Regulation of coronafacoyl phytotoxin production in the potato common scab pathogen *Streptomyces scabies*

by

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Abstract

The genus *Streptomyces* consists of hundreds of species of Gram-positive, filamentous bacteria, which have a complex developmental life cycle. Many specialized metabolites of clinical, agricultural and biotechnological value are produced by *Streptomyces*. Very few *Streptomyces* species have been shown to be pathogenic to plants. The best characterized pathogenic species is *Streptomyces scabies*, which is the main causative agent of potato common scab (CS) disease. CS is characterized by the formation of lesions on the potato tuber surface, which negatively impact the market value of the affected potatoes, leading to significant losses for growers.

S. scabies and other CS-causing pathogens produce thaxtomin A, the key virulence factor involved in CS disease. *S. scabies* also produces *N*-coronafacoyl-L-isoleucine (CFA-L-Ile), which is a member of the coronafacoyl family of phytotoxins. The coronafacoyl phytotoxin biosynthetic gene cluster in *S. scabies* consists of 15 genes, of which 13 are enzyme-encoding genes and are co-transcribed. The remaining two genes are divergently co-transcribed from the biosynthetic genes. The first gene, *scab79591*/*cfaR*, encodes a PAS-LuxR family regulator that activates the transcription of the enzyme-coding genes. The second gene, *scab79581*/*orf1*, encodes a ThiF family protein of unknown function.

This thesis examines the regulation of CFA-L-Ile biosynthesis in *S. scabies* and the role of the CfaR and ORF1 proteins. Our results show that CfaR is the principle regulator controlling expression of the coronafacoyl phytotoxin biosynthetic genes and CFA-L-Ile production, while ORF1 augments phytotoxin production in a CfaR dependent manner and may function as a “helper” of CfaR. Bioinformatics analysis suggests that ORF1 may

catalyze AMPylation of an unknown target molecule. In addition, this thesis addresses the effects of the plant-derived molecules cellobiose and suberin on CFA-L-Ile production as well as the role of CFA-L-Ile in controlling its own production. While cellobiose and suberin both induce thaxtomin A production, our results suggest that these molecules inhibit CFA-L-Ile production in *S. scabies*. *In vitro* binding assays showed that CFA-L-Ile inhibits the binding of CfaR to its target site in the coronafacoyl phytotoxin biosynthetic gene cluster. This suggests that CFA-L-Ile production is subjected to negative feedback regulation in *S. scabies*.

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Table of Contents

Abstract……...II

Acknowledgements……IV

Table of Contents……...VI

List of Figures……XIV

List of Tables……XVIII

List of Abbreviations and Symbols……XIX

List of Appendices……XXIII

Chapter 1: Introduction and Overview……1

1.1 General Features of *Streptomyces*……..1

1.2 Specialized Metabolism in *Streptomyces* Species……..3

1.3 Regulation of Specialized Metabolism in *Streptomyces*…….……5

1.3.1 CSRs of specialized metabolism…….....5

1.3.1.1 ActII-ORF4: the CSR for actinorhodin production……...…6

1.3.1.2 A cascade of CSRs controlling tylosin production……7

1.3.1.3 RedZ: a multi-functional CSR……..9

1.3.2 Pleiotropic regulators of specialized metabolism……..10

1.3.2.1 Two component systems (TCSs)……10

1.3.2.1.1 The AfsQ1/Q2 regulatory system……11

1.3.2.1.2 The AfsK-AfsR-AfsS regulatory System……...12

1.3.2.1.3 The PhoR-PhoP system sensing phosphate starvation……12

1.3.2.2 Global regulators of specialized metabolism and morphological differentiation: *bld* genes……..……13

1.3.3 Regulation of specialized metabolism by small molecules……15

1.3.3.1 GBL regulatory cascades……15

1.3.3.2 PI factor……..17

1.3.3.3 Crosstalk and feedback control of specialized metabolite biosynthesis…..18

1.3.4 The stringent response to nitrogen, phosphate and carbon limitation…….19

1.3.5 Carbon catabolite repression of specialized metabolism in *Streptomyces*….20

1.4 Plant Pathogenicity in the Genus *Streptomyces*……21

1.5 Known or Predicted Virulence Factors Produced by *S. scabies*……23

1.5.1 Thaxtomins……23

1.5.2 Nec1……..26

1.5.3 Coronafacoyl phytotoxins……26

1.5.3.1 Coronafacoyl phytotoxin biosynthesis in *S. scabies*……...27

1.5.3.2 Regulation of coronafacoyl phytotoxin production in *S. scabies*……28

1.5.3.3 Role of coronafacoyl phytotoxins in *S. scabies* pathogenicity……30

1.6 Thesis Objectives and Outline……..31

1.7 References……34

1.8 Figures and Tables……67

Co-Authorship Statement……...76

Chapter 2: Regulation of coronafacoyl phytotoxin production by the PAS-LuxR family regulator CfaR in the common scab pathogen *Streptomyces scabies*……78

2.1 Abstract……78

2.2 Introduction……..79

2.3 Materials and Methods……82

2.3.1 Bacterial strains, cultivation and maintenance……...82

2.3.2 Plasmids, primers and DNA manipulation……83

2.3.3 Construction of protein expression plasmids……..……84

2.3.4 Protein overexpression and purification……84

2.3.5 Total RNA isolation……...85

2.3.6 Reverse transcription PCR……86

2.3.7 Primer extension analysis……..86

2.3.8 Electrophoretic mobility shift assay (EMSA)……87

2.3.9 Glutaraldehyde cross-linking of CfaR proteins……..88

2.3.10 Construction of *cfaR*, *orf1* and *cfaR+orf1* overexpression plasmids……88

2.3.11 Extraction and analysis of CFA-L-Ile production……89

2.3.12 Bioinformatics analysis……...89

2.4 Results and Discussion……90

2.4.1 CfaR binds to a single site located in the *cfa1* promoter region……..90

2.4.2 The CfaR PAS domain is required for DNA binding and protein dimerization *in vitro*……92

2.4.3 Activation of coronafacoyl phytotoxin production by CfaR is enhanced by ORF1……..94

2.4.4 Phylogenetic analysis indicates that CfaR is a novel member of the PAS-LuxR protein family……..…….95

2.5 Concluding Remarks……97

2.6 References……98

2.7 Figures and Tables……..106

2.8 Supplementary Materials……118

Chapter 3: ORF1 is a ThiF-family protein that promotes coronafacoyl phytotoxin production and pathogenicity in the common scab pathogen *Streptomyces scabies*…….122

3.1 Abstract……..122

3.2 Introduction……123

3.3 Materials and Methods……...126

3.3.1 Bacterial strains, cultivation and maintenance……126

3.3.2 Plasmids, primers and DNA manipulation……...128

3.3.3 Bioinformatics analyses……..128

3.3.4 RNA isolation and analysis of gene expression……...129

3.3.5 Construction of the *S. scabies* Δ*txtA*/*orf1*, Δ*txtA*/*cfaR* and Δ*txtA*/*cfaR*/*orf1* mutant strains……...129

3.3.6 Genetic complementation of the *orf1* deletion mutant……130

3.3.7 Construction of *Streptomyces* gene overexpression plasmids harbouring the *ermE*p*, *kasO*p* and SP44 promoters……..130

3.3.8 eGFP fluorescence assay……132

3.3.9 Purification of HIS_6-CfaR from *S. scabies*……..133

3.3.10 Western blot analysis……134

3.3.11 Mass spectrometry (MS) analysis of the purified HIS_6-CfaR……134

3.3.12 Overproduction of HIS_6-CfaR and ORF1-HIS_6 in *E. coli*……135

3.3.13 Detection of CFA-L-Ile production by *S. scabies* strains……...……135

3.3.14 Detection of thaxtomin A production by *S. scabies* strains……136

3.3.15 Potato tuber bioassays……...136

3.3.16 Statistical analyses……137

3.4 Results……137

3.4.1 ORF1 is a member of the ThiF protein family……..137

3.4.2 ORF1 is a positive regulator of coronafacoyl phytotoxin production in *S. scabies*……..139

3.4.3 Development of plasmids for protein overexpression and purification in *Streptomyces*……141

3.4.4 Purification and analysis of CfaR expressed in the presence and absence of ORF1……143

3.4.5 Overproduction of CfaR and ORF1 in *E. coli*……..144

3.4.6 Increased production of CFA-L-Ile enhances the virulence of *S. scabies* during infection of potato tuber tissue……146

3.5 Discussion……..148

3.6 References……..152

3.7 Figures and Tables……..163

3.8 Supplementary Materials……186

Chapter 4: Effect of microbial and plant-derived molecules on the production of coronafacoyl phytotoxins in the common scab pathogen *Streptomyces scabies*……...196

4.1 Abstract……..196

4.2 Introduction……197

4.3 Materials and Methods……...200

4.3.1 Bacterial strains, cultivation and maintenance……200

4.3.2 Analysis of CFA-L-Ile production by *S. scabies*……..202

4.3.3 Analysis of thaxtomin A production by *S. scabies*……...202

4.3.4 Dry cell weight measurement and HPLC result standardization……..202

4.3.5 Identification of known and predicted CebR binding sites in the *S. scabies* 87-22 genome……203

4.3.6 Overexpression of histidine-tagged CfaR in *E. coli* and protein purification……...203

4.3.7 Electrophoretic mobility shift assay (EMSA)……..204

4.3.8 Statistical analyses……...204

4.4 Results……205

4.4.1 Effect of cellobiose on CFA-L-Ile production in *S. scabies*……205

4.4.2 The CFA-L-Ile biosynthetic gene cluster lacks putative CebR binding sites……..206

4.4.3 Effect of potato suberin on CFA-L-Ile production……...207

4.4.4 Effect of potato tuber tissue or juice on CFA-L-Ile production……209

4.4.5 CFA-L-Ile inhibits the DNA binding activity of CfaR……210

4.5 Discussion……211

4.6 References……217

4.7 Figures and Tables……227

4.8 Supplementary Materials……236

Chapter 5: Summary and Future Directions……248

5.1 Summary……248

5.2 Future Directions……252

5.3 References……253

List of Figures

Figure 1.1. Regulation of tylosin production in *Streptomyces fradiae*……..……...67

Figure 1.2. Regulation of RED, ACT and CDA production by *redZ* in *Streptomyces coelicolor*……... 68

Figure 1.3. Model of GBL regulatory cascades in *Streptomyces* spp. 69

Figure 1.4. Positive feedback control of daunorubicin biosynthesis in *Streptomyces peucetius.* 70

Figure 1.5. Negative feedback control of nosiheptide biosynthesis in *Streptomyces actuosus.* 71

Figure 1.6. Crosstalk regulation of chloramphenicol and jadomycin biosynthesis in *Streptomyces venezuelae*..... 72

Figure 1.7. Structure of CFA, CFA-L-Ile and COR..... 73

Figure 1.8. Organization of the *cfa* biosynthetic gene cluster from *Pseudomonas syringae* pv. tomato DC3000 and the *cfa*-like biosynthetic gene cluster from *Streptomyces scabies* 87-22 74

Figure 2.1. Organization of the *Streptomyces scabies* 87-22 coronafacoyl phytotoxin biosynthetic gene cluster and domain structure of the CfaR protein. 106

Figure 2.2. $CfaR^{full}$-HIS_6 binds to a single site within the *cfaR-cfa1* intergenic region. 107

Figure 2.3. Sequence logo of PAS-LuxR protein binding sites. 109

Figure 2.4. Mapping the transcription start site of *cfa1*. 110

Figure 2.5. The CfaR PAS domain is required for DNA binding and protein dimerization. 112

Figure 2.6. Coronafacoyl phytotoxin production is greatly enhanced by overexpression of both *cfaR* and *orf1*..... 114

Figure 2.7. Phylogenetic analysis of PAS-LuxR family proteins from *Streptomyces* and other actinomycetes. 115

Figure 3.1. Coronafacoyl phytotoxin biosynthetic gene cluster in *S. scabies*. 163

Figure 3.2. Phylogenetic analysis of ORF1 and other ThiF family proteins from actinomycetes..... 164

Figure 3.3. ORF1 contains a potential ATP binding site. 165

Figure 3.4. Semi-quantitative RT-PCR analysis showing enhanced gene transcription by overexpression of CfaR and CfaR+ORF1 in *S. scabies* 87-22. 167

Figure 3.5. Analysis of CFA-L-Ile production in different Δ*txtA*/*orf1* mutant isolates (A) and in the Δ*txtA*/*cfaR*, Δ*txtA*/*orf1* and Δ*txtA*/*cfaR*/*orf1* mutant strains (B).169

Figure 3.6. Complementation of the *orf1* deletion mutant. 171

Figure 3.7. Coronafacoyl phytotoxin production is enhanced by overexpressing *cfaR* from the SP44 and *kasO*p* promoters.. 173

Figure 3.8. Overproduction and purification of HIS_6-CfaR from *S. scabies* strains expressing (lane 1) or lacking (lane 2) ORF1. 175

Figure 3.9. Mass spectrometry analysis of HIS_6-CfaR purified from *S. scabies*..... 176

Figure 3.10. Overproduction of HIS-tagged CfaR and ORF1 proteins in different *E. coli* host strains. 177

Figure 3.11. Overexpression of *cfaR* and *orf1* enhances the virulence phenotype of *S. scabies* 87-22. 179

Figure 3.12. Overexpression of *cfaR* and *orf1* causes enhanced potato tissue hypertrophy by *S. scabies* Δ*txtA*. 180

Figure 3.13. Effects of pure thaxtomin A ± CFA-L-Ile on potato tuber tissue. 181

Figure S3.1. Structure of the SP44, *kasO*p* and *ermE*p* promoter fragments used in this study. 186

Figure S3.2. Construction of *Streptomyces* overexpression plasmids used in this study. 187

Figure S3.3. Semi-quantitative reverse-transcription PCR analysis showing gene expression in *S. scabies* wild type (87-22) and the Δ*txtA* mutant strain. 189

Figure S3.4. Fluorescence assay for detecting promoter activities in *Streptomyces*. 190

Figure S3.5: Thaxtomin A production in OBB medium by *Streptomyces* strains. 191

Figure 4.1. Effect of suberin and/or cellobiose on CFA-L-Ile production in *S. scabies* Δ*txtA*/pRLDB51-1. 227

Figure 4.2. Concentration-dependent effect of cellobiose on CFA-L-Ile production in *S. scabies*. 228

