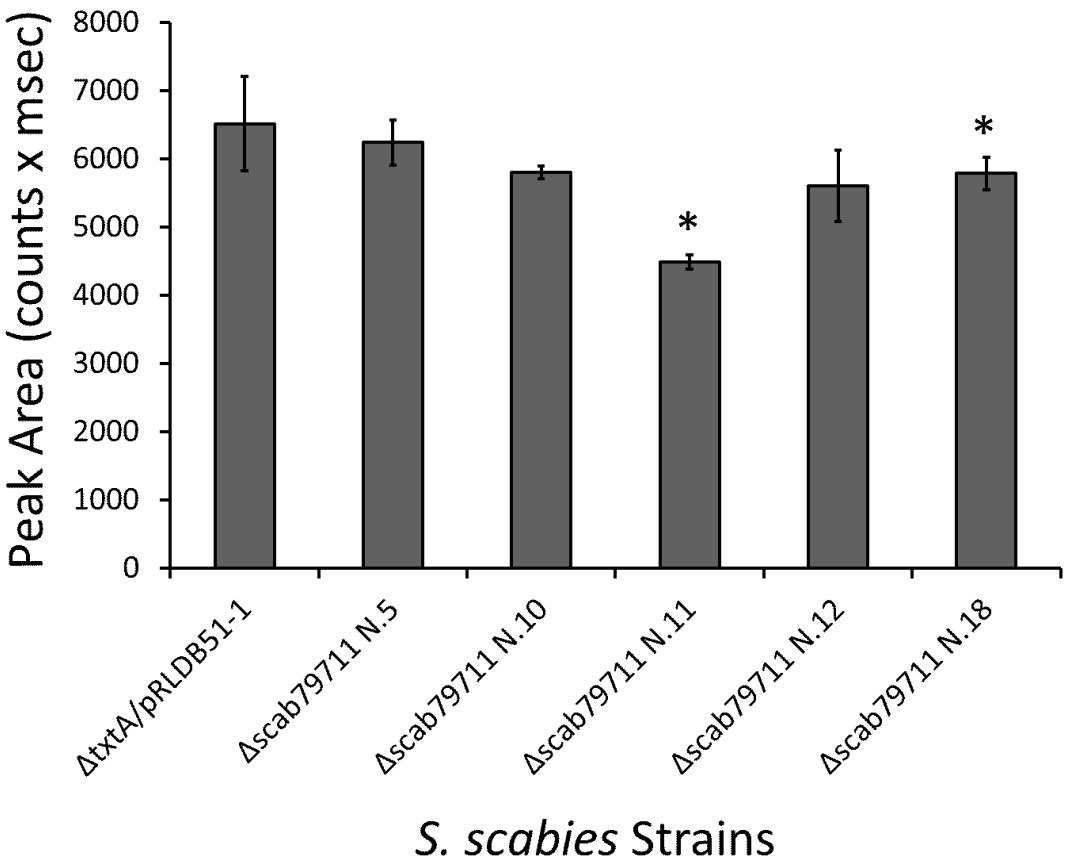

Figure 2.13: Relative production levels of the CFA-L-Ile COR-like metabolite in the Δ*txtA/*pRLDB51-1 strain and the Δ*scab79711* mutant isolates. The bars indicate the mean production level from triplicate cultures of each strain, and error bars indicate the standard deviation from the mean. Mutant production levels that were determined to be significantly different (* $p < 0.05$) from that of the Δ*txtA/*pRLDB51-1 strain are indicated.

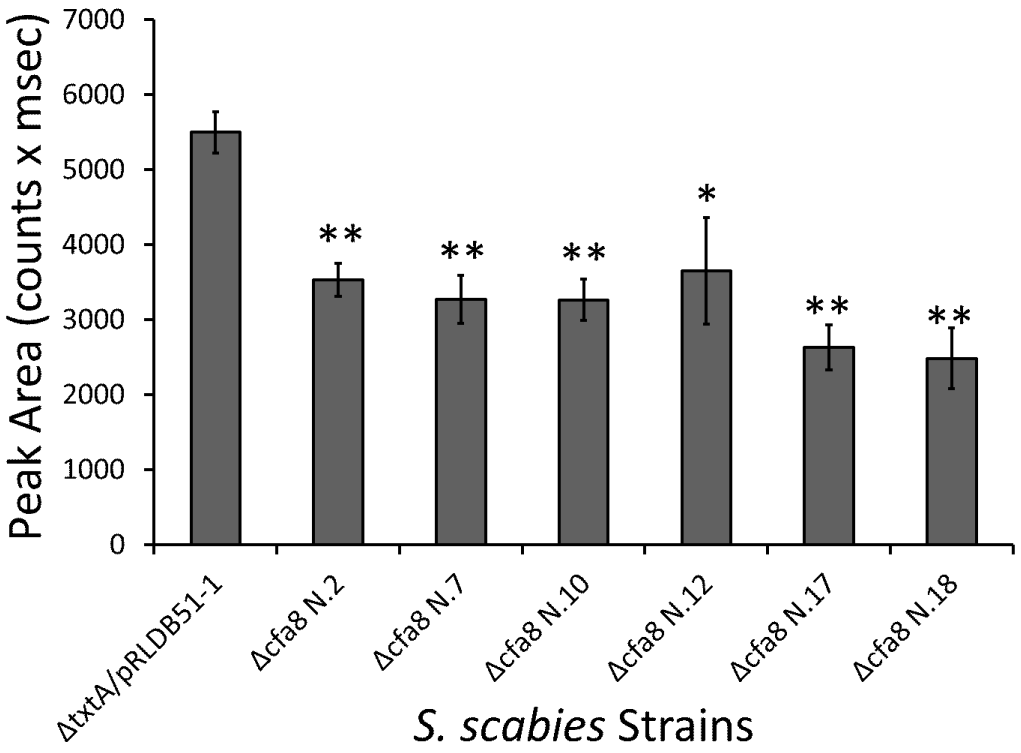

Figure 2.14: Relative production levels of the CFA-L-Ile COR-like metabolite in the Δ*txtA/*pRLDB51-1 strain and the Δ*cfa8* mutant isolates. The bars indicate the mean production level from triplicate cultures of each strain, and error bars indicate the standard deviation from the mean. Mutant production levels that were determined to be significantly different (* $p < 0.05$; ** $p < 0.01$) from that of the Δ*txtA/*pRLDB51-1 strain are indicate.

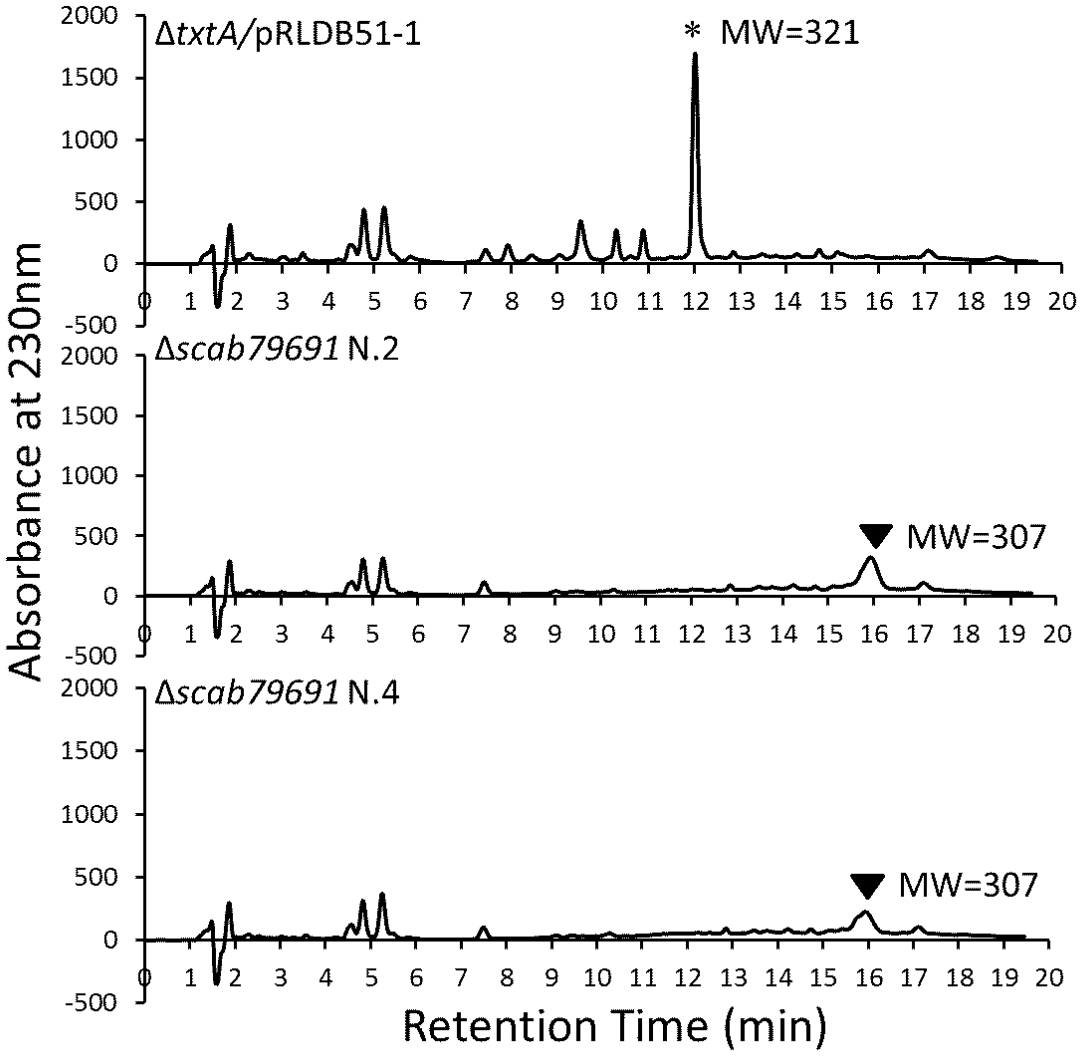

Figure 2.15: HPLC analysis of the acidic culture extracts from the *S. scabies* Δ*txtA*/pRLDB51-1 strain and the Δ*scab79691* mutant (isolate N.2 and N.4). The peak representing the CFA-L-Ile is indicated with (*), while the putative intermediate accumulating in the Δ*scab79691* mutant cultures (isolate N.2 and N.4) is indicates with (▼). The molecular mass (in Da) of CFA-L-Ile and of the accumulated metabolite, as determined by LC-MS, is indicated.

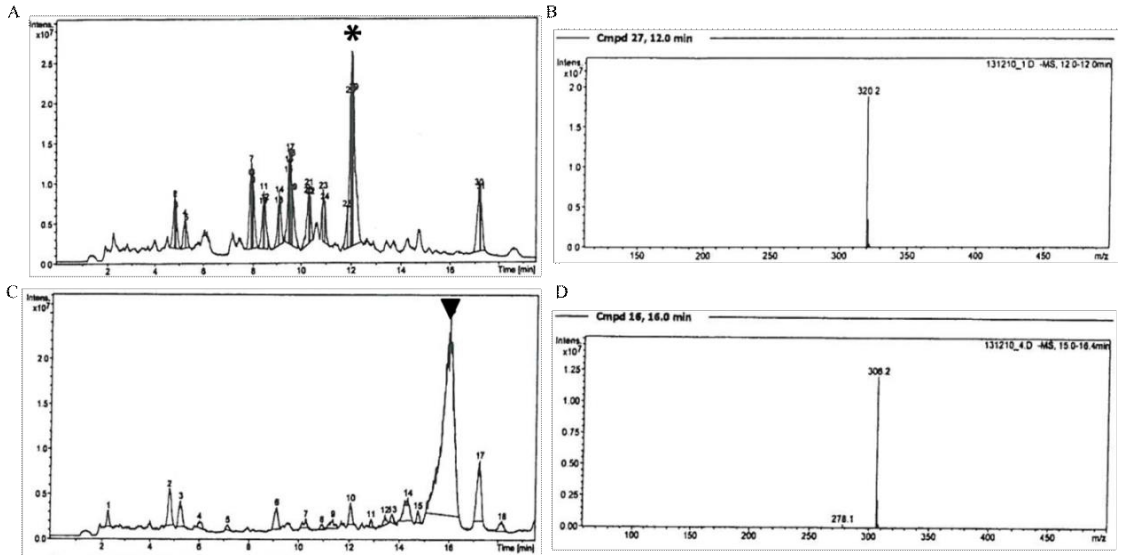

Figure 2.16: LC-MS analysis of the *S. scabies* the Δ*txtA*/pRLDB51-1 and Δ*scab79691* (isolate N.2) strains. Total ion current chromatograms were obtained for the Δ*txtA*/pRLDB51-1 (A) and Δ*scab79691* (C) strains, with (*) indicating the CFA-L-Ile peak in the Δ*txtA*/pRLDB51-1 extract and (▼) indicating the accumulated metabolite in the Δ*scab79691* mutant extract. The extracted-ion chromatogram for the CFA-L-Ile peak (B) and the accumulated metabolite peak (D) are also shown along with the corresponding *m/z* in negative ion mode $[M-H]^-$.

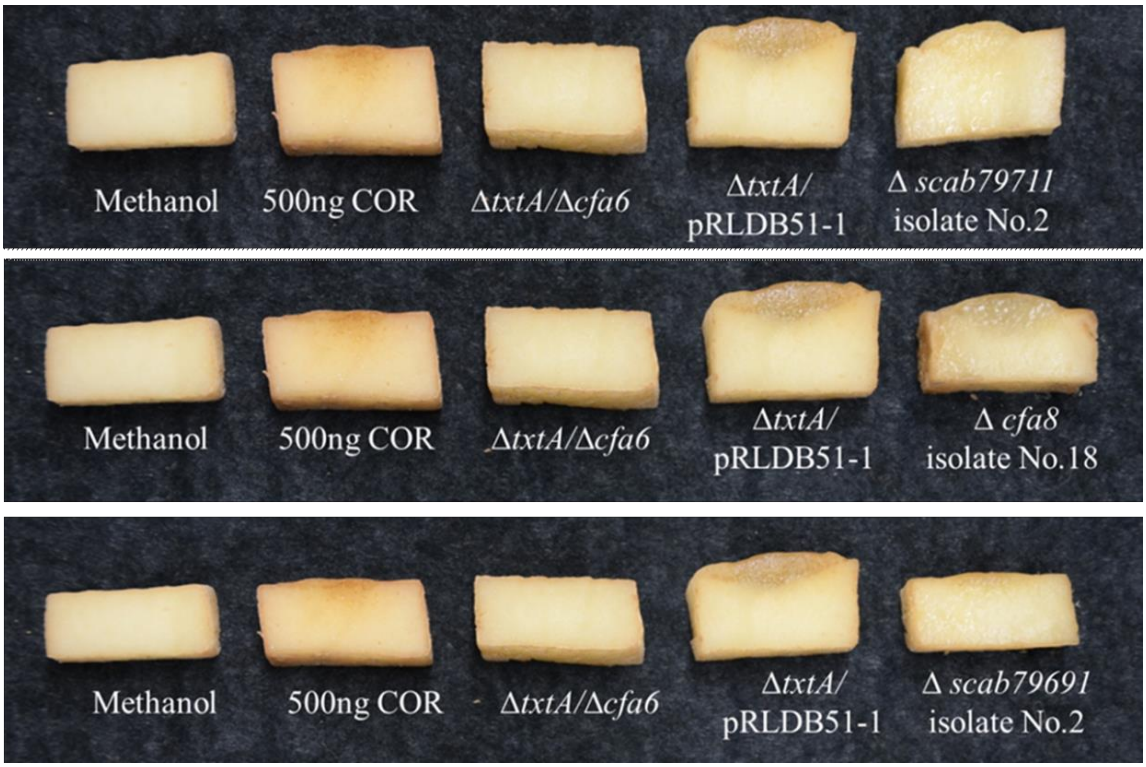

Figure 2.17: Bioactivity of the *S. scabies* Δ*scab79711* (isolate N.2)*,* Δ*cfa8* (isolate N.18) and Δ*scab79691* (isolate N.2) organic acidic culture extracts on potato tuber tissue. Pure authentic COR and extract from the Δ*txtA*/pRLDB51-1 strain served as positive controls, whereas methanol and extract from a COR-like metabolite deficient strain Δ*txtA*/Δ*cfa6* were included as negative controls.

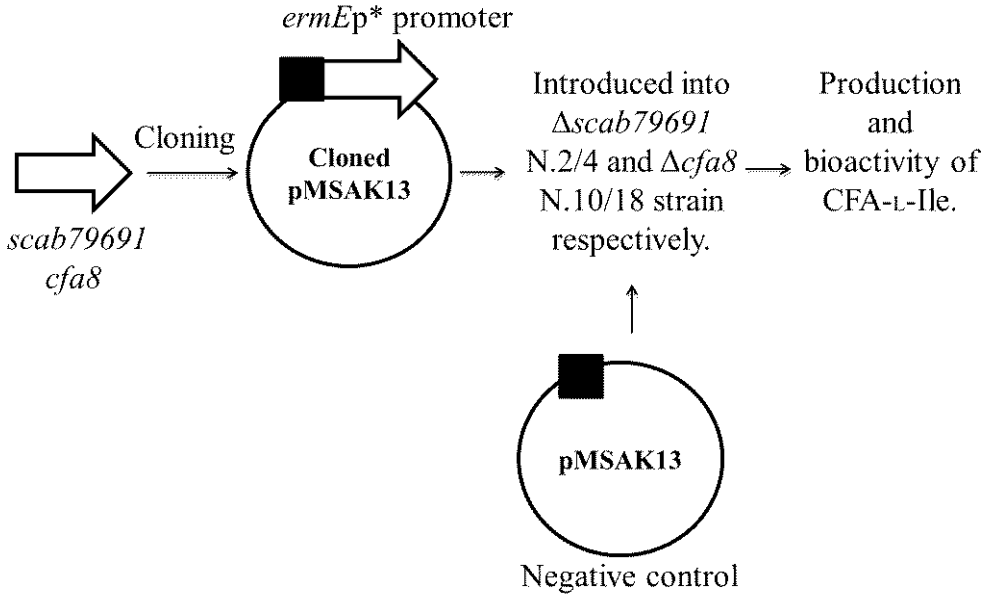

Figure 2.18: Strategy for genetic complementation of the *S. scabies* Δ*scab79691* and Δ*cfa8* deletion mutants. The *scab79691* and *cfa8* gene were first cloned into the pGEM-T EASY vector. After sequencing, the clones were digested with NdeI and XhoI to release the inserts. Plasmid pMSAK13 was digested with the same enzymes and was ligated separately with each gene insert. Positive clones (pMSAK13/*scab79691*, pMSAK13/*cfa8*), along with the empty pMSAK13 vector, were subsequently introduced into the corresponding *S. scabies* mutant strain by conjugation with *E. coli*, and the resulting exconjugants were analyzed for COR-like metabolite production. The black box indicates the strong, constitutive promoter *ermE*p* in the pMSAK13 vector.