Figure 4.3. Concentration-dependent effect of suberin on CFA-L-Ile production in *S. scabies*..... 230

Figure 4.4. Effect of potato peel/juice on CFA-L-Ile production in *S. scabies* Δ*txtA*/pRLDB51-1 232

Figure 4.5. Analysis of CfaR-HIS_6 DNA binding activity in the presence of CFA-L-Ile and related metabolites. 233

Figure S4.1: Induction of thaxtomin A production by cellobiose in *S. scabies* 87-22. .. 236

Figure S4.2. Induction of thaxtomin A production by suberin in *S. scabies* 87-22..... 238

List of Tables

Table 1.1. Examples of GBL regulatory cascades in *Streptomyces* 75

Table 2.1. Bacterial strains and plasmids used in this study..... 116

Table S2.1. Oligonucleotides used in this study..... 118

Table S2.2. Accession numbers of PAS-LuxR homologues used for phylogenetic trees 120

Table 3.1. Bacterial strains and plasmids used in this study..... 182

Table S3.1. Oligonucleotides used in this study. 192

Table S3.2. Accession numbers of ORF1 homologues used for the phylogenetic analysis. 194

Table S3.3. Rare codon frequency in the *orf1* gene..... 195

Table 4.1. Bacterial strains and plasmids used in this study..... 235

Table S4.1. CebR site distribution in *S. scabies* 87-22 genome……239

List of Abbreviations and Symbols

aa: amino acids

ACT: actinorhodin

ARR: atypical response regulator

AS: acid scab

AtrA: actinorhodin-associated transcriptional regulator

bld: bald

cbs: CebR-binding site

CCR: carbon catabolite repression

CDA: calcium-dependent antibiotic

c-di-GMP: cyclic diguanylate

Cam: chloramphenicol

CFA: coronafacic acid

CFA-L-Ile: coronafacoyl-L-isoleucine

Cfl: coronafacate ligase

CMA: coronamic acid

COR: coronatine

CPK: cryptic polyketide

CS: common scab

CSR: cluster-situated regulators

DCW: dry cell weight

DMSO: dimethyl sulfoxide

DNR: daunorubicin

eGFP enhanced green fluorescent protein

EMSA: electrophoretic mobility shift assay

EtBr: ethidium bromide

FAM: carboxyfluorescein

FPLC: fast protein liquid chromatography

GBL: γ-butyrolactone

GlcNAc: *N*-acetylglucosamine

GST: glutathione *S*-transferase

HK: histidine kinase

HPLC: high-performance liquid chromatography

HTH: helix-turn-helix

IM-2: (2R,3R,1'R)-2-1'-hydroxybutyl-3-hydroxymethyl- γ -butanolide

IPTG: isopropyl 1-thio-β-D-glucopyranoside

JA: jasmonic acid

JA-L-Ile: jasmonyl-L-isoleucine

Jd: jadomycin

LB: Luria-Bertani

MM: methylenomycin

MS: mass spectrum

neo3: kanamycin resistant gene

OBB: oat bran broth

OTC: oxytetracycline

PAGE: polyacrylamide gel electrophoresis

PAI: pathogenicity island

PAS: PER-ARNT-SIM

PBS: phosphate buffered saline

PKS: polyketide synthase

PMA: potato mash agar

(p)ppGpp: guanosine pentaphosphate

pv: pathovars

RED: undecylprodigiosin

RR: response regulator

RT-PCR: reverse transcription-PCR

SA: salicylic acid

SARP: *Streptomyces* antibiotic regulatory protein

SCB: *S. coelicolor* butanolide

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SFMB: soy flour mannitol broth

spp.: species (plural)

sp.: species (singular)

TCS: two-component system

TEV: tobacco etch virus

TMA: volatile trimethylamine

TSB: trypticase soy broth

TSS: transcription start site

UV: ultraviolet

VB: virginiae butanolide

VM: virginiamycin M

VS: virginiamycin S

Δ: deletion

List of Appendices

Appendix 1:

Bignell DR, Fyans JK, Cheng Z. Phytotoxins produced by plant pathogenic *Streptomyces* species. J Appl Microbiol. 2014 Feb;116(2):223-35.

Bignell DRD, Cheng Z, Bown L. The coronafacoyl phytotoxins: structure, biosynthesis, regulation and biological activities. Antonie Van Leeuwenhoek. 2018 May;111(5):649-666.

Chapter 1: Introduction and Overview

1.1 General Features of *Streptomyces*

Streptomyces is a genus of Gram-positive, filamentous *Actinobacteria* that is composed of hundreds of species. These bacteria are found predominantly in soil and decaying vegetation where they degrade complex organic polymers and contribute to nutrient recycling in the environment (Hodgson, 2000). Streptomycetes are also found in marine environments (Deepa et al., 2012; Lin et al., 2014; Ian et al., 2014; Cho & Kim, 2012; Biswas et al., 2017; Hakvåg et al., 2008; Dalisay et al., 2013). Their genomes consist of large, linear chromosomes and have a high GC content (Hopwood, 2006). Some species have large linear and circular plasmids (Gomez-Escribano et al., 2015; Mingyar et al., 2018; Ortseifen et al., 2015) and small plasmids as well (Zhou et al., 2012).

Streptomycetes are distinct from other bacteria in that they have a complex developmental life cycle that involves the formation of dormant unicellular spores, which are thought to allow dispersion of the organisms in the environment (McCormick & Flärdh, 2012). The life cycle involves multicellular growth that can be divided into a number of different stages, beginning with spore germination (Elliot et al., 2008). When a spore encounters an appropriate source of nutrients, growth begins with the emergence of one or more filamentous cells known as “vegetative hyphae”. The hyphae grow by atypical tip extension and by branching, and eventually a complex network of intertwined hyphae is formed and is referred to as the “vegetative mycelium”. Under circumstances of nutrient depletion, the growth of the vegetative hyphae ceases and new structures begin to form

called aerial hyphae, which grow up into the air and impart a fuzzy appearance to the colony surface. At this stage, the vegetative hyphae undergo lysis, providing nutrients to support the growth of the aerial hyphae. The aerial hyphae are non-branching and eventually undergo septation, which subdivides the hyphae into ~40-60 compartments. Each compartment contains a single copy of the organism's genome. This is followed by a number of steps that culminate in the formation of the mature spores, which can disperse to new locations where fresh nutrient sources may be available.

Besides the traditional *Streptomyces* life cycle, a novel form of *Streptomyces* development, called "exploratory growth", has recently been identified in some species (Jones & Elliot, 2017). Exploratory growth is mediated by production of volatile trimethylamine (TMA) and by an increase in pH, which does not typically happen under normal laboratory condition when *Streptomyces* species are cultured alone. When cultured with yeast, which consumes glucose in the medium, *Streptomyces venezuelae* starts to produce and release TMA, resulting in a pH increase in the medium. Then, exploring nonbranching vegetative hyphae start growing rapidly and moving across solid surfaces, such as rocks and polystyrene barriers. Exploratory growth represents a form of interkingdom interactions and appears to be a response to glucose starvation. The mechanism of exploratory growth is not yet understood (Jones & Elliot, 2017).

Streptomyces produce numerous specialized metabolites (also known as secondary metabolites, see Section 1.2), as well as industrially important enzymes (glucose isomerase, transglutaminase, etc.), which have been used in the fermentation industry (Horinouchi,

2007). In addition, the widely available enzymes for specialized metabolism have been used in combinatorial biosynthesis, where artificial gene clusters are formed to biosynthesize novel compounds (Horinouchi, 2007).

1.2 Specialized Metabolism in *Streptomyces* Species

Specialized metabolism is the mechanism of producing organic compounds that are not required for growth, development or reproduction under laboratory conditions, but likely provide a selective advantage to the producing organism within its natural habitat (Berdy, 2005; Moore et al., 2014; O'Brien & Wright, 2011). These compounds usually have a small molecular weight (<3kDa) and are named specialized (or secondary) metabolites to distinguish them from primary metabolites, which are essential for growth and survival (Berdy, 2005; Moore et al., 2014; O'Brien & Wright, 2011).

Specialized metabolites are usually derived from primary metabolites and/or their associated metabolic intermediates (Gunatilaka & Wijeratne, 2000). Specialized metabolites of the same class originate from a common biosynthetic pathway and are modified by unique enzyme-catalyzed pathways to form compounds with extremely diverse chemical structures (O'Brien & Wright, 2011). Specialized metabolites produced by microbes include terpenoids, polyketides, alkaloids, peptides, carbohydrates, steroids and lipids (O'Brien & Wright 2011).

Streptomyces bacteria are renowned for their ability to produce many specialized metabolites of clinical, agricultural and biotechnological value. Nearly two-thirds of the clinically-useful antibiotics are produced by *Streptomyces* spp. or are derived from

Streptomyces specialized metabolites (Mohanraj & Sekar, 2013). It is estimated that streptomycetes are capable of producing approximately 100,000 antimicrobial compounds in total (Procópio et al., 2012; Watve et al., 2001). Antibiotic compounds produced by *Streptomyces* spp. include molecules with anti-bacterial (Procópio et al., 2012), anti-fungal (Lyu et al., 2017; Raytapadar & Paul, 2001; Wang et al., 2013a) and anti-viral activities (Manimaran et al., 2018; Nakagawa et al., 1981; Wei et al., 2014). In addition, *Streptomyces* spp. are able to produce specialized metabolites with bioactivities that are immunosuppressant (Muramatsu & Nagai, 2013), anti-parasitic (Pimentel-Elardo et al., 2010), herbicidal (Hahn et al., 2009), insecticidal (Berdy, 2005) and anti-cancer (Noomnual et al., 2016). The genes involved in specialized metabolite biosynthesis are usually clustered together within *Streptomyces* genomes. Such clusters typically consist of genes encoding the biosynthetic enzymes as well as genes involved in the regulation of production, metabolite export and resistance in the case of antibacterial metabolites (van Wezel & McDowall, 2011).

The ecological functions of specialized metabolites are not known in many instances, though molecules with antimicrobial activity may provide a selective advantage to producing organisms by killing other microbes that are competing for the limited nutrients in the environment (O'Brien & Wright, 2011). Specialized metabolites are also thought to mediate symbiotic interactions between *Streptomyces* and other organisms, including invertebrates and plants (Seipke et al., 2012). For example, attine ants, which depend on a cultivated fungus for food, employ *Streptomyces* to produce candicidin, antimycin and other possible antifungal(s) to avoid unwanted fungi (Seipke et al., 2011).

Antibiotics produced by *Streptomyces* are also thought to provide protection to plants against pathogens, while exudates produced by plant roots can be used by the *Streptomyces* bacteria as a nutrient source for promoting growth and development (Bosso et al., 2010).

1.3 Regulation of Specialized Metabolism in *Streptomyces*

Specialized metabolism in *Streptomyces* is controlled at multiple levels. The most basic level involves cluster-situated regulators (CSRs), which directly control the expression of the genes within the associated biosynthetic gene cluster (van Wezel & McDowall, 2011). In turn, the expression of CSR-encoding genes may be controlled by one or more pleiotropic regulators, which regulate multiple gene clusters and pathways. Hormone-like signalling molecules such as γ-butyrolactone (GBL) autoregulators and PI factor can also play a role in modulating the production of one or more specialized metabolites, as can intermediates and/or end products of specialized metabolism (Niu et al., 2016). Nutritional or environment factors can additionally influence specialized metabolism in *Streptomyces* spp. (Martín et al., 2011; Ruiz et al., 2010).

1.3.1 CSRs of specialized metabolism

CSRs are typically located within specialized metabolite biosynthetic clusters and regulate the enzyme-coding genes of the same cluster in which they are situated. CSRs can be positive activators or negative repressors and are considered pathway or cluster specific in that they usually only control the expression of genes required for production of a specific specialized metabolite. Single or multiple CSRs have been found in many biosynthetic gene clusters in *Streptomyces* spp., and there is convincing evidence that the

cellular concentration of a CSR is the primary factor dictating the level of biosynthetic gene expression and metabolite production (van Wezel & McDowall, 2011). CSRs belong to multiple protein families, and common protein families represented among CSRs are summarized elsewhere (Ju et al., 2017). Some examples of CSRs are discussed below.

1.3.1.1 ActII-ORF4: the CSR for actinorhodin production

Streptomyces coelicolor A3(2) produces actinorhodin (ACT), an aromatic polyketide metabolite with weak antibiotic activity (Itoh et al., 2007). The *act* gene cluster is 22 kb in length and consists of 22 genes encoding the ACT biosynthetic enzymes as well as regulatory genes and genes responsible for export of the antibiotic (Okamoto et al., 2009). The *actII*-ORF4 gene encodes a protein of the *Streptomyces* antibiotic regulatory protein (SARP) family (Wietzorrek & Bibb, 1997), members of which are associated with specialized metabolite biosynthetic gene clusters in diverse actinomycetes (Liu et al., 2013). This family consists of the first identified pathway-specific regulatory proteins in *Streptomyces* (Wietzorrek & Bibb, 1997). SARPs contain an N-terminal winged helix-turn-helix (HTH) domain, which typically binds to direct heptameric DNA repeats, and a C-terminal domain involved in activating transcription. Both pathway-specific regulators and pleiotropic regulatory proteins (e.g. AfsR) have been found to be the SARP family members (Tanaka et al., 2007).

ActII-ORF4 binds to some intergenic regions within the *act* cluster and thus activates gene expression in this cluster (Arias et al., 1999; Liu et al., 2013). Overexpression of *actII*-ORF4 contributes to enhanced ACT production, confirming that

ActII-ORF4 is a transcriptional activator (Hindra et al., 2010; Sohoni et al., 2014). Expression of *actII*-ORF4 is controlled by many regulatory proteins and signals (Liu et al., 2013). For example, (p)ppGpp (guanosine pentaphosphate) synthesis induces transcription of *actII*-ORF4 (Hesketh et al., 2001). AtrA, a regulator that is not associated with any known antibiotic clusters, binds to the promoter region of *actII*-ORF4 and positively regulates gene expression and ACT production (Uguru et al., 2005). DasR, a GntR-family regulator controlling *N*-acetylglucosamine (GlcNAc) catabolism, represses *actII*-ORF4 expression by antagonizing AtrA (Świątek-Połatyńska et al., 2015). *actII*-ORF4 expression is regulated by AdpA (Ohnishi et al., 1999), a pleiotropic regulator of development and specialized metabolism. In addition, *actII*-ORF4 is also regulated by LexA [a transcriptional repressor responding to DNA damage (Kuzminov, 1999)], AbsA2 [a negative regulator of antibiotic production (McKenzie & Nodwell, 2007)], DraR (Yu et al., 2012) and AfsQ1 (Wang et al., 2013b) [both are response regulators of two component systems involved in antibiotic production], ROK7B7 [regulator of carbon catabolite repression, growth, and antibiotic production (Świątek et al., 2013)] and possibly GlnR [regulating nitrogen metabolism and specialized metabolism (Pullan et al., 2011)]. Furthermore, *actII*-ORF4 contains a TTA codon and is thus regulated by *bldA* (Fernandez-Moreno et al., 1991).