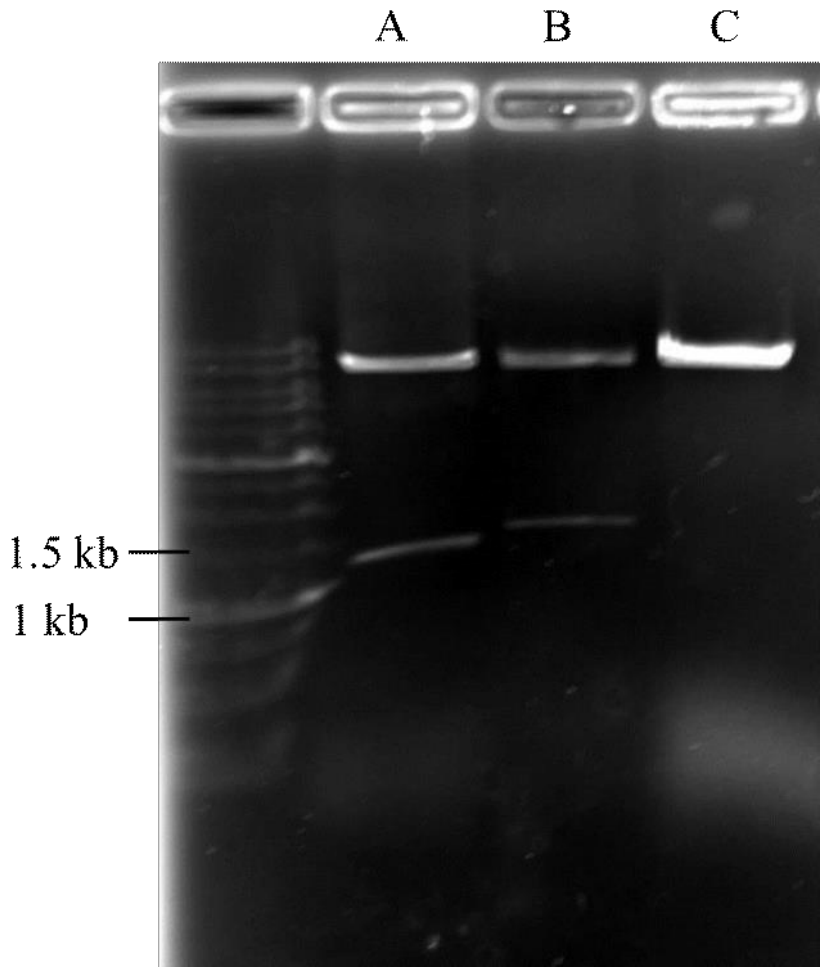

Figure 2.19: Verification of gene *scab79691* and *cfa8* complementation plasmids by restriction digestion. Plasmids pMSAK13/*scab79691* (A), pMSAK13/*cfa8* (B), pMSAK13 (C) were digested with NdeI and XhoI to release the cloned inserts, and the resulting products were analyzed by agarose gel electrophoresis. The expected size of *scab79691* insert is 1270bp and the *cfa8* insert is 1513bp. The bands (bp) of the 1kb marker are indicated in the first line.

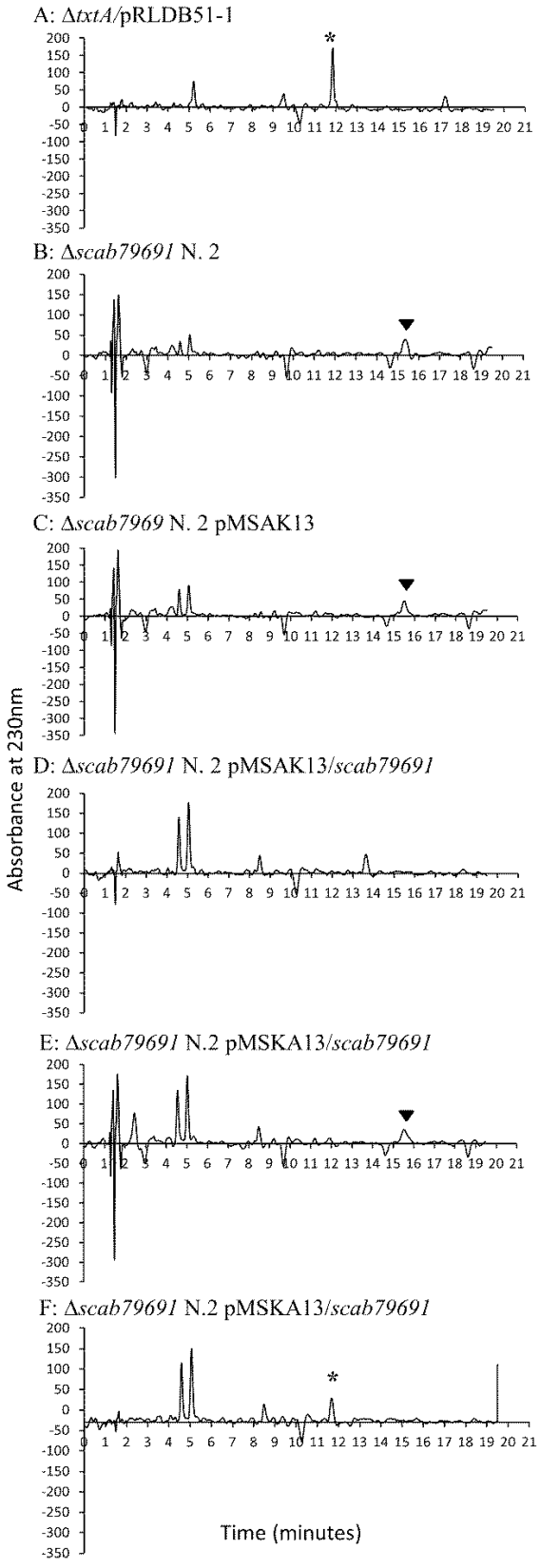
A: Δ*txtA*/pRLDB51-1
B: Δ*scab79691* N. 2
C: Δ*scab7969* N. 2 pMSAK13
D: Δ*scab79691* N. 2 pMSAK13/*scab79691*
E: Δ*scab79691* N.2 pMSKA13/*scab79691*
F: Δ*scab79691* N.2 pMSKA13/*scab79691*
Absorbance at 230nm
Time (minutes)

Figure 2.20: HPLC analysis of the acidic culture extracts from the *S. scabies* Δ*txtA*/pRLDB51-1 strain (A), Δ*scab79691* mutant (isolate N.2) strains (B), complemented Δ*scab79691* N.2 pMSAK13/*scab79691* strains (D-F) and its negative control Δ*scab79691* N.2 pMSAK13 strain (C). The peak representing CFA-L-Ile is indicated with (*), while the putative intermediate (Fig. 2.15-16) accumulating from the Δ*scab79691* mutant (N.2), the negative controls (Δ*scab79691* N.2 pMSAK13) and some complemented strain (Δ*scab79691* N.2 pMSAK13/*scab79691*) cultures are indicates with (▼).

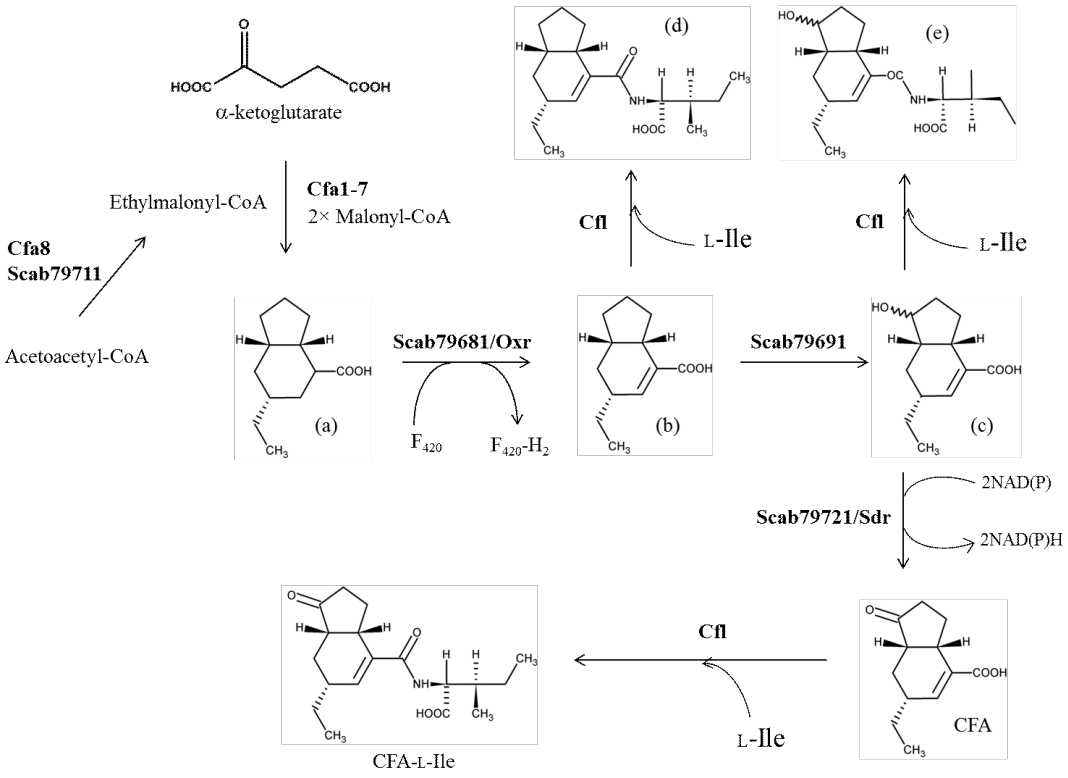

Figure 2.21: Proposed biosynthetic pathway for the CFA-L-Ile COR-like metabolite in *S. scabies*.