1.3.1.2 A cascade of CSRs controlling tylosin production

Tylosin, a macrolide antibiotic, was first identified in the fermentation products from *Streptomyces fradiae* (Corcoran et al., 1977). The *tyl* gene cluster consists of 43 contiguous genes and is 85 kb in size, occupying ~1% of the *S. fradiae* genome (Cundliffe,

2008). Six CSR-encoding genes are found within the *tyl* cluster, five of which, *tylP*, *tylQ*, *tylS*, *tylT* and *tylU*, are located together in a sub-cluster that is over 65 kb from the sixth regulatory gene, *tylR* (Cundliffe, 1999; Cundliffe, 2008). The regulation of tylosin production in *S. fradiae* is shown in Figure 1.1. TylR is a global activator of the tylosin biosynthetic pathway and occupies the lowest level in the genetic hierarchy controlling production in *S. fradiae.* Disruption of *tylR* abolishes the production of tylosin and its biosynthetic intermediates, indicating that it is essential for metabolite biosynthesis (Bate et al., 1999). Production of tylosin was also abolished in a disruption mutant of *tylS,* which encodes a SARP family protein (Bate et al., 2002). Transcription of *tylR* was shown to be dependent on *tylS* but not *vice versa*, suggesting that *tylS* controls *tylR* expression (Bate et al., 2002). Control of *tylR* expression also involves TylU, which has been proposed to function as a 'SARP-helper' protein that is needed to facilitate the activation of *tylR* transcription by TylS through an unknown mechanism (Bate et al. 2006). *tylU* is also under the control of *tylS* (Stratigopoulos et al., 2004). The *tylT* gene encodes a second predicted SARP family member, though it does not appear to be essential for tylosin production and its role is currently unknown (Bate et al., 2002).

Two negative repressors, TylQ and TylP, are also involved in regulating tylosin production in *S. fradiae* (Cundliffe, 2008). TylQ represses the expression of *tylR* during the early stages of fermentation, while in the later stages when tylosin is produced the *tylQ* gene is silent (Stratigopoulos & Cundliffe, 2002). This led to a model in which activation of *tylR* gene expression and tylosin production requires that *tylQ* be switched off or reduced in expression (Cundliffe, 2008). TylP is a predicted GBL receptor protein that resembles

the A-factor binding protein ArpA of *Streptomyces griseus* (Section 1.3.3.1). TylP inhibits expression from its own promoter as well as from the *tylS* and *tylQ* promoters by binding to a partially palindromic sequence (called "PARE" sites) within each promoter (Bignell et al., 2007; Stratigopoulos & Cundliffe, 2002). The DNA binding activity of TylP was shown to be inhibited by one or more small molecule ligands that are extractable from stationary-phase cultures of *S. fradiae*, and the accumulation of this still unidentified ligand(s) may serve as the signal for initiating the regulatory cascade leading to antibiotic biosynthesis (Bignell et al., 2007; Stratigopoulos & Cundliffe, 2002)

1.3.1.3 RedZ: a multi-functional CSR

Although CSRs are mainly thought to be pathway-specific, there is evidence that some may also control other biosynthetic gene clusters, and thus may function as pleiotropic regulators (Huang et al., 2005). The best characterized *Streptomyces* sp., *S. coelicolor*, produces five antibiotics: ACT, undecylprodigiosin (RED), methylenomycin (MM), calcium dependent antibiotic (CDA) (Bibb, 1996) and a cryptic polyketide antibiotic (CPK) (Liu et al., 2013). Within the *red* biosynthetic gene cluster is a CSR-encoding gene called *redZ*, which encodes an atypical response regulator (ARR) that differs from conventional response regulators in that its activity is not controlled by phosphorylation (Liu et al., 2013). RedZ activates the expression of a second CSR-encoding gene, *redD*, which encodes a SARP family regulator that directly activates the expression of the RED biosynthetic genes (Fig. 1.2) (White & Bibb, 1997). Interestingly, Huang and colleagues showed that constitutive expression of *redZ* upregulates the production of ACT and CDA in addition to RED, and this was associated with a dramatic

increase in the expression of AfsS, a 'higher level' pleiotropic regulator of specialized metabolism that is highly conserved in *Streptomyces* spp. and is not associated with any biosynthetic gene cluster (Fig. 1.2) (Huang et al., 2005). This suggests that cross-regulation between a CSR and a higher level regulator can modulate the production of specialized metabolites in some instances (Huang et al., 2005).

1.3.2 Pleiotropic regulators of specialized metabolism

1.3.2.1 Two component systems (TCSs)

Unlike CSRs, pleiotropic regulators are considered 'higher level' regulators in that they usually control multiple biosynthetic gene clusters and pathways in response to extracellular signals or cellular development (Huang et al., 2005). In *Streptomyces*, many pleiotropic regulators of specialized metabolism belong to TCSs, which typically consist of (1) a membrane-bound histidine protein kinase (HK) that senses environmental stimuli, and (2) a corresponding response regulator (RR) that mediates cellular responses through transcriptional regulation of target genes (Rodríguez et al., 2013). The HK usually has an N-terminal ligand-binding domain, and a C-terminal kinase domain which is responsible for the autophosphorylation and the phosphorelay to the target RR (Stock et al., 2000). Compared with other bacteria, *Streptomyces* spp. harbour a large number of TCSs, most likely due to the complexity of the environments where these organisms are found. The number of HKs and RRs encoded within a *Streptomyces* genome can range from several dozens to over a hundred (Rodríguez et al., 2013). It has been reported that 67 TCSs exist in *S. coelicolor* (Hutchings et al., 2004), of which at least 10 have been shown to influence

specialized metabolism in that organism (Liu et al., 2013). Some examples of TCSs implicated in the regulation of specialized metabolism are discussed below.

1.3.2.1.1 The AfsQ1/Q2 regulatory system

A DNA fragment from *S. coelicolor* containing *afsQ1* was able to induce the production of pigmented antibiotics in *Streptomyces lividans*, leading to the identification of the AfsQ1/Q2 TCS, in which AfsQ1 is a RR and AfsQ2 is a HK (Ishizuka et al., 1992). The trigger activating the AfsQ1/Q2 system is predicted to be a nutritional signal, although the real signal has not been identified yet (Rodríguez et al., 2013). It has been shown that AfsQ1/Q2 only activated antibiotic production in a defined medium with glutamate as the only carbon source but not in a rich medium. In *S. coelicolor*, AfsQ2 appears to be the only HK to phosphorylate AfsQ1. The active form of AfsQ1 binds to the promoter regions of CSR-encoding genes, such as *actII*-ORF4 of the ACT pathway, *cdaR* of the CDA pathway and *redZ* of the RED pathway, and activates antibiotic biosynthesis (Shu et al., 2009). The AfsQ1/Q2 system has two roles in CPK biosynthesis. AfsQ1/Q2 promotes CPK production by inducing the expression of genes such as *cpkA*/*cpkD* (Wang et al., 2013b). On the other hand, AfsQ1/Q2 positively regulates *sigQ*, which encodes a putative sigma factor and repressor of antibiotic production. The AfsQ1/Q2 system also regulates nutrient metabolism (Shu et al., 2009). AfsQ1 functions as a transcriptional repressor of nitrogen assimilation but an activator of phosphate and carbon metabolism (Wang et al., 2013b). The AfsQ1/Q2 regulatory system is widely conserved in *Streptomyces* (Daniel-Ivad et al., 2017).

1.3.2.1.2 The AfsK-AfsR-AfsS regulatory System

The AfsK-AfsR-AfsS regulatory system in *S. coelicolor* was first identified when it was introduced into *S. lividans,* where it stimulated the production of ACT and RED (Horinouchi et al., 1983). In this system, AfsK is a serine/threonine kinase and undergoes self-activation through autophosphorylation (Tomono et al., 2006). KbpA, a repressor, binds to the unphosphorylated form of AfsK and inhibits the autophosphorylation of AfsK (Umeyama & Horinouchi, 2001). AfsK phosphorylates AfsR (Hong et al., 1991; Matsumoto et al., 1994), which shows similarity to SARP-family proteins (Horinouchi et al., 1986). AfsR is also phosphorylated by two other kinases, AfsL and PkaG (Sawai et al., 2004), and thus serves as an integrator of intracellular and extracellular signals (Horinouchi, 2003). AfsR regulates multiple downstream target genes, one of which is *afsS* (Lee et al., 2002). *afsS* encodes a small sigma factor-like protein that activates the expression of several specialized metabolite biosynthetic gene clusters, including the *act* and *red* clusters in *S. coelicolor* (Santos-Beneit et al., 2011b).

The *afsK*/*afsR*/*afsS* gene set is conserved in several *Streptomyces* species, indicating that the AfsK-AfsR-AfsS system is a common mechanism regulating specialized metabolism in these bacteria (Parajuli et al., 2005; Umeyama et al., 1999).

1.3.2.1.3 The PhoR-PhoP system sensing phosphate starvation

The PhoR-PhoP system was recently reviewed by Liu and colleagues (Liu et al., 2013). This system is widely distributed in bacteria, is activated under phosphate starvation conditions, and it controls both specialized metabolism and morphological differentiation

in *Streptomyces* spp. PhoR, the membrane HK, senses the signal and then self-phosphorylates and phosphorylates the RR, PhoP. The phosphorylated PhoP binds to promoters, thereby activating or repressing gene expression (Allenby et al., 2012). PhoR-PhoP is known to modulate the production of antibiotic metabolites in *Streptomyces* spp., including activating and repressing ACT and RED in *S. coelicolor* (Santos-Beneit et al., 2009) and in S. *lividans* (Sola-Landa et al., 2003; Santos-Beneit et al., 2011a), respectively, and repressing pimaricin in *Streptomyces natalensis* (Mendes et al., 2007). Candicidin in *S. griseus* (Asturias et al., 1994) and oxytetracycline (OTC) in *Streptomyces rimosus* (Petković et al., 2017) are induced by phosphate starvation possibly through the PhoR-PhoP system, although there is no direct evidence to support this theory. Notably, in *S. lividans*, the role of the PhoR-PhoP system is different than in *S. coelicolor*. Deletion of *phoP* strongly increased production of ACT and RED, which is normally turned off in *S. lividans* (Sola-Landa et al., 2003). The differences in the effects of PhoP on ACT and RED production in these two *Streptomyces* spp. is probably not directly due to PhoP, but instead is probably caused by differences in the downstream genes that are regulated by PhoP (Allenby et al., 2012).

1.3.2.2 Global regulators of specialized metabolism and morphological differentiation: *bld* genes

bld genes were first identified in *Streptomyces* mutants that failed to form aerial hyphae and thus had a shiny "bald" appearance compared with the fuzzy appearance of wild-type colonies (McCormick & Flärdh, 2012). At least 10 *bld* loci, *bldA, bldB, bldC, bldD, bldF, bldG, bldH, bldI, bldK,* and *bld261*, have been identified, some of which have

been extensively studied (Mishig-Ochiriin et al., 2003). The *bld* gene products are diverse (Bibb et al., 2000; Bignell et al., 2000; Molle & Buttner, 2000; Nodwell et al., 1996; Pope et al., 1998) and some *bld* genes influence both aerial hyphae formation and antibiotic production (Chater & Chandra, 2008; Eccleston et al., 2002; Pope et al., 1996; Pope et al., 1998).

One of the best characterized *bld* gene is *bldA*, which encodes the only tRNA that recognizes the rare UUA codon, and thus it plays a role in controlling the translation of TTA codon containing genes (Barka et al., 2016; Lawlor et al., 1987). *bldA* mutants of *S. coelicolor* are unable to produce ACT and RED (Hackl & Bechthold, 2015), and it has been shown that the CSR genes *actII*-ORF4 and *redZ* in the *act* and *red* gene clusters, respectively, contain a single TTA codon (O'Rourke et al., 2009). In *Streptomyces*, many specialized metabolite biosynthetic gene clusters contain TTA codons, which most often are associated with the CSR genes. The TTA codons tend to be located near the beginning of the coding regions in which they are found, suggesting a more effective way of translation regulation by *bldA* (Chater & Chandra, 2008).

Another well characterized *bld* gene is *bldD*, which encodes an XRE-family transcriptional regulator that is essential for both aerial hyphae formation and antibiotic production (den Hengst et al., 2010; Elliot et al., 1998). *bldD* holds the highest position in the *bld* regulatory hierarchy, controlling other *bld* genes (Molle & Buttner, 2000). It has been reported that BldD regulates ~167 transcriptional units and controls the expression of 42 other regulatory genes, including those involved in mediating specialized metabolism

(den Hengst et al., 2010). Recently, it was shown that the BldD protein in *S. venezuelae* binds cyclic diguanylate (c-di-GMP) to form a $BldD_2$-$(c\text{-}di\text{-}GMP)_4$ complex, which is necessary for the full DNA binding activity of BldD (Schumacher et al., 2017). c-di-GMP is a bacterial second messenger of growing recognition involved in the regulation of a number of complex physiological processes in diverse bacteria (Tamayo et al., 2007).

1.3.3 Regulation of specialized metabolism by small molecules

1.3.3.1 GBL regulatory cascades

A model of GBL regulatory cascades that occur in *Streptomyces* spp. is illustrated in Figure 1.3, and some examples of GBL regulatory systems that have been described are listed in Table 1.1. The A-factor regulatory cascade in *S. griseus* is a well characterized pathway that involves the regulation of both specialized metabolism and morphological differentiation by the A-factor extracellular signalling molecule (Hara et al., 1983; Mori, 1983; Ohnishi & Horinouchi, 2004). A-factor is a GBL autoregulator that is produced in response to environmental stimuli and can move freely within and between hyphae in *S. griseus*, thus serving as a messenger in cell to cell communication (Horinouchi & Beppu, 1993-1994). The interaction between A-factor and its cognate receptor has strict binding specificity, allowing the organism to recognize other cells of the same species (Miyake et al., 1989). This provides an advantage for the survival of the species as A-factor produced by one cell is recognized by others, therefore promoting sporulation and dispersal of organisms. A-factor has also been shown to function in quorum sensing (Bassler & Losick, 2006).