CHAPTER 3: Characterizing the biological activity of the *Streptomyces scabies* COR-like metabolites

3.1 Introduction

COR is a non-host-specific polyketide phytotoxin produced by at least five pathovars of the hemibiotrophic pathogen *P. syringae,* including *atropurpurea, glycinea, maculicola, morsprunorum,* and *tomato.* Though not essential for *P. syringae* pathogenicity, COR plays several important roles during the host infection process (Xin and He 2013). Infection by *P. syringae* begins with the pathogen colonizing and penetrating the plant surface. This process is facilitated by COR, which inhibits the closure of stomata (Lee et al. 2013). Stomata are tiny pores found in the epidermis of terrestrial plants, and the closure of such pores in response to PAMPs (Pathogen-associated molecular pattern) is considered an important innate defense mechanism against foliar pathogens (Melotto et al. 2006). It has been shown that COR-deficient mutants of *P. syringae* inoculated on the surface of wild-type *Arabidopsis thaliana* leaves are considerably reduced in their ability to cause infection, whereas such mutants can readily infect *A. thaliana* mutants that are deficient in stomatal closure (Melotto et al. 2006; Zeng et al. 2011; Zeng and He 2010), thereby illustrating the importance of COR in overcoming stomatal defenses. After successful invasion, COR also facilitates bacterial multiplication and persistence within the plant intercellular spaces by overcoming the apoplastic defenses (Zeng et al. 2011). Furthermore, COR contributes to disease symptom development by *P. syringae*. The primary symptom caused by COR is leaf chlorosis (yellowing), which can be observed in a variety of different plant species

(Bender et al. 1999a, b). Intense chlorosis in response to COR develops in soybean and tobacco leaf tissues, and less intense chlorosis forms in potato, tomato, pepper, cabbage and corn leaf tissues (Gnanamanickam et al. 1982; Uppalapati et al. 2005, 2007; Wangdi et al., 2010). The chlorosis symptom has been attributed to the ability of COR to induce the expression of chlorophyllase, which breaks down chlorophyll (Tsuchiya et al. 1999). In *Arabidopsis* and tomato, COR induces the production of anthocyanins, which result in the formation of a purple colour in the plant tissue (Feys et al., 1994; Uppalapati et al. 2005). Other symptoms attributed to COR include root stunting in *Arabidopsis* and tomato (Feys et al., 1994; Uppalapati et al. 2005), hypertrophy of potato tuber tissue (Völksch et al. 1989), ethylene emission (Ferguson and Mitchell 1985; Kenyon and Tuner, 1992) and tendril coiling in *Bryonia dioica* (Blechert et al. 1999). Furthermore, COR application to tomato leaves results in epidermal cell wall thickening, shrunken and more intensely stained chloroplasts, and deposition of proteinase inhibitor particles in plant cell vacuoles (Palmer and Bender 1995; Uppalapati et al. 2005).

It was previously noted by many that COR is structurally and functionally similar to the plant hormone JA, and in particular, the bioactive derivative JA-Ile (Fig. 3.1). JA is a plant growth regulator derived from the octadecanoid signaling pathway and is involved in controlling defense responses to biological stresses (Feys et al. 1994; Sembdner and Parthier 1993; Wasternack and Parthier 1997). When JA-Ile levels are low, JA-responsive genes are repressed by JAZ (Jasmonate zim domain) repressor proteins. In response to biological stresses, JA-Ile accumulates and binds to the COI1 F-box protein, which determines the target specificity of the SCF (Skp1-Cullin1-F-box)^{COI1} E3 ubiquitin ligase. Binding of JA-Ile to COI1 promotes the formation of a SCF^{COI1}–JA-Ile–JAZ ternary

complex and the subsequent ubiquitination and degradation of the JAZ proteins, leading to activation of JA-responsive gene expression (Katsir et al. 2008a, b). As a molecular mimic of JA-Ile, COR can also bind with high affinity to COI1 and promote the degradation of JAZ proteins and the activation of JA-responsive genes (Katsir et al. 2008a, b; Melotto et al. 2008). This, in turn, leads to suppression of SA (Salicylic acid)-dependent signalling pathways, which are critical for defense against *P. syringae* infection (Zhao et al. 2003; Brooks et al. 2005; Cui et al. 2005; Laurie-Berry et al. 2006). Remarkably, COR is more effective than JA-Ile in promoting SCF^{COI1} -JAZ interaction in *vitro*, although COR and JA-Ile are recognized by the same receptor (Katsir et al. 2008a). There is also evidence that COR suppresses plant defense responses to PAMPs by callose deposition inhibition in an SA signaling-independent manner (Geng et al. 2012).

S. scabies was recently shown to produce the COR-like metabolite CFA-L-Ile as a major product as well as other minor related metabolites, including CFA-D-Val (Fyans et al. 2014). CFA-L-Ile and other coronafacoyl-amino acid conjugates are produced in minor amounts by *P. syringae* (Bender et al. 1999a), and production of such molecules has also been reported for the plant pathogen *Xanthomonas capmpestris* pv *phormiicola* (Mitchell and Frey 1986; Mitchell 1985). The COR-like molecules produced by *P. syringae* and *X. capmpestris* are known to exhibit chlorosis-inducing activity (Bender et al. 1999a; Mitchell and Ford 1998), and like COR, the CFA-L-Val produced by *P. syringae* can cause changes in chloroplast ultrastructure, cell wall thickening, and accumulation of proteinase inhibitors, though it is not as toxic as COR (Uppalapati et al. 2005). Given that *S. scabies* culture extracts containing CFA-L-Ile and other minor COR-like metabolites are able to induce potato tuber tissue hypertrophy in a similar manner as COR (Fig. 2.17;

Altowairish 2014; Bignell et al. 2014), it is likely that the metabolites exhibit some or all of the other biological effects attributed to COR, though this has not been demonstrated. As such, the objective of this study was to further investigate the biological activity of the *S. scabies* COR-like metabolites using different plant bioassays.

3.2 Materials and methods

3.2.1 Bacterial strains, culturing conditions and maintenance

The *S. scabies* COR-like metabolite overproducing strain Δ*txtA/*pRLDB51-1 and the non-producing strains Δ*txtA/*Δ*cfa6* and Δ*cfl* (Table 2.1; Altowairish 2014) were used in this study. Strains were maintained as spore and mycelial stocks at -80°C as described in section 2.2.1.2. Growth of the strains for COR-like metabolite production was as described in section 2.2.1.2 except that the TSB seed cultures were sub-cultured into 25 ml of SFMB.

3.2.2 Chemical extraction of the COR-like metabolites

SFMB culture supernatants were extracted using chloroform as described previously (section 2.2.5.1). Extraction of uninoculated SFMB medium was also performed for use as a negative control in subsequent experiments. The resulting acidic extracts were redissolved in 2 mL of 100% v/v of HPLC-grade MeOH (Methanol).

3.2.3 Plant bioassays

3.2.3.1 Leaf infiltration bioassay using culture supernatants

SFMB culture supernatants were filter-sterilized using a syringe filter (0.2 μm pore size; VWR International), and 50 μL of each was infiltrated into leaves of 6-7 weeks old *Nicotiana benthamiana* plants. The infiltration was conducted by making a small incision on the upper side of a leaf using a syringe needle, and then placing the tip of a syringe (without a needle) containing culture supernatant against the leaf surface at the point of the incision. Next, the supernatant was injected while pressure was simultaneously applied to the underside of the leaf. This, in turn, allowed the supernatant to enter the intercellular space within the leaf in the vicinity of the point of injection. Each supernatant sample was infiltrated into the same leaf, and the infiltration was repeated on three leaves of the same plant and on three different plants for a total of 9 infiltrations per sample. Supernatant from an uninoculated SFMB culture served as the negative control while 3 nmol of COR (C8115; Sigma Aldrich Canada, Oakville, ON) served as the positive control. The plants were kept at room temperature (~22±2°C) under a 16 hour photoperiod for 7 days, after which the leaves were removed and photographed. The bioassay was performed three times.

3.2.3.2 Radish seedling bioassay using culture supernatants or organic culture extracts

Radish seeds (cv Cherry Belle; Heritage Harvest Seed, Carman, MB) were surface sterilized by treating with 40 mL of 70% v/v ethanol for 5 minutes with gentle agitation followed by 40 mL of 1% v/v sodium hypochlorite (Chlorox) with 0.1% v/v polysorbate 20 (ICN10316890, MP Biomedicals) for 10 minutes with gentle agitation. Next, the seeds were rinsed 8-10 times with sterile distilled water (~40 mL). The surface sterilized

radish seeds were placed into a deep Petri dish (89107-632, VWR International) containing Whatman #4 filter paper (90 mm) that had been wetted using 2 mL of sterile water containing nystatin (N6261-25MU; Sigma Aldrich Canada) at 50 μg/mL final concentration. The dish was wrapped with Parafilm and was incubated for ~24 hours at room temperature (~22±2°C) in the dark in order to allow seed germination to occur.

Filter-sterilized SFMB culture supernatants (5 mL) or organic culture extracts (0.5 mL of undiluted or 10-fold diluted extract) were pipetted into triplicate wells in 6-well tissue culture plates (BD Falcon). When organic extracts were used, the plates were left open in a biosafety cabinet to allow the solvent to evaporate completely, after which 5 mL of sterile water was added to the each well. Then, germinated seeds with a uniform hypocotyl and root length were selected, and four seeds were transferred to each well. Negative control wells contained either water, uninoculated SFMB medium, or extract from an uninoculated SFMB culture, while positive control wells containing 30 nmol of COR in water. The plates were wrapped with Parafilm and were incubated with gentle shaking (125 rpm) at room temperature (~22±2°C) for 4 days under a 16 hour photoperiod, after which the total length of each plant (root + shoot) was measured. The maximum and minimum measured seedling length in each treatment was omitted, and the remaining values were used to calculate the average seedling length per treatment. Statistically significant differences in seedling length were determined using the Student's *t*-test (tails: 2, type: 2) in Microsoft Excel. The bioassay was performed twice using supernatants and three times using organic extracts.

3.2.3.3 Radish seedling bioassay using pure CFA-L-Ile

Radish seedlings were surface sterilized and germinated as described in section 3.2.3.2. CFA-L-Ile, purified from *S. scabies* culture supernatant and dissolved in DMSO (provided by Dr. J. Fyans, Memorial University), was pipetted into triplicate wells in a 6-well tissue culture plate. Similarly, pure COR dissolved in 100% MeOH was pipetted into triplicate wells. Three different amounts of each metabolite (90, 9 and 0.9 nmol dissolved in 30 μL of solvent) were transferred to triplicate wells, and control wells contained 30 μL of the corresponding solvent (MeOH or DMSO), after which 5 ml of sterile water was added to the each well. Then, germinated seeds with uniform hypocotyl and root length were selected and transferred to the wells (4 plants per well). Incubation and processing of the plants was as described in section 3.2.3.2.

3.2.3.4 Analysis of anthocyanin production in radish seedlings

Anthocyanins were extracted and quantified from radish seedlings obtained in the bioassays described in sections 3.2.3.1 and 3.2.3.2. The method used is similar to the one described by Uppalapati et al. (2005) with some modifications. Three seedlings were placed into a tube containing 1 mL of a solution of 3M HCl:H_2O:MeOH (1:3:16 by volume). The tubes were agitated and then incubated at 4°C in the dark overnight. Next, the anthocyanin extracts (200 μL) were transferred to wells in a 96-well plate, and the absorbance at 530 and 653 nm (A_{530} and A_{653}) was measured in each well using a microplate Synergy H1 Hybrid Reader (BIOTEK, Winooski, Vermont, USA). Extracts were prepared using all 12 seedlings from each treatment resulting in three extracts per

treatment, and every extract was analyzed three times by the microplate reader. The anthocyanin levels were determined in each extract using the formula $A_{530} - 0.24A_{653}$ and were normalized using seedling wet weight. The average and standard deviation of the normalized anthocyanin levels for each treatment were determined, and the Student's *t* test (tails: 2, type: 2) in Excel was used to identify statistically significant differences between the treatments and the control. The bioassay was performed twice in total.

3.2.3.5 Potato tuber slice bioassay using pure CFA-L-Ile

This was performed as described previously in section 2.2.8 with some modifications mentioned below. Purified CFA-L-Ile (8, 16 and 32 nmol) dissolved in DMSO, and COR (8, 16 and 32 nmol) dissolved in MeOH, were applied to sterile paper disks on potato tuber slices. After 24 hours incubation, 25 µL of sterile water was added onto each disk, and the tuber slices were incubated for a further 4-6 days. Control slices were treated with MeOH or DMSO. Following incubation, the tuber slices were dissected and imaged. The bioassay was performed twice in total.

3.3 Results

3.3.1 The *S. scabies* COR-like metabolites are associated with necrosis of *N. benthamiana* leaf tissue

The induction of chlorosis in a variety of plant hosts is the primary disease symptom attributed to COR during *P. syringae* infections (Bender et al. 1999a, b). To determine whether the COR-like metabolites produced by *S. scabies* can also cause

chlorosis, a leaf infiltration bioassay was performed using *N. benthamiana* plants. *N. benthamiana* was chosen for this assay since COR is known to induce chlorosis in this plant (Uppalapati et al. 2011). Furthermore, the large, broad leaves of this plant make it a suitable host for infiltration assays. The assay was performed using filter-sterilized SFMB culture supernatants of a *S. scabies* COR-like metabolite overproducer (Δ*txtA/*pRLDB51-1) and of two COR-like metabolite non-producing strains (Δ*txtA*/Δ*cfa6* and Δ*cfl*). Supernatants, rather than organic culture extracts, were used in the assay in order to avoid any toxic effects due to the infiltration of organic solvents.

As shown in Figure 3.2, the *N. benthamiana* leaf tissue infiltrated with the Δ*txtA/*pRLDB51-1 culture supernatant did not show any obvious chlorosis, but instead it consistently showed brown, water-soaked necrosis. Leaf tissue infiltrated with the Δ*txtA*/Δ*cfa6* and Δ*cfl* culture supernatants also showed water-soaked necrosis surrounded by mild chlorosis in some instances; however, the effect of these supernatants was variable among the different replicates and was consistently less severe than the Δ*txtA/*pRLDB51-1 culture supernatant (Fig. 3.2B, C). Although infiltration with 3 nmol of COR did cause slight chlorosis in some replicates, the results were quite variable and in many cases, there was no apparent chlorosis induced by the COR as expected (Fig. 3.2D). It is likely that the concentration of COR used in this assay is not enough to induce chlorosis as 20 nmol of COR was reported to cause 5-10 mm chlorotic zone on tomato leaves (Uppalapati et al. 2005).

3.3.2 The *S. scabies* COR-like metabolites can cause stunting of radish seedlings

A radish seedling bioassay was next performed in order to determine whether the *S. scabies* COR-like metabolites can cause seedling stunting as previously described for COR (Feys et al. 1994; Uppalapati et al. 2005). The assay was performed using SFMB culture supernatants of the Δ*txtA/*pRLDB51-1, Δ*txtA*/Δ*cfa6* and Δ*cfl* strains as well as two different concentrations (undiluted and 10-fold diluted) of acidic organic extracts prepared from the culture supernatants. The results using supernatants (data not shown) and culture extracts (Figs. 3.3 and 3.4) were consistent and showed that the *S. scabies* COR-like metabolites can indeed cause stunting of the radish seedlings. Treatment with the Δ*txtA/*pRLDB51-1 culture extract caused significant stunting as compared to the mock (water) treated plants, with the undiluted extract being comparable to the COR treatment (Figs. 3.3 and 3.4). Although significant stunting was also observed with the undiluted Δ*txtA*/Δ*cfa6* and Δ*cfl* extracts, the effect was not as severe as with the undiluted Δ*txtA/*pRLDB51-1 culture extract (Figs. 3.3 and 3.4).

The stunting activity of the primary *S. scabies* COR-like metabolite, CFA-L-Ile, was also investigated in order to directly compare the bioactivity of this metabolite to that of COR. Equimolar amounts of each metabolite (90, 9 and 0.9 nmol) were used in the radish seedling bioassay, and as shown in Fig. 3.5, the growth of seedlings treated with all three concentrations of COR showed a statistically significant reduction when compared to the solvent (methanol) control plants, whereas only seedlings treated with 90 nmol of CFA-L-Ile showed a statistically significant reduction in growth when compared to the solvent (DMSO) control. Furthermore, the treatment with 90 nmol of CFA-L-Ile did not cause the same amount of seedling stunting as observed with any of the COR treatments

(Fig. 3.5). The results therefore demonstrate that CFA-L-Ile can cause stunting of radish seedlings at nanomolar concentrations; however it is less toxic than COR in its bioactivity.

3.3.3 Analysis of anthocyanin production in response to the COR-like metabolites

Next, the ability of the COR-like metabolites to induce anthocyanin production was investigated in a radish seedling bioassay. Figure 3.6 shows that treatment with filter-sterilized supernatant from cultures of the Δ*txtA/*pRLDB51-1 strain resulted in a significantly higher level of anthocyanins in radish seedlings compared to treatment with the Δ*txtA*/Δ*cfa6* and Δ*cfl* supernatants as well as treatment with uninoculated SFMB supernatant. It should be pointed out, however, that there was a high degree of variation in the results from one experiment to the next, and it was very difficult to consistently demonstrate a positive association between the presence of the COR-like metabolites and an increase in anthocyanin production. The assay was also performed using culture extracts (data not shown) as well as pure CFA-L-Ile (Fig. 3.7), and again, there was no consistent correlation between anthocyanin production and the presence of the COR-like metabolites. Treatment with 90 nmol of the pure CFA-L-Ile did cause an increase in anthocyanin production as compared to the solvent (DMSO) control, although the results were not deemed to be statistically significant. Though treatment with 0.9 nmol of COR did cause a significant increase in anthocyanin production compared to the solvent (MeOH) control (Fig. 3.7), it is noteworthy that the effect of COR on anthocyanin production was also found to be highly variable from one experiment to the next in this

bioassay.