A-factor is synthesized by the AfsA GBL synthase (Horinouchi et al., 1989). When the concentration of A-factor reaches a critical level, it binds the GBL receptor protein, ArpA, and causes the release of ArpA from the promoter region of the *adpA/bldH* gene, thereby activating *adpA/bldH* expression. This activation of *adpA/bldH* is A-factor concentration dependent and is an all-or-nothing switch (Horinouchi, 2002; Kato et al., 2004). AdpA/BldH is a member of the AraC/XylS protein family and a key transcriptional activator of specialized metabolism and morphological development in *S. griseus* and in other *Streptomyces* spp. (McCormick & Flärdh, 2012; Nguyen et al., 2003; Ohnishi et al., 1999; Takano et al., 2003). The *strR* gene, encoding the CSR for streptomycin biosynthesis in *S. griseus*, is activated by AdpA/BldH, and this leads to activation of the *str* biosynthetic genes (Tomono et al., 2005). AdpA/BldH also indirectly activates the grixazone CSR, GriR (Higashi et al., 2007), and other possible pathways in *S. griseus* (Yamazaki et al., 2004).

GBL regulatory cascades have also been described in other *Streptomyces* spp. In *S. coelicolor*, biosynthesis of the signaling molecules, *S. coelicolor* butanolides (SCBs) is catalyzed by ScbA (D'Alia et al., 2011; van Wezel & McDowall, 2011). A GBL receptor protein, ScbR, is a repressor of the *cpkO* [also known as *kasO* (Takano et al., 2005)] gene (Liu et al., 2013). *cpkO* encodes a SARP family activator for CPK biosynthetic gene expression (Gottelt et al., 2012). SCBs bind to ScbR, thereby relieving the repression of *cpkO* and activating CPK biosynthesis (Liu et al., 2013). In *Streptomyces virginiae*, virginiae butanolide (VB) is synthesized by BarS1 (Shikura et al., 2002). VB binds to the receptor, BarA, to regulate expression of *vmsR*, which encodes the SARP activator required for the biosynthesis of both virginiamycin M (VM) and virginiamycin S (VS) (Kawachi et

al., 2000). VmsR is an upper-level regulator that activates expression of two CSRs, *vmsS* (required for both VM and VS production) and *vmsT* (required for VM production only) (Pulsawat et al., 2009). In *Streptomyces lavendulae*, IM-2 [(2R,3R,1′R)-2-1′-hydroxybutyl-3-hydroxymethyl-γ-butanolide] (Hashimoto et al., 1992) is synthesized by FarX, which is an AfsA-family protein (Kitani et al., 2010). IM-2 binds to the receptor, FarA (Kitani et al., 1999; Kitani et al., 2001), to activate showdomycin and minimycin production (Hashimoto et al., 1992), though it remains unclear how FarA activates the production of these antibiotics. Recent genome sequencing of *Streptomyces showdoensis* revealed a showdomycin biosynthetic gene cluster containing a predicted regulatory gene called *sdmJ*, which encodes a HTH-type transcriptional regulator (Palmu et al., 2017). Whether this regulatory gene is conserved in *S. lavendulae* and whether it is under control of FarA remain to be determined.

1.3.3.2 PI factor

Another example of a quorum sensing autoinducer eliciting specialized metabolite production is PI factor synthesized by *S. natalensis*. The chemical structure of PI factor has been determined to be 2,3-diamino-2,3-bis(hydroxymethyl)-1,4-butanediol (Recio et al., 2004). Both PI factor and A-factor can induce pimaricin production in *S. natalensis*; however, PI factor is not able to complement an A-factor deficiency in *S. griseus* (Recio et al., 2004). This result indicates that *S. natalensis* may possess a PI factor pathway and a similar pathway to the A-factor cascade of *S. griseus*. *S. natalensis* is not able to synthesize A-factor but is able to recognize it from other *Streptomyces* spp. (Recio et al., 2004).

1.3.3.3 Crosstalk and feedback control of specialized metabolite biosynthesis

In some specialized metabolite biosynthetic pathways, the end products or intermediates of the pathway can influence the expression of the biosynthetic genes by interacting with one or more CSRs. An example of this can be seen with the biosynthesis of daunorubicin (DNR) in *Streptomyces peucetius* (Fig. 1.4) (Otten *et al.*, 1995). There are three CSR-encoding genes controlling metabolite production: *dnrO*, *dnrN* and *dnrI* (Ajithkumar & Prasad, 2010; Jiang & Hutchinson, 2006). *dnrO* encodes a TetR-like protein that binds to its own promoter and represses gene expression. DNR or rhodomycin D, one of the intermediates, can bind to DnrO, and this leads to de-repression of *dnrO* and activation of the divergently transcribed gene *dnrN*, which encodes an ARR (Bourret, 2010; Hickey et al., 2011). DnrN in turn activates *dnrI*, which encodes a SARP family protein that turns on expression of the DNR biosynthetic genes (Jiang & Hutchinson, 2006).

Nosiheptide is a thiopeptide antibiotic produced by *Streptomyces actuosus* (Mocek et al., 1993). Its biosynthetic gene cluster consists of 15 enzyme-encoding genes, of which 12 are co-transcribed in one direction and the other three are co-transcribed in the opposite direction (Badding et al., 2017; Yu et al., 2009). *nosP*, the only CSR in the gene cluster, encodes a SARP family protein that controls the expression of all enzyme-encoding genes by binding to the intergenic region between *nosM* and *nosL*. The ribosomally synthesized precursor peptide of nosiheptide contains an N-terminal 37-amino acid leader peptide and a 13 amino acid C-terminal core peptide. During posttranslational modification of the precursor, the core peptide is modified to form a macrocyclic skeleton of the end product, while the leader peptide is cleaved (Yu et al., 2009). Both the cleaved leader peptide and

the end product serve as ligands of NosP, negatively regulating its activity. This is the first example of SARP family protein binding small molecule ligands (Fig. 1.5) (Li et al., 2018).

The end product regulation of CSRs is not only limited to one pathway as crosstalk between different pathways within the same organism can also occur. In *S. venezuelae*, the primary jadomycin CSR, JadR1, modulates the biosynthesis of both jadomycin (Jd) and chloramphenicol (Cam) (Liu et al., 2013). During early stages of growth, the *jadR1* gene is repressed by a second CSR called JadR2, and this results in repression of Jd production and de-repression of Cam biosynthesis. Binding of either Jd or Cam to JadR2 causes the repression of *jadR1* to be relieved and the accumulation of JadR1, which then activates Jd production and suppresses Cam biosynthesis (Fig. 1.6) (Xu et al., 2010).

1.3.4 The stringent response to nitrogen, phosphate and carbon limitation

In *Streptomyces* spp. and in other bacteria, the accumulation of (p)ppGpp signals the start of the stringent response, which allows organisms to survive periods of nutrient limitation (Hauryliuk et al., 2015). In *S. coelicolor,* (p)ppGpp synthesis is catalyzed by the (p)ppGpp synthetase RelA in response to nitrogen and amino acid starvation (Chakraburtty & Bibb, 1997). A *relA* mutant of *S. coelicolor* was unable to produce (p)ppGpp as well as RED and ACT under conditions of nitrogen starvation, and expression of the *actII*-ORF4 and *redD* genes was also reduced in the mutant, suggesting a direct role for (p)ppGpp in activating antibiotic biosynthesis (Sun et al., 2001). In addition to inhibiting protein synthesis and DNA replication, (p)ppGpp also interacts with RNA polymerase in recognizing promoters in *Escherichia coli*. This causes transcriptional inhibition of some

genes but activation of others, including specialized metabolite biosynthetic genes (Srivatsan & Wang, 2008). Similar process has been reported in *S. coelicolor*, where (p)ppGpp synthesis inhibits the expression of genes required for growth while activating those genes involved in stationary phase processes, including specialized metabolism (Hesketh et al., 2007).

1.3.5 Carbon catabolite repression of specialized metabolism in *Streptomyces*

Specialized metabolism is also controlled by carbon source regulation in *Streptomyces*. In *Streptomyces,* carbon catabolite repression (CCR) is one of the most important control mechanisms that allow the bacteria to utilize a preferred carbon source when exposed to multiple choices. The presence of glucose inhibits the expression of genes involved in the utilization of alternative carbon sources (Kwakman & Postma, 1994). In *Streptomyces* spp., extracellular glucose also represses the production of many specialized metabolites, including ACT in *S. coelicolor,* cephamycin C in *Streptomyces clavuligerus,* streptomycin in *S. griseus,* and chloramphenicol in *S. venezuelae*, by down-regulating enzyme coding genes involved in antibiotic biosynthesis (van Wezel & McDowall, 2011). The production of ACT in *S. coelicolor* is controlled by GlcNAc, which stimulates antibiotic production under nutrient depletion conditions and inhibits production when nutrient sources are available. GlcNAc, once inside *Streptomyces* cells, is modified and acts as a signalling molecule for the global transcription factor DasR, a GntR family protein. DasR binds to the *act* biosynthetic gene cluster and represses gene expression and ACT production, and this repression is relieved upon binding of the GlcNAc signalling molecule to DasR (Sánchez et al., 2010).

In *Streptomyces*, *bldB* encodes a DNA-binding protein that is required for antibiotic production and aerial hyphae formation (Pope et al., 1998). A *bldB* null mutant of *S. coelicolor* failed to produce antibiotics and to form aerial hyphae when cultured on different carbon sources (van Wezel & McDowall, 2011). Other *bld* gene deletion mutants were also unable to produce antibiotics; however, production could be restored when the mutants were cultured on media containing non-repressing (such as mannitol) carbon sources (van Wezel & McDowall, 2011). These results indicate that antibiotic production is subjected to CCR in *S. coelicolor*. It has been reported that glucokinase activity is required for CCR as CCR was relieved in a *bldA* deletion mutant when *glkA*, a glucokinase-encoding gene, was also deleted (van Wezel & McDowall, 2011). Interestingly, expression of the CPK biosynthetic genes in *S. coelicolor* is repressed by glucose at the protein level (Pawlik et al., 2010) but is stimulated by glucose at the mRNA level (Romero-Rodríguez et al., 2016).

1.4 Plant Pathogenicity in the Genus *Streptomyces*

Among the several hundred *Streptomyces* spp. that have been described, only a dozen or so are known to be plant pathogens. These species cause diseases in a variety of economically-important crops, the most important being common scab (CS) of potato (*Solanum tuberosum*) (Bignell et al., 2014b). CS is characterized by the formation of superficial, deep-pitted or raised lesions on the potato tuber surface, and these lesions negatively impact the quality and market value of the affected potato crop. CS has been rated among the top five important diseases by potato growers in the USA (Slack, 1991), and it has been estimated to have caused economic losses to Canadian growers of between

$15.3 and 17.3 million Canadian dollars in 2002 (Hill & Lazarovits, 2005). In Tasmania, Australia, estimated losses of $3.66 million Australian dollars per acre have been reported, which works out to about 4% of the industry value (Wilson, 2004). In addition, CS pathogens may also affect the potato crop yield by causing delayed emergence and decreased tuber size (Hiltunen et al., 2005).

Streptomyces scabies (syn. *S. scabiei*) is the first described pathogenic species, is the best characterized, and it is the main CS-causing species in North America (Dees & Wanner, 2012). Other CS-causing species include *Streptomyces turgidiscabies*, *Streptomyces europaeiscabiei*, *Streptomyces stelliscabiei*, *Streptomyces luridiscabiei*, *Streptomyces puniciscabiei* and *Streptomyces niveiscabiei* (Wanner, 2009). *Streptomyces acidiscabies* causes a very similar disease called acid scab (AS), which is identical to CS except that it occurs in acid soils where CS is typically suppressed (Bignell et al., 2014b). Pathogenic *Streptomyces* spp. that cause CS or AS have no tissue or host specificity (Loria et al., 2006), although expanding tissue is sensitive to infection (Khatri et al., 2010; Khatri et al., 2011). The host range includes root crops such as radish, beet, carrot, and parsnip, in addition to potato (Goyer & Beaulieu, 1997). *S. scabies* and other scab pathogens can also cause disease on seedlings of monocot and dicot plants in the lab, leading to root and shoot stunting and tissue chlorosis and necrosis (Leiner et al., 1996; Loria et al., 1997).

Some control strategies for CS have been developed, among which the use of resistant potato cultivars appears to be the most reliable method (Dees & Wanner, 2012). The mechanism of the resistance, however, is not fully understood and there are no

commercial potato cultivars that are completely resistant to CS. Overall, there is currently no single strategy that can control CS effectively and consistently, and thus a better understanding of the associated pathogens and their virulence determinants is required.

1.5 Known or Predicted Virulence Factors Produced by *S. scabies*

1.5.1 Thaxtomins

All scab-causing *Streptomyces* spp. produce a family of phytotoxic specialized metabolites known as thaxtomins, of which thaxtomin A is predominant (King & Calhoun, 2009). Thaxtomin A is a cyclic dipeptide (2,5-diketopiperazine) metabolite that is derived from L-phenylalanine and L-4-nitrotryptophan. A positive correlation between thaxtomin A production and the pathogenicity of scab-causing streptomycetes has been observed (Goyer et al., 1998; King et al., 1991; Kinkel et al., 1998; Loria et al., 1995), and mutant strains of *S. scabies* and *S. acidiscabies* that lack the ability to produce thaxtomin A do not cause typical disease symptoms (Healy et al., 2000; Joshi & Loria, 2007b). Although the exact function of thaxtomin A in plant pathogenicity is unknown, several lines of evidence suggest that it targets cellulose synthesis in the plant host (Bischoff et al., 2009; Duval & Beaudoin, 2009; Fry & Loria, 2002; Scheible et al., 2003) and, therefore, may be required for the penetration of plant tissue during host colonization (Loria et al., 2008). Recently, Bischoff and colleagues showed that treatment with thaxtomin A reduces the crystalline cellulose and increases pectins and hemicelluloses in the cell walls of *Arabidopsis* (Bischoff et al., 2009). The treatment with thaxtomin A also altered the expression of genes involved in cellulose synthesis. In addition, genes related to callose deposition, a plant

defense mechanism, were also upregulated in response to thaxtomin A. These results support the proposed function of thaxtomin A as influencing cellulose synthesis in host cells (Bischoff et al., 2009).