3.3.4 The *S. scabies* CFA-L-Ile COR-like metabolite causes hypertrophy of potato tuber tissue

Results from this thesis (see Fig. 2.17) as well as from other studies in our lab (Altowairish 2014; Bignell et al. 2014) have demonstrated that *S. scabies* culture extracts containing the COR-like metabolites can induce hypertrophy of potato tuber tissue, an effect that has been described for COR (Völksch et al. 1989). To further study the hypertrophy-inducing activity of the CFA-L-Ile primary COR-like metabolite as compared to COR, a potato tuber slice bioassay was performed using equimolar amounts of COR and CFA-L-Ile. Figure 3.8 shows that treatment with 16 nmol of COR and CFA-L-Ile led to the formation of hypertrophic outgrowths on the potato tissue; however, the amount of hypertrophy observed was much less with the CFA-L-Ile treatment than with the COR treatment. Similar results were observed using higher and lower (8 and 32 nmol) amounts of each metabolite (data not shown). Therefore, though CFA-L-Ile exhibits the same effect as COR on potato tuber tissue, it is not as toxic as COR in its bioactivity.

3.4 Discussion

The COR phytotoxin from *P. syringae* is known to cause a variety of different effects against different plant hosts, including tissue hypertrophy, seedling stunting, anthocyanin production and chlorosis (Bender et al. 1999a,b). The aim of this study was to better characterize the bioactivity of the *S. scabies* COR-like metabolites in order to determine whether the metabolites exhibit some or all of the same bioactivities described

for COR and whether the primary metabolite, CFA-L-Ile, exhibits the same level of toxicity as COR. Previous studies showed that COR can cause stunting of tomato and *Arabidopsis* seedlings (Feys et al., 1994; Uppalapati et al. 2005), and results from the current study indicate that the *S. scabies* COR-like metabolites exhibit the similar effect against radish seedlings. Treatment of radish plants with culture extracts containing the COR-like metabolites caused comparable stunting as the COR control (Fig. 3.3 and 3.4). However in this study, bioassays using equimolar amounts of COR and CFA-L-Ile showed that the ability of CFA-L-Ile to cause seedling stunting is less than that of COR (Fig. 3.5). Similar results were observed in the potato tuber slice bioassay where an equimolar amount of CFA-L-Ile caused reduced tuber tissue hypertrophy compared to COR (Fig. 3.8). The finding that CFA-L-Ile is not as toxic as COR is in agreement with previous reports which showed that COR is the most toxic coronafacoyl compound produced by *P. syringae* (Shiraishi et al. 1979; Uppalapati et al. 2005). The fact that the *S. scabies* culture extracts containing the COR-like metabolites showed similar seedling stunting and tissue hypertrophy – inducing activity as COR in the radish (Fig. 3.3 and 3.4) and potato tuber (Fig. 2.17; Altowairish 2014; Bignell et al. 2014) bioassays, respectively, may be due to the presence of very high amounts of CFA-L-Ile in the extracts, or perhaps the other minor COR-like metabolites in the extracts are also required for optimal bioactivity against plant tissues.

This study also addressed whether or not the *S. scabies* COR-like metabolites are able to cause the production of anthocyanins, an effect that has been observed in tomato and *Arabidopsis* plants in response to COR (Feys et al., 1994; Uppalapati et al. 2005). Anthocyanins are natural pigments produced by plants as a hallmark of stress induced by

pathogens, herbivores and UV light (Shan et al. 2009). Although results obtained in this study suggest that anthocyanin production does occur in response to the COR-like metabolites in radish seedlings (Fig. 3.6 and 3.7), the results from replicate experiments were too inconsistent to conclusively say this. It is worth pointing out, though, that inconsistent results were also observed in the bioassays with the COR control, suggesting that the assay used was not ideal for testing this bioactivity. Red skin radish (*Raphanus savitus* L. var. "Cherry Belle") used in the bioassay contains a high level of anthocyanins naturally (Papetti et al. 2014), and this may be the reason why there was no obvious and reproducible increase in anthocyanins in response to COR and CFA-L-Ile. Therefore, to study the effect of the COR-like metabolites on anthocyanin production, tomato and *Arabidopsis* plants, which are both naturally low in anthocyanins, could be used so that any changes can be more readily observed.

The primary symptom induced by COR is chlorosis, the severity of which varies depending on the plant host (Gnanamanickam et al. 1982; Uppalapati et al. 2005, 2007; Wangdi et al., 2010). Chlorosis-inducing activity has also been reported for other coronafacoyl compounds (Mitchell 1985), and it was anticipated that a similar effect would be observed in response to the *S. scabies* COR-like metabolites. However, in *N. benthaminana* infiltration bioassays performed in this study, no chlorosis was observed in response to culture supernatants containing the COR-like metabolites, and instead the liquid- infiltrated tissue became water-soaked and necrotic (Fig. 3.2), an effect that is reminiscence of the hypersensitive response in plants (Heath 2000). A similar effect could also be observed in tissues infiltrated with culture supernatants lacking the COR-like metabolites, though the severity of tissue damage was always less than that observed

when the COR-like metabolites were present (Fig. 3.2). It is likely that the observed tissue damage is in response to other known or predicted virulence factors such as TomA and Nec1 that are likely present within the supernatants, and that the COR-like metabolites enhance the severity of the tissue damage that is caused by these other virulence factors. The chlorosis-inducing activity of the *S. scabies* COR-like metabolites, therefore, will require further investigation, most likely by testing aqueous solutions of the pure metabolites rather than complex culture supernatants.

COR is known to function as a molecular mimic of bioactive JA derivatives such as JA-Ile, thereby stimulating JA-mediated signaling pathways in the plant host (Katsir et al. 2008a, b). JA/ET-mediated signaling pathways play a critical role in controlling host defense responses to necrotrophic pathogens, which kill host tissue early during infection in order to obtain nutrients from the dead or dying host cells (Glazebrook 2005). In contrast, SA-mediated signaling pathways are generally regarded as being important for inducing defense responses to biotrophic pathogens, which feed on living plant tissue without causing host cell death (Glazebrook 2005). SA- and JA/ET-mediated signaling pathways exhibit antagonistic crosstalk such that activation of SA-mediated pathways leads to suppression of JA/ET-mediated pathways, and vice versa (Derksen et al. 2013). Previous reports have provided convincing evidence that SA-mediated defense response pathways are important for defense against *P. syringae* (Brooks et al. 2005; Laurie-Berry et al. 2006), and it has been proposed that COR is produced by *P. syringae* in order to take advantage of the antagonistic interactions between JA/ET- and SA- mediated signaling pathways in order to suppress the defense responses that are relevant to *P. syringae* (Xin and He 2013). As *P. syringae* is considered a hemibiotrophic pathogen

since it exhibits a biotrophic phase during the early stages of infection and a necrotrophic phase during the later stages (Xin and He 2013), it is likely that production of COR is critical for overcoming host defenses early during the infection process.

The structural and phenotypic similarities shared by COR and the *S. scabies* COR-like metabolites suggest that the COR-like metabolites may also have a role in suppressing SA-mediated defense pathways during pathogen infection. Whether SA-mediated signaling pathways play an important role in host defense against *S. scabies* infection, however, is currently unknown, and warrants further investigation. It also cannot be ruled out that the COR-like metabolites have additional roles during pathogen infection, and therefore further investigations into the function of the metabolites during host-pathogen interactions is needed.

3.5 References

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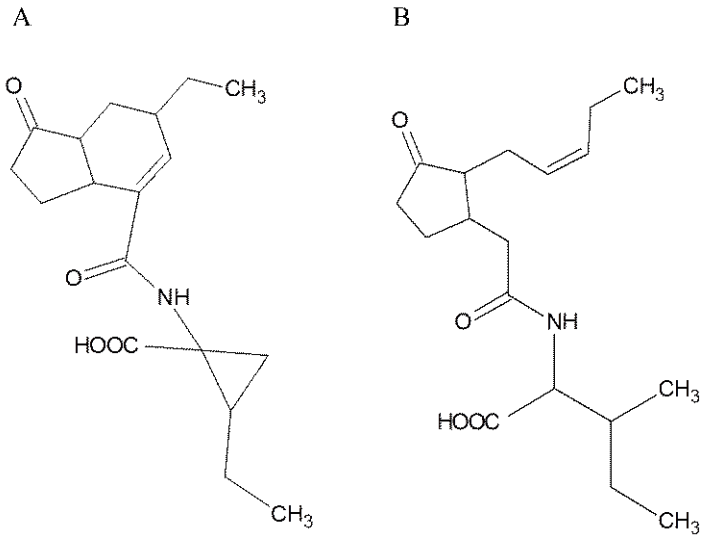

Figure 3.1: Structure of the COR phytotoxin (A) and the plant signalling molecule JA-Ile (B).