The thaxtomin biosynthetic gene cluster consists of five enzyme-coding genes (reviewed in Bignell et al., 2014b). *txtA* and *txtB* encode non-ribosomal peptide synthetases for the synthesis of the thaxtomin backbone. TxtC, a P450 monooxygenase, is required for post-cyclization hydroxylation reactions. TxtD is a nitric oxide synthase that produces the nitric oxide used by the TxtE cytochrome P450 to generate the L-4-nitrotryptophan substrate. The function of *txtH* is not clear. *txtR* encodes an AraC/XylS family transcriptional regulator that functions as the CSR controlling thaxtomin production (Joshi et al., 2007b). Deletion of *txtR* leads to loss of thaxtomin biosynthetic gene expression and metabolite production, indicating that TxtR is a positive activator of thaxtomin production (Joshi et al., 2007b). The thaxtomin biosynthetic gene cluster is conserved in pathogenic *Streptomyces* spp., including *S. scabies*, *S. turgidiscabies* and *S. acidiscabies*. It is worth mentioning that the thaxtomin biosynthetic gene cluster is localized on a pathogenicity island (PAI) that is conserved in pathogenic *Streptomyces* spp. and can be mobilized among different *Streptomyces* spp. (Kers et al., 2005; Zhang et al., 2018).

Recent studies into the regulation of thaxtomin A production in *S. scabies* have revealed that the biosynthesis of the phytotoxin is induced by cellobiose and cellotriose, which are the smallest units of cellulose (Johnson et al., 2007). Cellobiose and cellotriose induce the transcription of *txtR* through an upper level regulator called CebR, which is a

repressor of cellulose/cello-oligosaccharides/cellobiose utilization in *Streptomyces* spp. (Schlösser et al., 1999; Marushima et al., 2009). When cello-oligosaccharides are absent, CebR binds to two CebR boxes, called *cbs* (CebR-binding site), one of which is present in the intergenic region between *txtR* and *txtA*, and one which is located within the *txtB* gene. Binding CebR to these sites inhibits expression of *txtA*, *txtB* and *txtR* (Francis et al., 2015). Cellobiose, and to a lesser extent cellotriose, is able to impair the binding of CebR to the *cbs* sites, and this leads to activation of thaxtomin biosynthetic gene expression (Francis et al., 2015). Cellobiose is produced and released during cellulose synthesis in suspension-cultured *Populus alba* cells (Ohmiya et al., 2000), and cellotriose was shown to be released from rapidly growing suspension-cultured tobacco cells (Johnson et al., 2007). This has led to the hypothesis that cello-oligosaccharides serve as indicators of expanding plant tissues, which are the primary infection site for *S. scabies* and other scab-causing pathogens (Johnson et al., 2007). Production of thaxtomin A in response to released cello-oligosaccharides, together with the filamentous nature of *S. scabies*, may enable the penetration of expanding tissues and the intercellular and intracellular colonization of the plant host (Loria et al., 2008). The production of thaxtomin A is also affected by suberin, which is a lipid biopolymer, composed of monomers including aliphatic long-chain α, ω-diacids, ω-hydroxyacids and glycerol (Graça & Santos, 2007; Graça, 2015). In potatoes, suberin is present in the cell walls of the periderm (Järvinen et al., 2009). A study by Beauséjour and colleagues showed that thaxtomin A production can be stimulated by the addition of suberin to a minimal medium, and that the amount of thaxtomin A produced varied greatly among different strains of *S. scabies* (Beausejour et al., 1999). In a follow

up study by Lerat and colleagues, thaxtomin biosynthetic gene expression and metabolite production were shown to be more strongly induced by the addition of both cellobiose and suberin to a minimal medium compared to the addition of either suberin or cellobiose alone (Lerat et al., 2010). This suggests that suberin and cellobiose exhibit a synergistic effect on thaxtomin A production in this organism. Currently, it is unclear how suberin stimulates thaxtomin A biosynthesis, though there is evidence that it is able to promote the onset of specialized metabolite production in multiple *Streptomyces* spp. (Joshi et al., 2007b; Lerat et al., 2010; Lerat et al., 2012).

1.5.2 Nec1

The *nec1* gene is conserved in most scab-causing *Streptomyces* spp. and encodes a secreted protein that has no homologues in public database and which causes necrosis on potato tuber tissue (Bukhalid et al., 1998). Deletion of the *S. turgidiscabies nec1* gene resulted in a severe reduction in virulence on various plant hosts and compromised the ability of the pathogen to aggressively colonize plant tissues (Joshi et al., 2007a). The exact function of Nec1 in disease development has yet to be determined; however, it has been suggested that it may function to suppress the host defense response early in the infection process (Joshi et al., 2007a).

1.5.3 Coronafacoyl phytotoxins

The *S. scabies* 87-22 genome was fully sequenced in 2005 (https://www.sanger.ac.uk/resources/downloads/bacteria/streptomyces-scabies.html). Analysis of the sequence revealed the presence of a specialized metabolite biosynthetic

gene cluster that was predicted to synthesize a member of the coronafacoyl family of phytotoxins. Coronafacoyl phytotoxins are known or suspected to be produced by a number of different plant pathogenic bacteria, including several pathovars (pv) of the Gram-negative plant pathogen *Pseudomonas syringae* (Bignell et al., 2018). Members of this phytotoxin family consist of the polyketide metabolite coronafacic acid (CFA) linked via an amide bond to an amino acid or amino acid derivative (Fig. 1.7) (Bender et al., 1999a). At least seven different coronafacoyl phytotoxins have been described, of which coronatine (COR) (Fig. 1.7) is the best characterized and is the most toxic (Bignell et al., 2018). COR is the main coronafacoyl phytotoxin produced by *P. syringae* and is composed of CFA linked to the ethylcyclopropyl amino acid coronamic acid (CMA), which is derived from L-*allo*-isoleucine (Bender et al., 1999a). Recently, *S. scabies* was shown to be able to produce CFA-L-Ile (Fig. 1.7), which is also produced by *P. syringae* in minor amounts (Mitchell & Young, 1985). Other minor coronafacoyl phytotoxins are also produced by *S. scabies*; however, the organism is unable to produce COR due to the absence of the CMA biosynthetic genes from the genome (Bignell et al., 2010; Fyans et al., 2015).

1.5.3.1 Coronafacoyl phytotoxin biosynthesis in *S. scabies*

The biosynthetic gene cluster responsible for synthesis of CFA-L-Ile in *S. scabies* consists of at least 15 genes, of which nine are homologous to genes found within the CFA biosynthetic gene cluster in *P. syringae* (Fig. 1.8A) (Bignell et al., 2010b). Among the genes conserved in both organisms are the *cfa1-5* genes, which in *P. syringae* are believed to synthesize the 2-carboxy-2-cyclopentenone intermediate, and the *cfa6-7* genes, which encode large, multi-modular PKS enzymes that generate the CFA backbone (Bender et al.,

1999b; Rangaswamy et al., 1998a; Rangaswamy et al., 1998b). In addition, the *cfl* gene is conserved in both species and encodes an enzyme that catalyzes the ligation of CFA to its amino acid partner during the final stage of phytotoxin biosynthesis (Fyans et al., 2015). The *cfa8* gene found in both gene clusters encodes a predicted crotonyl-CoA carboxylase/reductase that is thought to be involved in the production of the ethylmalonyl-CoA extender unit used for CFA polyketide biosynthesis (Bignell et al., 2010). Interestingly, the *S. scabies* biosynthetic gene cluster contains four additional biosynthetic genes that are absent from the *P. syringae* gene cluster (Fig. 1.8), and recent studies from our lab have confirmed that three of these genes (*oxr, sdr, CYP107AK1*) are required for the biosynthesis of CFA (Bown et al., 2016; Bown et al., 2017). As homologues of these genes are not found anywhere in the genome of *P. syringae,* it appears as though *S. scabies* and *P. syringae* use distinct biochemical pathways to produce the same family of phytotoxins (Bown et al., 2016; Bown et al., 2017).

1.5.3.2 Regulation of coronafacoyl phytotoxin production in *S. scabies*

The biosynthetic genes involved in CFA-L-Ile production are transcribed as a single, polycistronic mRNA from a promoter region upstream of the *cfa1* gene (Bignell et al., 2010b). Divergently transcribed from *cfa1* is another gene designated *scab79591* (herein referred to as *cfaR*; Fig. 1.8), which has been shown to function as a positive transcriptional activator since overexpression of *cfaR* resulted in enhanced expression of the *cfa* biosynthetic genes and enhanced CFA-L-Ile production (Bignell et al., 2010; Fyans et al., 2015).

The CfaR protein shows similarity to a family of actinobacterial transcriptional regulators that are characterized by an N-terminal PAS (PER-ARNT-SIM) domain and a C-terminal LuxR-type DNA binding domain (Taylor & Zhulin, 1999; Fuqua et al., 1994). PAS domains occur in all kingdoms of life and typically function as signal sensor domains for detecting a variety of chemical and physical stimuli (Möglich et al., 2009; Taylor & Zhulin, 1999). In response to such stimuli, PAS domains regulate the activity of effector domains such as DNA binding domains or domains involved in catalysis (Möglich et al., 2009; Taylor & Zhulin, 1999). In *Streptomyces,* members of the PAS-LuxR family are known to function as CSRs and control the production of specialized metabolites such as pimaricin (also called natamycin), filipin and aureofuscin (Anton et al., 2007; Santos-Aberturas et al., 2011; Vicente et al., 2014; Wei et al., 2011; Wu et al., 2013). Interestingly, the *cfaR* gene in *S. scabies* was shown to be co-transcribed with a downstream gene designated *scab79581*/*orf1*, which encodes a 637 amino acid protein with a predicted ThiF-family domain (Bignell et al., 2010). The co-transcription of *scab79581*/*orf1* with *cfaR* suggests that both gene products may function in the regulation of coronafacoyl phytotoxin biosynthesis, though the exact role of the ORF1 protein remains unknown.

In addition to *cfaR,* other genes also appear to regulate the production of CFA-L-Ile in *S. scabies*. Deletion of the *bldA* gene, which as discussed previously encodes the only tRNA that efficiently translates the rare UUA codon in *Streptomyces* mRNA (Chater, 2006), led to a reduction in expression of the CFA-L-Ile biosynthetic genes (Bignell et al., 2010b). An analysis of the *cfaR* coding sequence revealed the presence of a single TTA codon, suggesting that CfaR is not efficiently translated in the *bldA* mutant (Bignell et al., 2010).

Other *bld* genes such as *bldD, bldG* and *bldH* were also shown to control the expression of *cfaR* and/or *cfa1* in *S. scabies* (Bignell et al., 2014a).

1.5.3.3 Role of coronafacoyl phytotoxins in *S. scabies* pathogenicity

Most studies of coronafacoyl phytotoxins have focused on COR since it is the predominant family member produced by *P. syringae* and other plant pathogenic *Pseudomonas* spp. and is the most toxic phytotoxin (Bignell et al., 2018). Although COR is not essential for the pathogenicity of *Pseudomonas* spp., it functions as an important virulence factor in these organisms (Xin & He, 2013). Studies have shown that it enables the pathogen to overcome host stomatal defenses in order to gain access to the internal plant tissues. Once inside the plant, COR promotes the multiplication and persistence of the pathogen within the plant apoplast, and COR contributes directly to disease symptom development during pathogen infection (Xin & He, 2013). COR functions as a molecular mimic of jasmonyl-L-isoleucine (JA-L-Ile), the most bioactive form of the plant hormone jasmonic acid (JA) (Fonseca et al., 2009; Katsir et al., 2008). JA-L-Ile is an important signalling molecule that controls various biological processes, including defense against herbivores and necrotrophic pathogens (Bignell et al., 2018). Stimulation of the JA-L-Ile signalling pathway by COR leads to repression of the salicylic acid (SA) signalling pathway, which is the main pathway in plants regulating defense against hemibiotropic pathogens like *P. syringae* (Xin & He, 2013). Thus, the production of COR by *P. syringae* provides an adaptive advantage by allowing the pathogen to manipulate the hormone signalling networks in order to overcome host defenses during infection (Gimenez-Ibanez et al., 2016).

Recent studies on CFA-L-Ile produced by *S. scabies* have shown that it displays similar effects on host plant tissues as COR, though it is not as toxic (Fyans et al., 2015). Promoter reporter studies demonstrated that the promoter region driving expression of the CFA-L-Ile biosynthesis genes is active during colonization of plant tissues, and a Δ*cfa6* deletion mutant strain of *S. scabies* was shown to be reduced in virulence in a tobacco seedling bioassay, though the organism was still pathogenic (Bignell et al., 2010). Together, these results suggest that CFA-L-Ile production may enhance the virulence phenotype of *S. scabies* as observed with COR-producing *Pseudomonas* spp. Whether it does so by contributing to host invasion and/or suppression of plant defense responses during infection is currently unknown; however, the structural similarities between COR, CFA-L-Ile and JA-L-Ile suggest that CFA-L-Ile might also function as a JA-L-Ile mimic and induce JA signaling pathways during pathogen infection.

1.6 Thesis Objectives and Outline

CS is a challenging disease for the potato industry worldwide, and traditional control strategies are largely ineffective or unreliable (Wanner & Kirk, 2015). The development of better management strategies for the disease has been hindered in part by a lack of understanding of the mechanisms used by pathogenic *Streptomyces* spp. to colonize and infect their plant hosts, though recent research is beginning to shed more light onto this. Work in our lab has been focusing on the molecular mechanisms of pathogenicity in the best characterized CS pathogen, *S. scabies.* In particular, our lab has been interested in the CFA-L-Ile phytotoxin that is produced by *S. scabies* and which is thought to contribute to the virulence phenotype of the organism. Prior to beginning my thesis work,

there was very little known regarding how the production of this specialized metabolite is regulated in *S. scabies.* A gene (*scab79591*/*cfaR*) encoding a PAS-LuxR family regulator controlling the production of CFA-L-Ile had been identified within the biosynthetic gene cluster, and genetic studies had demonstrated that it functions as the CSR controlling expression of the biosynthetic genes. In addition, *cfaR* was shown to be co-transcribed with a downstream gene (*scab79581/orf1*), the function of which was unknown (Bignell et al., 2010). While it had been shown that the plant-derived molecules cellobiose, cellotriose and suberin can stimulate the production of the thaxtomin A pathogenicity factor in *S. scabies,* there was nothing known regarding molecules that can stimulate the production of CFA-L-Ile. Promoter reporter studies had demonstrated that the promoter driving expression of the CFA-L-Ile biosynthetic genes is active when *S. scabies* is colonizing plant tissues, suggesting that one or more plant-derived molecules may serve as a signal for activating CFA-L-Ile biosynthetic gene expression and metabolite production (Bignell et al., 2010).