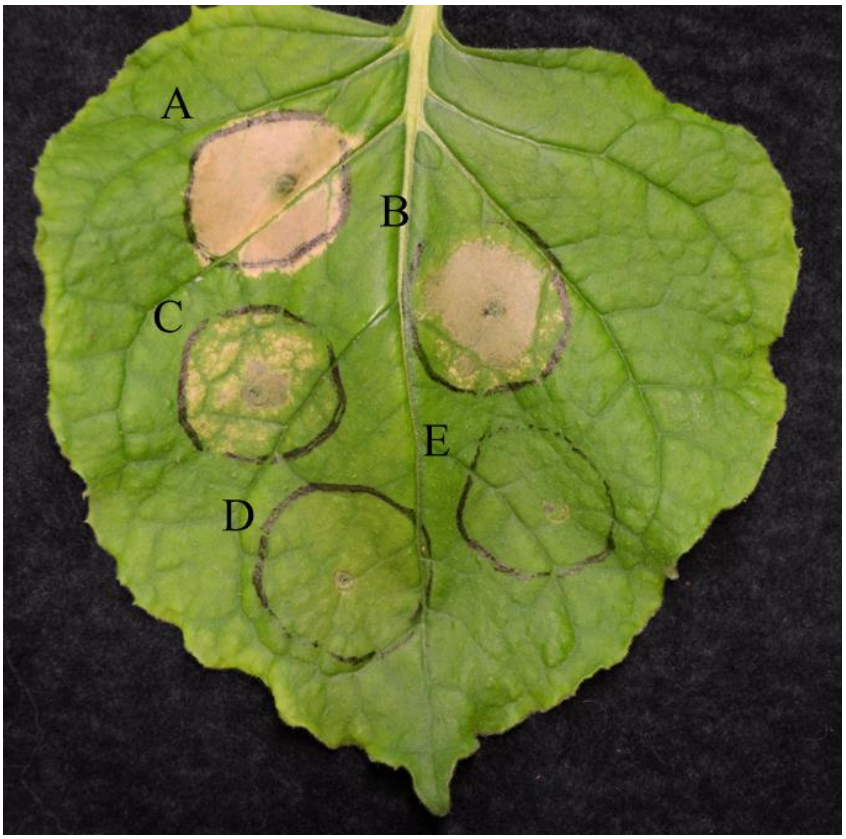

Figure 3.2: Infiltration bioassay showing the effect of the *S. scabies* COR-like metabolites on leaf plant tissue. Leaves of 6-7 week old *Nicotiana benthamiana* plants were infiltrated with filter-sterilized culture supernatant of the COR-like metabolite overproduction strain (A: Δ*txtA*/pRLDB51-1) and of the COR-like metabolite non-producing strains (B: Δ*txtA*/Δ*cfa6* and C: Δ*cfl*). Infiltration with 3 nmol of pure COR (D) was performed as a positive control while infiltration with supernatant from an uninoculated SFMB culture (E) was used as a negative control. The circled regions on the leaf represent the area of liquid infiltration for each treatment.

Figure 3.3: Radish seedling bioassay showing the effect of the *S. scabies* COR-like metabolites on seedling size. Seedlings were mock treated with water (J) or were treated with organic extract from SFMB cultures of *S. scabies* Δ*txtA/*pRLDB51-1 (A: undiluted; B: 10 fold diluted), Δ*txtA*/Δ*cfa6* (C: undiluted; D: 10-fold diluted) and Δ*cfl* (E: undiluted; F: 10-fold diluted). Treatment with extract from an uninoculated SFMB culture (G: undiluted; H: 10-fold diluted) served as an additional negative control, while treatment with 30 nmol of pure COR (I) served as a positive control. White bar indicates 1 cm.

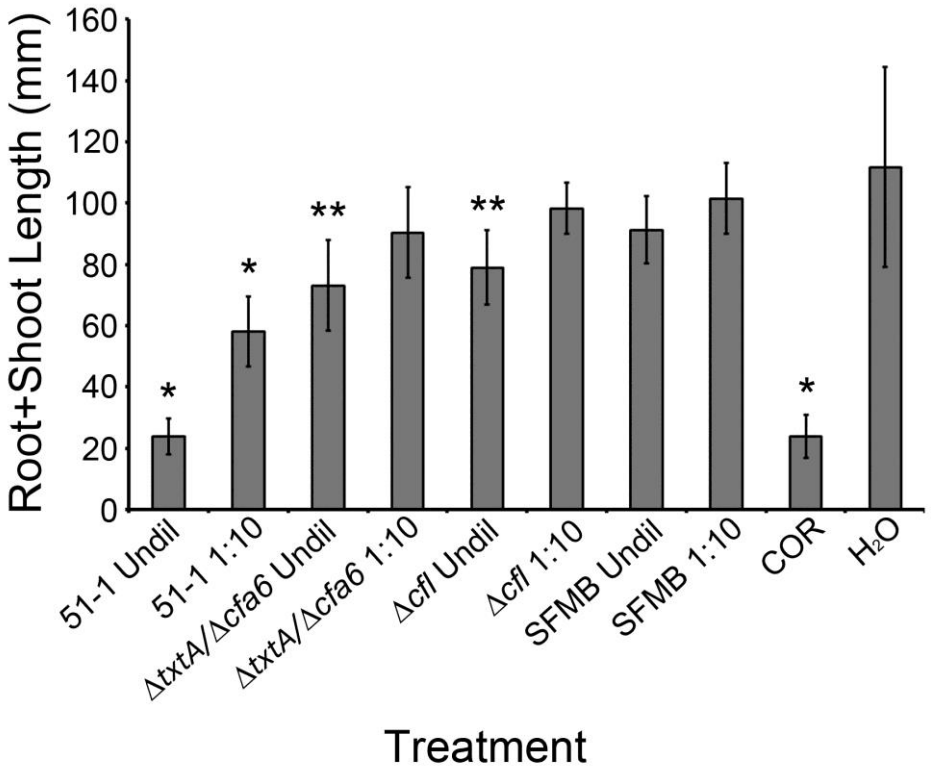

Figure 3.4: Quantification of radish seedling stunting by the *S. scabies* COR-like metabolites. The mean root and shoot length for 10 radish seedlings per treatment is shown, and the error bars represent the standard deviation from the mean. Seedlings were mock treated with water (H_2O) or were treated with organic extract from SFMB cultures of *S. scabies* Δ*txtA/*pRLDB51-1 (undiluted and 10 fold diluted), Δ*txtA/*Δ*cfa6* (undiluted and 10-fold diluted) and Δ*cfl* (undiluted and 10-fold diluted). Treatment with extract from an uninoculated SFMB culture (undiluted and 10-fold diluted) served as an additional negative control, while treatment with 30 nmol of pure COR served as a positive control. Treatments that produced a statistically significant result compared to the mock (H_2O) treatment are indicated by * ($p \leq 0.001$) or ** ($p \leq 0.01$).

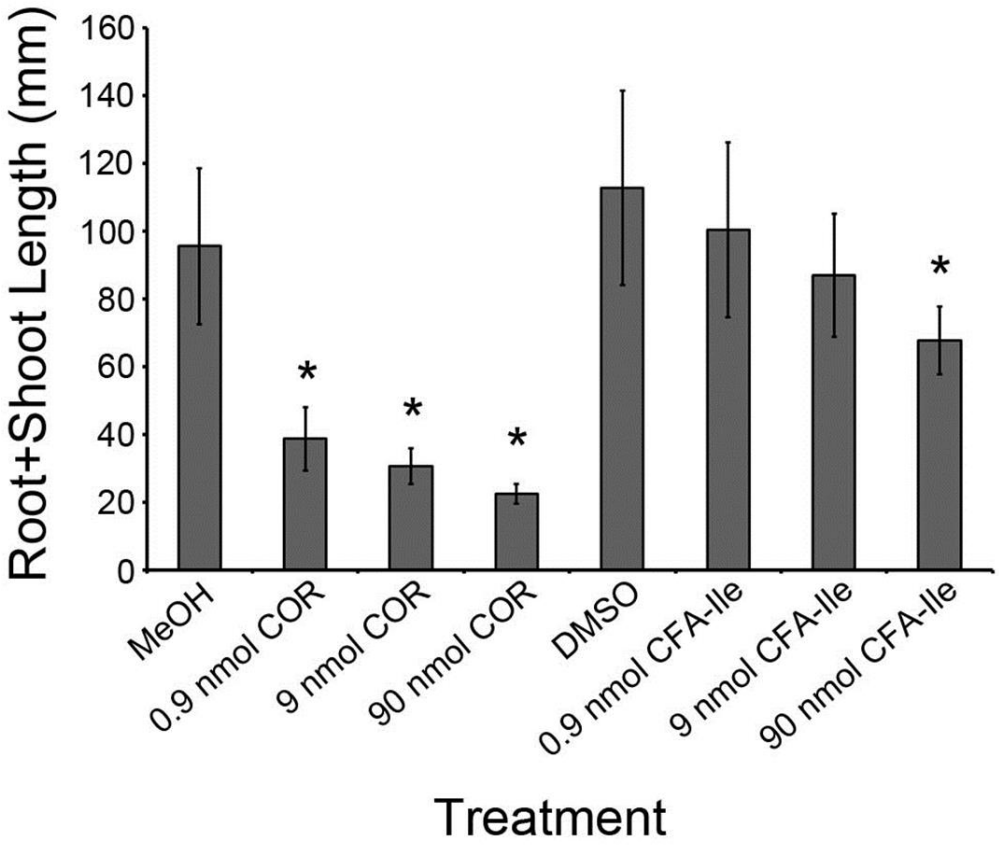

Figure 3.5: Radish seedling bioassay showing the effect of different amounts (0.9, 9 and 90 nmol) of pure COR (dissolved in MeOH) and CFA-L-Ile (dissolved in DMSO) on seeding root and shoot length. The mean root and shoot length for 10 radish seedlings per treatment is shown, and the error bars represent the standard deviation from the mean. Treatments that produced a statistically significant result compared to the relevant solvent control treatments (MeOH and DMSO) are indicated by * ($p \leq 0.001$).

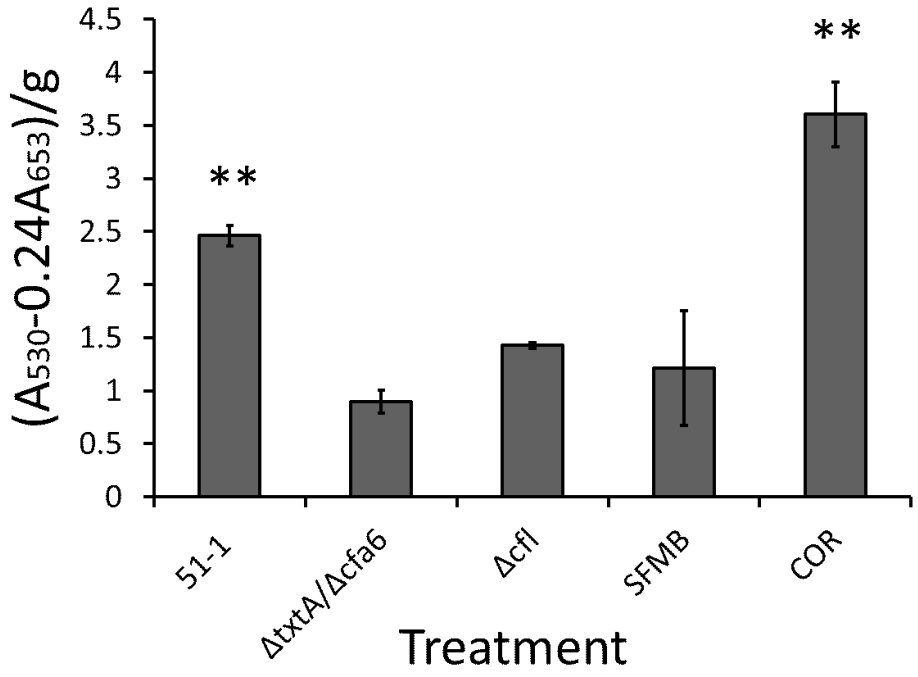

Figure 3.6: Effect of culture supernatant of the COR-like metabolite overproduction strain (Δ*txtA/*pRLDB51-1) on radish anthocyanin production. Pure COR (30 nmol) was used as a positive control while treatment with supernatant from COR-like metabolite non-producing strains (Δ*txtA*/Δ*cfa6* and Δ*cfl*) and an uninoculated SFMB culture were used as negative controls. Treatments that produced a statistically significant result compared to the mock (SFMB culture supernatant) treatment are indicated by ** ($p \leq 0.05$).

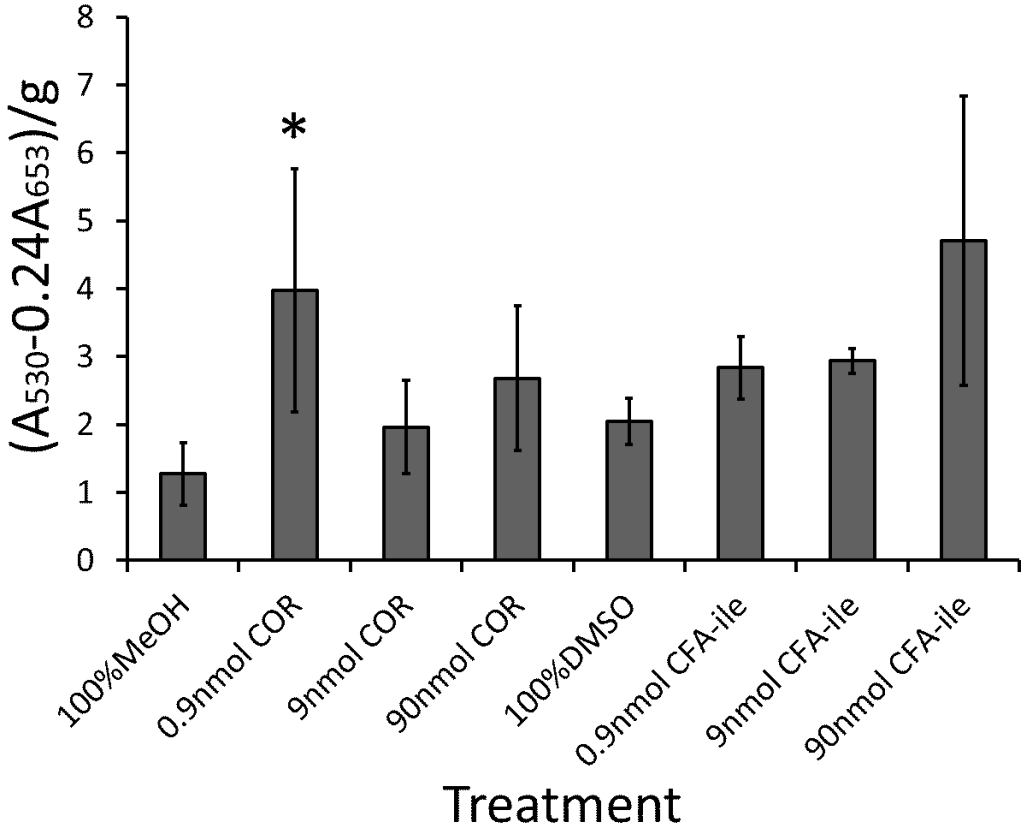

Figure 3.7: Effect of CFA-L-Ile on radish anthocyanin production. Radish seedlings treated with different amounts (0.9, 9 and 90 nmol) of pure COR (dissolved in MeOH) and CFA-L-Ile (dissolved in DMSO). MeOH and DMSO were used as a negative control for the COR and CFA-L-Ile treatments, respectively. The Student's *t*-test indicated a significant difference between the treatment with 0.9 nmol of COR and the corresponding negative control treatment ($p \leq 0.05$).

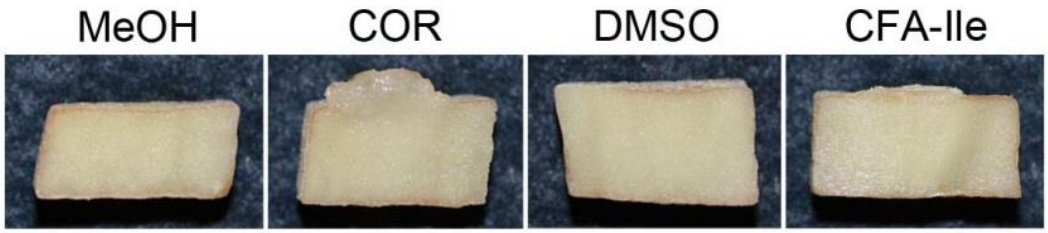

Figure 3.8: Potato tuber slice bioassay showing the induction of tissue hypertrophy by equimolar amounts (16 nmol) of COR (dissolved in MeOH) and CFA-L-Ile (dissolved in DMSO). Treatment with the metabolite solvents served as negative controls.

CHAPTER 4: Concluding Remarks

4.1 Summary and future directions

The research described in this thesis provides valuable insights into the biosynthesis and function of the COR-like metabolites, which are important for the plant pathogenic phenotype of *S. scabies.* In Chapter 2, it was demonstrated that the *cfa8* gene, which encodes a predicted CCR, is required for optimal production of CFA-L-Ile, and this is likely because it is important for providing a sufficient supply of ethylmalonyl-CoA for CFA biosynthesis. It was also shown that the *scab79691* gene*,* which encodes a predicted CYP450, is essential for CFA-L-Ile production, and it was proposed that this enzyme may participate directly in CFA biosynthesis by the introduction of a hydroxyl group. This result is of particular significance since a homologue of the Scab79691 protein does not exist in *P. syringae,* and it suggests that *P. syringae* and *S. scabies* may use different biosynthetic pathways to produce the same family of phytotoxins. In Chapter 3, it was revealed that CFA-L-Ile from *S. scabies* displays some of the same toxic effects as COR against different plant hosts, though COR is more toxic in its bioactivity. This suggests that the *S. scabies* COR-like metabolites may have a similar role as COR in manipulating plant defense responses during infection, thereby allowing the pathogen to colonize and grow within the plant host.

A number of questions stemming from this research remain and can serve as the basis for future studies into the *S. scabies* COR-like metabolites. For example, the identity of the putative intermediate accumulating in the Δ*scab79691* mutant remains to be

determined, and such information is critical for validating the proposed role of Scab79691 in the biosynthetic pathway for production of CFA-L-Ile. The proposed function of Scab79691 can also be validated by *in vitro* characterization of the enzyme activity following protein overexpression and purification. In addition, the genetic complementation of both the Δ*cfa8* and Δ*scab79691* mutants warrants further investigation since this will confirm that the observed phenotype of the mutants is due to the deletion of the target gene. The role of CFA-L-Ile in inducing anthocyanin production and chlorosis also should be further investigated to determine whether this metabolite shares all of the same bioactivities as COR. Given that COR promotes the formation of COI1-JAZ complexes and the ubiquination and degradation of JAZ proteins in order to upregulate JA-responsive genes (Katsir et al. 2008a, b), it would be interesting to determine whether CFA-L-Ile has a similar function. This could be accomplished using plant gene expression studies in order to identify the defense pathway(s) that is activated in response to CFA-L-Ile, as well as *in vitro* protein interaction studies to examine the effect of CFA-L-Ile on the formation of COI1-JAZ complexes (Katsir et al. 2008b). Of particular interest would be a comparison of the relative activities of COR, CFA-L-Ile and JA-Ile in promoting the formation of COI1-JAZ complexes since it has been reported that COR is ~1000 times more active than JA-Ile in this activity (Katsir et al. 2008b).

Knowledge of the biosynthesis and bioactivity of the *S. scabies* COR-like metabolites contributes to the basic understanding of how *S. scabies* is able to infect a plant host and cause disease. The ultimate goal of research on plant pathogenic *Streptomyces* spp. is to use the knowledge gained towards the development of control

strategies that are more reliable and effective for scab disease management in agricultural settings. In addition, such knowledge could have broader applications to the study of other host-pathogen systems in agriculture and in human or animal health.

4.2 References

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