This thesis is presented as three research chapters, each of which examines different aspects of the regulatory mechanisms controlling CFA-L-Ile production in *S. scabies*. The primary objectives and outline of each chapter are as follows.

Chapter 2: Regulation of coronafacoyl phytotoxin production by the PAS-LuxR family regulator CfaR in the common scab pathogen *Streptomyces scabies*

The aim of this chapter was to further investigate the mechanism by which CfaR activates the expression of the coronafacoyl phytotoxin biosynthetic genes in *S. scabies*. The CfaR protein was overexpressed and purified from *E. coli*, and its DNA binding

activity was characterized *in vitro.* Truncated versions of CfaR were generated in order to examine the role of the PAS and LuxR domains in the function of the protein. Furthermore, the role of the downstream *scab79581/orf1* gene in CFA-L-Ile production was investigated by constructing plasmids that overexpress the gene together with or without *scab79591*/*cfaR.* The results of this work were published in 2015 in the journal PLoS ONE (Cheng et al., 2015).

Chapter 3: ORF1 is a ThiF-family protein that promotes coronafacoyl phytotoxin production and pathogenicity in the common scab pathogen *Streptomyces scabies*

The aim of this chapter was to further study the *orf1* gene, which encodes a predicted ThiF-family protein. A deletion mutant of *orf1* was constructed in order to determine whether the gene is essential for CFA-L-Ile production, and gene expression analyses were conducted to determine the effects of *orf1* overexpression on phytotoxin biosynthetic gene expression. The ability of ORF1 to modify the CfaR protein post-translationally in *S. scabies* was also explored, and the effects of overexpression of both CfaR+ORF1 on the virulence phenotype of *S. scabies* was investigated. The results of this chapter will be submitted for publication in the near future.

Chapter 4: Effect of microbial and plant-derived molecules on the production of coronafacoyl phytotoxins in the common scab pathogen *Streptomyces scabies*

The aim of this chapter was to study the effects of different small molecules on the biosynthesis of CFA-L-Ile in *S. scabies.* The plant-based molecules cellobiose and suberin are known inducers of the primary virulence factor thaxtomin A, and we investigated

whether such compounds can also stimulate the production of CFA-L-Ile in liquid cultures of *S. scabies*. In addition, we examined whether the CFA-L-Ile end product can influence its own production by modulating the DNA binding activity of the CfaR transcriptional activator. This work will be submitted for publication in the near future.

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1.8 Figures and Tables

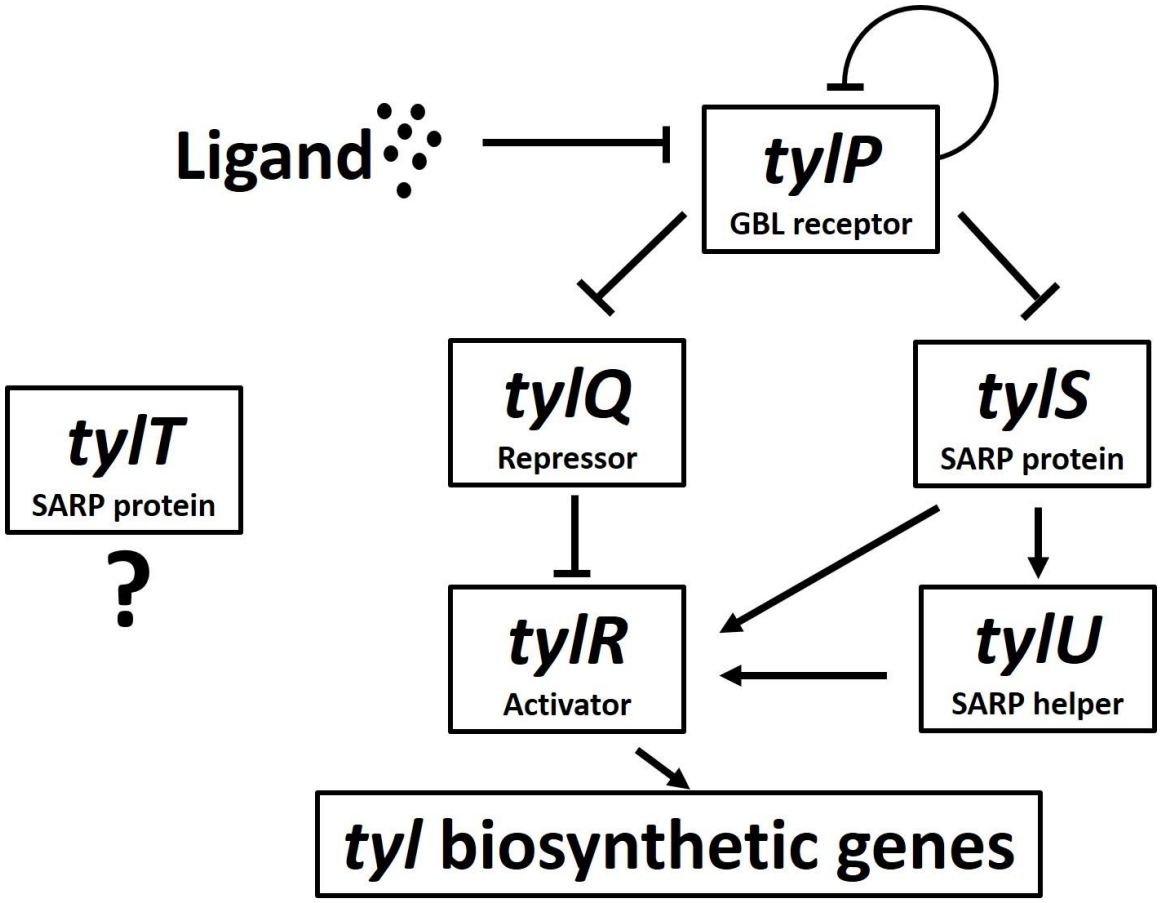

Figure 1.1. Regulation of tylosin production in *Streptomyces fradiae*. The arrows show positive effects and blunt end lines indicate negative effects. The question mark beneath *tylT* indicates that this gene is not essential for tylosin production and its role is currently unknown.

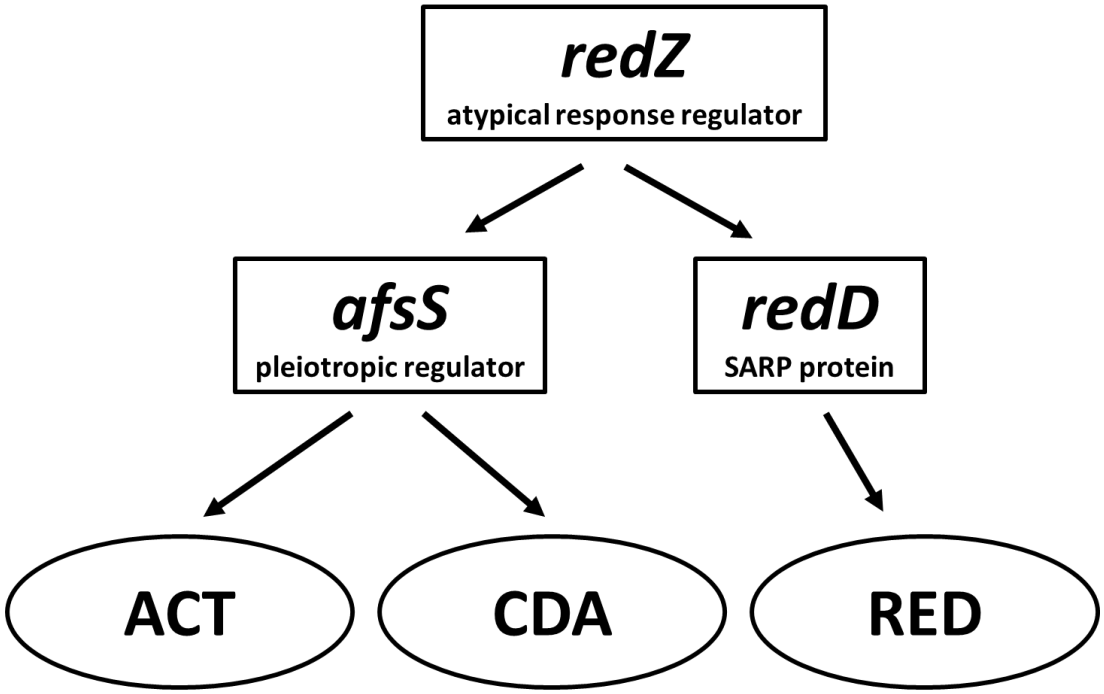

Figure 1.2. Regulation of RED, ACT and CDA production by *redZ* in *Streptomyces coelicolor*. RED: undecylprodigiosin; ACT: actinorhodin; CDA: calcium dependent antibiotic. The arrows show positive effect.

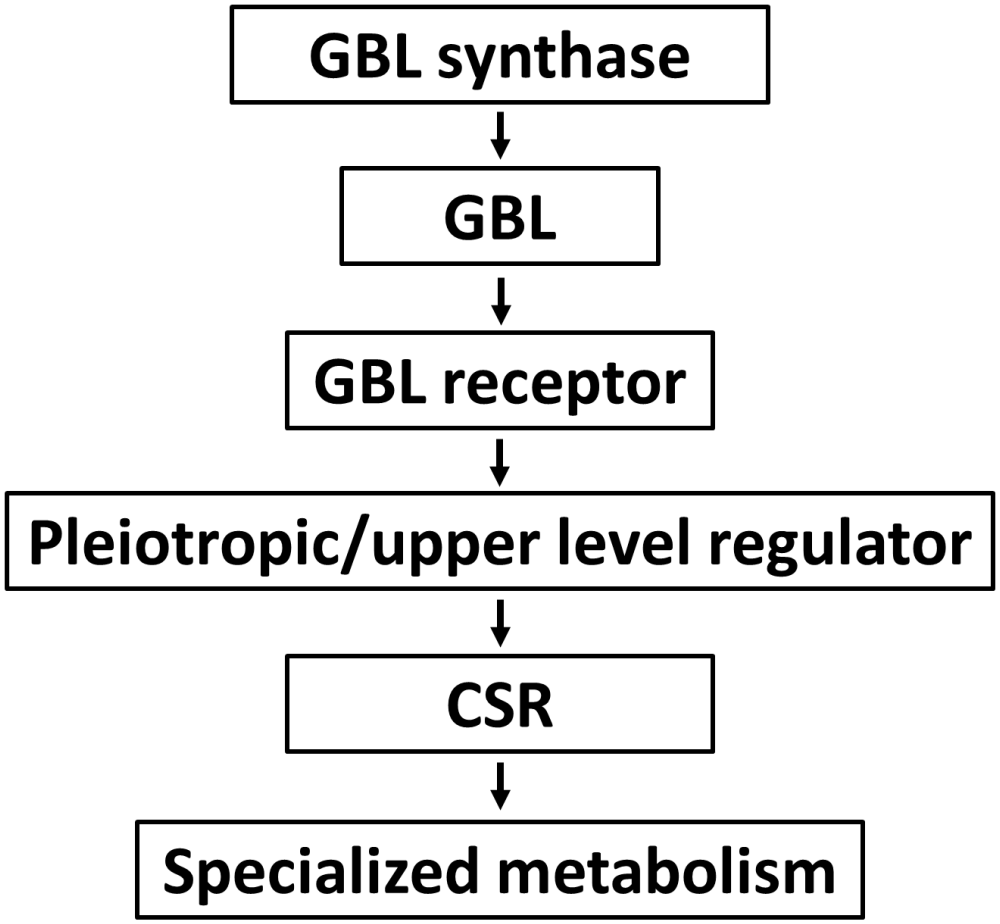

Figure 1.3. Model of GBL regulatory cascades in *Streptomyces* spp. The arrows show hierarchical relationship.

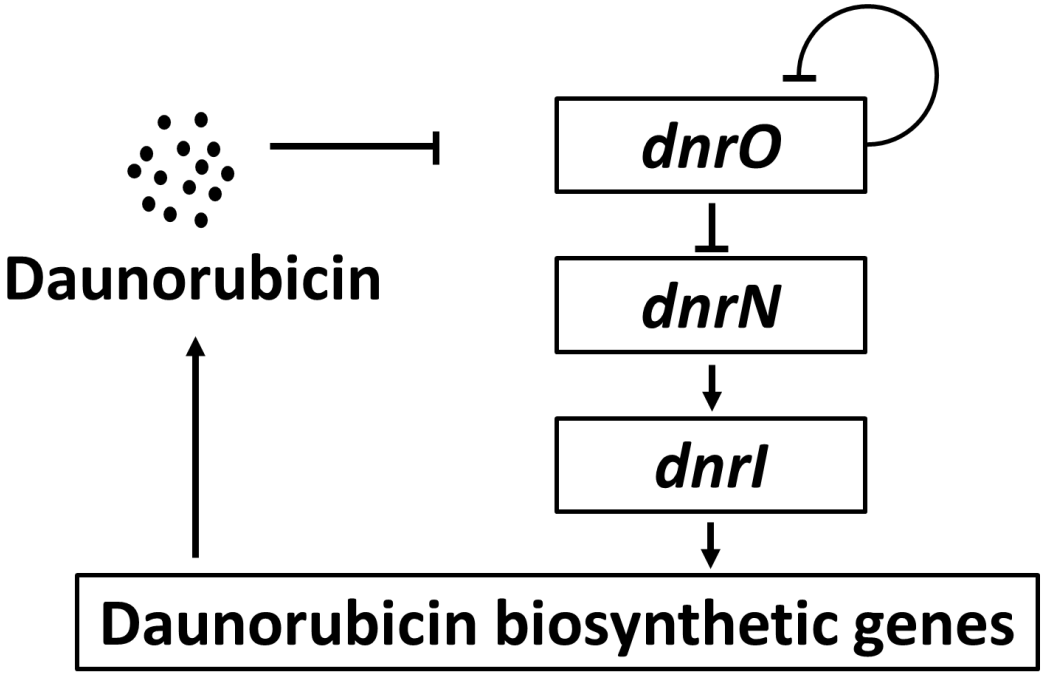

Figure 1.4. Positive feedback control of daunorubicin biosynthesis in *Streptomyces peucetius*. The arrows indicate positive effects and blunt end lines indicate negative effects.

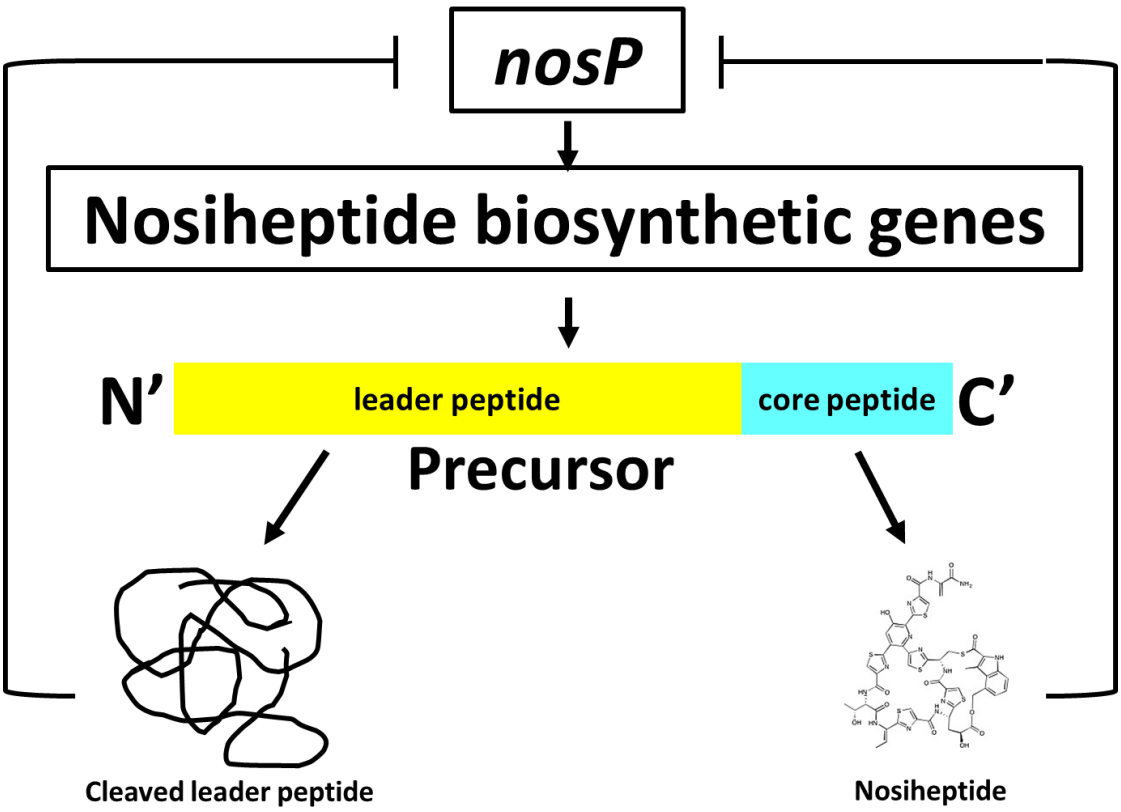

Figure 1.5. Negative feedback control of nosiheptide biosynthesis in *Streptomyces actuosus*. The arrows indicate positive effects and blunt end lines indicate negative effects.

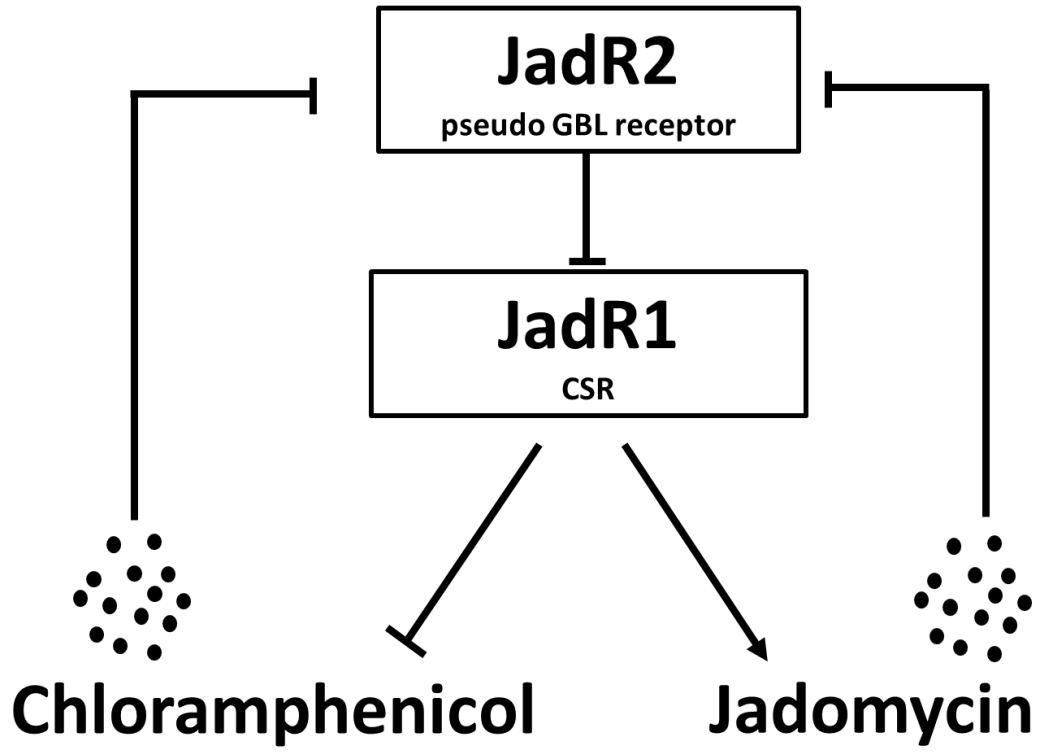

Figure 1.6. Crosstalk regulation of chloramphenicol and jadomycin biosynthesis in *Streptomyces venezuelae*. The arrows indicate positive effects and blunt end lines indicate negative effects.

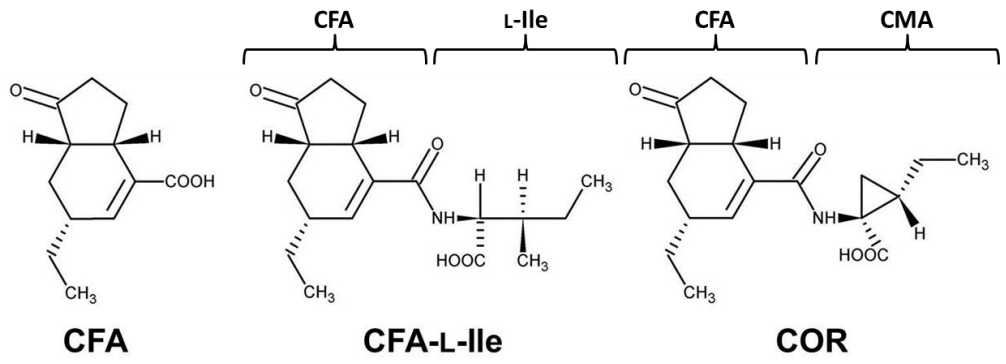

Figure 1.7. Structure of CFA, CFA-L-Ile and COR.

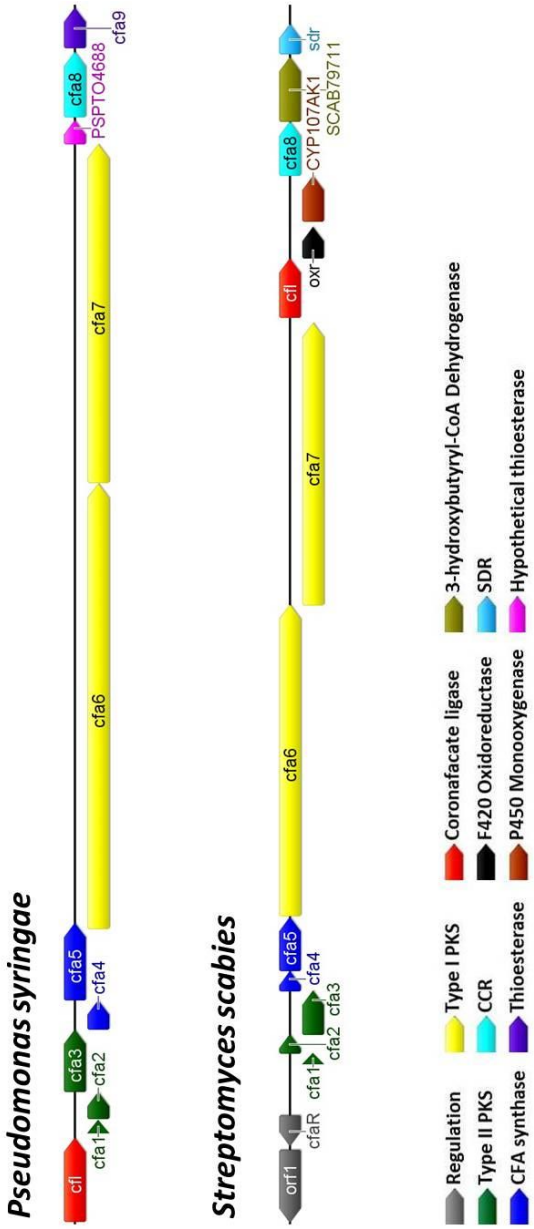

Figure 1.8. Organization of the *cfa* biosynthetic gene cluster from *Pseudomonas syringae* pv. tomato DC3000 and the *cfa*-like biosynthetic gene cluster from *Streptomyces scabies* 87-22. Related genes are in the same color, and the known or predicted functions are indicated below.

Table 1.1. Examples of GBL regulatory cascades in *Streptomyces*

***Streptomyces* spp.**	**Signalling molecule synthase**	**Signalling molecule**	**GBL receptor**	**Pleiotropic/upper level regulator**	**CSR**	**Specialized metabolite**
S. griseus	AfsA	A-factor	ArpA	AdpA	GriR	Grixazone
					StrR	Streptomycin
S. coelicolor	ScbA	SCB	ScbR	n/a*	CpkO	CPK
S. virginiae	BarS1	VB	BarA	VmsR	VmsS	Virginiamycin M and S
					VmsT	Virginiamycin M
S. lavendulae	FarX	IM-2	FarA	**?**	SdmJ (?)	Showdomycin
				?	**?**	Minimycin

*ScbR directly regulates CpkO

Co-Authorship Statement

Chapter 2 is a version of a manuscript published in PLoS One [Cheng Z, Bown L, Tahlan K, Bignell DR. Regulation of coronafacoyl phytotoxin production by the PAS-LuxR family regulator CfaR in the common scab pathogen *Streptomyces scabies*. PLoS One. 2015 Mar 31;10(3):e0122450.]. The initial study concept was designed by D. Bignell, and the experimental methodology was conceived and designed by D. Bignell, Z. Cheng and K. Tahlan. Z. Cheng conducted all of the described experimental work with the exception of the HPLC analysis of CFA-L-Ile production, which was conducted by L. Bown. The primer extension analysis was conducted at The Centre for Applied Genomics (Toronto, Canada) using samples prepared by Z. Cheng. Data analysis was conducted by Z. Cheng and D. Bignell, and reagents, materials and analysis tools were contributed by D. Bignell and K. Tahlan. The manuscript was drafted and prepared by Z. Cheng and D. Bignell, and editorial input was provided by the co-authors.

Chapter 3 is a manuscript in preparation for submission. The initial study concept and experimental methodology were conceived and designed by Z. Cheng and D. Bignell. With some exceptions as described below, Z. Cheng conducted all of the described experimental work and data analyses. L. Bown conducted the HPLC analysis of CFA-L-Ile production, and B. Piercey constructed the ORF1 protein 3D model and performed all of the statistical analyses. Mass spectrometry analysis of the CfaR protein was performed at the SPARC Biocentre at the Toronto Hospital for Sick Children (Toronto, Canada). The manuscript was written by Z. Cheng and D. Bignell, with editorial input provided by the other co-authors.

Chapter 4 is a manuscript in preparation for submission. The initial study concept and experimental methodology were designed by Z. Cheng and D. Bignell. Z. Cheng conducted all of the described experimental work and data analyses except for the HPLC analysis of CFA-L-Ile production, which was conducted by L. Bown, and the statistical analyses, which were performed by B. Piercey. The manuscript was written by Z. Cheng with editorial input by D. Bignell.

Appendix 1 contains a version of a review published in Journal of Applied Microbiology [Bignell DR, Fyans JK, Cheng Z. Phytotoxins produced by plant pathogenic *Streptomyces* species. J Appl Microbiol. 2014 Feb;116(2):223-35.] and a version of a review published in Antoine van Leeuwenhoek [Bignell DRD, Cheng Z, Bown L. The coronafacoyl phytotoxins: structure, biosynthesis, regulation and biological activities. Antonie Van Leeuwenhoek. 2018 May;111(5):649-666.]. This appendix is included to demonstrate scholarly contributions not covered in the main body of the thesis.

Chapter 2: Regulation of coronafacoyl phytotoxin production by the PAS-LuxR family regulator CfaR in the common scab pathogen *Streptomyces scabies*

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2.1 Abstract

Potato common scab is an economically important crop disease that is characterized by the formation of superficial, raised or pitted lesions on the potato tuber surface. The most widely distributed causative agent of the disease is *Streptomyces scabies*, which produces the phytotoxic secondary metabolite thaxtomin A that serves as a key virulence factor for the organism. Recently, it was demonstrated that *S. scabies* can also produce the phytotoxic secondary metabolite coronafacoyl-L-isoleucine (CFA-L-Ile) as well as other related metabolites in minor amounts. The expression of the biosynthetic genes for CFA-L-Ile production is dependent on a PAS-LuxR family transcriptional regulator, CfaR, which is encoded within the phytotoxin biosynthetic gene cluster in *S. scabies*. In this study, we show that CfaR activates coronafacoyl phytotoxin production by binding to a single site located immediately upstream of the putative -35 hexanucleotide box within the promoter region for the biosynthetic genes. The binding activity of CfaR was shown to require both the LuxR and PAS domains, the latter of which is involved in protein homodimer formation. We also show that CFA-L-Ile production is greatly enhanced in *S. scabies* by overexpression of both *cfaR* and a downstream co-transcribed gene, *orf1*. Our results provide important insight into the regulation of coronafacoyl phytotoxin production, which is thought to contribute to the virulence phenotype of *S. scabies*. Furthermore, we provide

evidence that CfaR is a novel member of the PAS-LuxR family of regulators, members of which are widely distributed among actinomycete bacteria.

2.2 Introduction

The genus *Streptomyces* consists of hundreds of species of Gram-positive filamentous actinobacteria that are recognized for their ability to produce a large variety of useful secondary metabolites, including many medically and agriculturally important compounds (Berdy, 2005). In addition, some species are notable for their ability to cause important crop diseases such as potato common scab (CS), which is characterized by the formation of superficial, erumpent (raised) or pitted lesions on the potato tuber surface (Dees & Wanner, 2012). Such lesions negatively impact the quality and market value of the potato tubers and cause significant economic losses to potato growers. In Canada, losses associated with CS during the 2002 growing season were estimated at $15.3-17.3 million dollars (Hill & Lazarovits, 2005), and in Australia, the disease has been estimated to cause losses of approximately 4% of the total industry value (Wilson, 2004). Furthermore, it has been reported that CS can also decrease the overall yield of the potato crop and increase the number of smaller tubers in the yield (Hiltunen et al., 2005).

Streptomyces scabies (syn. *S. scabiei*) is the best characterized and most widely distributed *Streptomyces* spp. that causes CS disease (Dees & Wanner, 2012). The key virulence factor produced by *S. scabies* and other CS-causing pathogens is a phytotoxic secondary metabolite called thaxtomin A, which functions as a cellulose synthesis inhibitor (Bischoff et al., 2009; Duval & Beaudoin, 2009; Fry & Loria, 2002; King & Calhoun, 2009; Scheible et al., 2003). It has been shown by several groups that there is a positive

correlation between the pathogenicity of scab-causing organisms and the production of the thaxtomin A phytotoxin (Goyer et al., 1998; Healy et al., 2000; King et al., 1991; Kinkel et al., 1998; Loria et al., 1995). Recently, it was demonstrated that *S. scabies* strain 87-22 also produces metabolites that are structurally related to the coronatine (COR) phytotoxin, which contributes to the virulence phenotype of the Gram-negative plant pathogen *Pseudomonas syringae* (Fyans et al., 2015). COR functions in promoting the invasion and multiplication of *P. syringae* within the plant host, it contributes to disease symptom development during *P. syringae* infection, and it enhances the disease susceptibility of the plant in uninfected regions (Xin & He, 2013). In *P. syringae*, COR is produced by linking coronafacic acid (CFA) to coronamic acid (CMA), a reaction that is thought to be catalyzed by the coronafacate ligase (Cfl) enzyme (Bender et al., 1999). Although *S. scabies* lacks the ability to produce COR due to the absence of the CMA biosynthetic genes, it does harbour homologues of genes involved in CFA biosynthesis as well as a *cfl* homologue (Bignell et al., 2010). Recent work from our laboratory demonstrated that this organism produces the coronafacoyl compound CFA-L-Ile as a major product along with other related molecules in minor amounts (Fyans et al., 2015). Furthermore, mutational studies in *S. scabies* combined with bioactivity studies of the pure CFA-L-Ile molecule support the notion that this molecule functions as a phytotoxin and contributes to the virulence phenotype of *S. scabies* (Bignell et al., 2010; Fyans et al., 2015).

The biosynthetic gene cluster for production of the coronafacoyl phytotoxins in *S. scabies* is composed of at least 15 genes (Fig. 2.1A), of which 13 are co-transcribed as a single polycistronic mRNA transcript (Bignell et al., 2010). The remaining two genes are

oriented in the opposite direction to the other genes and are co-transcribed as a separate transcript (Bignell et al., 2010). The first gene in this two-gene operon, *scab79591* (herein referred to as *cfaR*), encodes a 265 amino acid protein belonging to the PAS-LuxR family of transcriptional regulators, which are only found in the actinomycetes. Members of this family contain an N-terminal PAS (PER-ARNT-SIM) domain and a C-terminal LuxR-type domain (Fig. 2.1B), and are often associated with secondary metabolite biosynthetic gene clusters. PAS domains belong to a sensing module superfamily that recognize stimuli such as light, oxygen, redox potential or ligands in order to modulate the regulatory activity of the corresponding protein in which they are present (Taylor & Zhulin, 1999). The LuxR-type domain is named after the *Vibrio fischeri* LuxR protein where the domain was first identified, and it contains a helix-turn-helix (HTH) motif that is typically involved in binding to specific DNA sequences called *lux*-boxes within target promoter(s) for transcription activation (Fuqua et al., 1994). The best characterized PAS-LuxR family member is PimM, which in *Streptomyces natalensis* binds to eight promoters and activates expression of the biosynthetic genes for production of the polyene antifungal antibiotic pimaricin (also known as natamycin) (Anton et al., 2007; Santos-Aberturas et al., 2011b). Other family members that have been described include PteF, which controls the production of filipin in *Streptomyces avermitilis* (Vicente et al., 2014), AURJ3M, which is a positive activator of aureofuscin biosynthesis in *Streptomyces aureofuscus* (Wei et al., 2011) and SlnM, which activates production of natamycin in *Streptomyces lyticus* (Wu et al., 2014). In *S. scabies*, the CfaR protein has been shown to function as a transcriptional activator for CFA-L-Ile phytotoxin production (Bignell et al., 2010; Fyans et al., 2015);

however, there is currently no information as to how the protein regulates phytotoxin production.

In this study, we set out to characterize the mechanism of regulation of coronafacoyl phytotoxin biosynthetic gene expression by the CfaR protein. We show that the protein binds to a single imperfect palindromic sequence located immediately upstream of the putative -35 hexanucleotide box in the *cfa1* promoter region, which drives the expression of the biosynthetic genes for phytotoxin production. We also show that the DNA binding activity of CfaR depends on both the LuxR and PAS domains, and that the PAS domain is required for the formation of CfaR homodimers. Furthermore, we demonstrate that a high level of CFA-L-Ile production occurs in *S. scabies* when *cfaR* is overexpressed together with the downstream co-transcribed gene, *orf1*. This, together with phylogenetic analyses of CfaR and other PAS-LuxR proteins, indicates that CfaR is a novel member of the PAS-LuxR family of transcriptional regulators.

2.3 Materials and Methods

2.3.1 Bacterial strains, cultivation and maintenance

Bacterial strains used in this study are listed in Table 2.1. *Escherichia coli* strains were routinely cultivated in Luria-Bertani (LB) Lennox medium (Fisher Scientific, Canada) at 37°C unless otherwise stated. Where required, the LB medium was supplemented with kanamycin or apramycin (Sigma Aldrich, Canada) at 50 μg/mL final concentration, or with chloramphenicol (MP Biomedicals North America, USA) at 25 μg/mL final concentration. *E. coli* strains were maintained at -80°C in 20% v/v glycerol (Sambrook & Russell, 2001).

S. scabies strains were routinely cultured at 25°C or 28°C on potato mash agar (PMA; 5% w/v mashed potato flakes, 2% w/v agar) solid medium or in trypticase soy broth (TSB; BD Biosciences, Canada), nutrient broth (BD Biosciences, Canada) and soy flour mannitol broth (SFMB) liquid media (Kieser et al., 2000). When necessary, the growth medium was supplemented with apramycin or thiostrepton (Sigma Aldrich, Canada) at 50 or 25 μg/mL final concentration, respectively. Seed cultures for RNA extraction were prepared by inoculating 50 μL of a *S. scabies* spore stock into 5 mL of TSB followed by incubation for 24-48 hr until dense mycelial growth was obtained. The seed cultures (0.5 mL) were subsequently used to inoculate 25 mL of SFMB in 125 mL flasks, which were incubated at 25°C and 200 rpm for 4 days. Cultures for small scale CFA-L-Ile extraction were prepared by inoculating TSB seed cultures (200 μL) into 5 mL of SFMB in 6 well plates (Fisher Scientific, Canada) and then incubating at 25°C and 125 rpm for 7 days. *S. scabies* strains were maintained at -80°C as spore suspensions in 20% v/v glycerol (Kieser et al., 2000).

2.3.2 Plasmids, primers and DNA manipulation

Plasmids used in this study are listed in Table 2.1. Plasmids were manipulated in *E. coli* using standard procedures (Sambrook & Russell, 2001). All oligonucleotides used in reverse transcription, PCR, sequencing and electrophoretic mobility shift assays were purchased from Integrated DNA Technologies (USA) and are listed in Table S2.1. DNA sequencing was performed by The Centre for Applied Genomics (TCAG; Canada). *Streptomyces* genomic DNA was isolated from mycelia harvested from 2-day old nutrient

broth cultures using the DNeasy Blood & Tissue Kit as per the manufacturer's protocol (QIAgen Inc, Canada).

2.3.3 Construction of protein expression plasmids

Three forms of the *cfaR* gene, one encoding the full length protein ($CfaR^{full}$), one encoding the first 140 amino acids of the protein with the PAS domain ($CfaR^{\Delta LuxR}$), and one encoding the C-terminal 174 amino acids of the protein and harbouring the LuxR domain ($CfaR^{\Delta PAS}$), were amplified by PCR using Phusion DNA Polymerase (New England Biolabs, Canada) according to the manufacturer's instructions, except that DMSO (5% v/v final concentration) was included in the reactions. The resulting products were digested with NdeI and HindIII (New England Biolabs, Canada) and were ligated into similarly digested pET30b to generate the C-terminal 6 × HIS-tagged full length and truncated CfaR expression plasmids. The constructed expression plasmids were sequenced to confirm the fidelity of the inserts, after which they were transformed into *E. coli* BL21(DE3) cells using the one step method (Chung et al., 1989).

2.3.4 Protein overexpression and purification

For expression of $CfaR^{full}$-HIS_6 and $CfaR^{\Delta LuxR}$-HIS_6, the *E. coli* cells were grown at 28°C in 500 mL of LB containing kanamycin until an OD_{600} of 0.6 was reached, after which isopropyl 1-thio-β-D-glucopyranoside (IPTG) was added to a final concentration of 1 mM and the cells were incubated for an additional 4 h. To express $CfaR^{\Delta PAS}$-HIS_6, cells were grown at 25°C to an OD_{600} of 0.6, after which they were induced with IPTG (0.25 mM final concentration) and were incubated for an additional 5 h. The cells were harvested

and resuspended in buffer consisting of 20 mM sodium phosphate, 500 mM sodium chloride and 30 mM imidazole (pH 7.4), and were lysed using a French press (SLM Instruments Inc., USA). The soluble proteins were purified using an ÄKTA pure FPLC system with a HiTrap IMAC FF 1 mL column at 4°C according to the manufacturer's recommendations (GE Healthcare, Canada). The collected fractions were analyzed by SDS-PAGE on a 12% gel, and those fractions containing protein were pooled and desalted by FPLC using a HiTrap Desalting 5 mL column (GE Healthcare, Canada). The protein concentration in each preparation was determined by the Bradford method (Bradford, 1976), and the proteins were stored at -80°C in buffer containing 20 mM sodium phosphate, 150 mM NaCl and 20% glycerol (pH = 7.8).

2.3.5 Total RNA isolation

S. scabies mycelia from 4-day old SFMB cultures were harvested by centrifugation, and approximately 0.5 g of the cell pellet was placed into a sterile 2 mL microcentrifuge tube. Total RNA was isolated using an innuPREP Bacteria RNA Kit and a SpeedMill PLUS tissue homogenizer (Analytik Jena AG, Germany) as per the manufacturer's instructions. The resulting RNA samples were treated with DNase I (New England Biolabs, Canada) as directed by the manufacturer to remove trace amounts of genomic DNA, after which the DNase-treated RNA samples were quantified using a P300 Nanophotometer (Implen Inc., USA) and were stored at -80°C.

2.3.6 Reverse transcription PCR

Reverse transcription (RT) was performed using SuperScript III reverse transcriptase (Life Technologies, Canada) with 500 ng of DNase-treated total RNA and 2 pmol of the gene-specific primer DRB674. Reactions were set up as per the manufacturer's instructions and were incubated at 55°C for 1 hr. A negative control reaction in which no reverse transcriptase enzyme was added was included to verify the absence of genomic DNA in the RNA samples. PCR was performed using 2 µL of the cDNA template and the primer pairs DRB674-DRB253, DRB674-DRB254a and DRB674-DRB255. Amplification was conducted using Taq DNA polymerase (New England Biolabs, Canada) as per the manufacturer's protocol except that the reactions included 5% v/v DMSO. The resulting PCR products were analyzed by electrophoresis using a 1% w/v agarose gel and 1× Tris Borate EDTA (TBE) buffer and were visualized by staining with ethidium bromide.

2.3.7 Primer extension analysis

Primer extension was performed using a 6-carboxyfluorescein (FAM)-labeled primer, DRB674, as previously described (Palmer et al., 2010) with modifications. Briefly, a 15 µL reaction containing 40 µg of DNase - treated RNA and 0.6 pmol of 5′-FAM-labeled primer was incubated at 65°C for 5 min and then chilled on ice. Next, 3 µL of SuperScript III reverse transcriptase (600U), 1.5 µL of RNaseOUT Recombinant Ribonuclease Inhibitor (Life Technologies, Canada), 3 µL of dNTPs (10 mM each), 1.5 µL of 0.1M dithiothreitol (DTT) and 6 µL of 5× First-Strand Buffer (Life Technologies, Canada) were added to the reaction, and the reaction was incubated at 55°C for 2 hr. An extra 1 µL of

SuperScript III reverse transcriptase (200U) was added after 1 hr of incubation. Then, the reaction was heated at 70°C for 15 min, after which 1 µL (5U) of RNase H (New England Biolabs, Canada) was added and the reaction was incubated at 37°C for 30 min. This was followed by phenol/chloroform extraction and ethanol precipitation of the cDNA. The resulting cDNA pellet was air dried and then sent to TCAG for DNA sizing analysis. The primer extension analysis was performed twice in total.

2.3.8 Electrophoretic mobility shift assay (EMSA)

The DNA probes used for EMSAs were amplified by PCR and were gel-purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Canada). In addition, two pairs of long oligonucleotides (40 nt each), LC12-LC13 and LC14-LC15, were synthesized and used as probes in EMSAs. The complementary oligonucleotides LC12 and LC13 were used to generate probe 1 (P1) and contained the putative CfaR binding site, while the complementary pair LC14 and LC15 were used to generate the negative control probe (P2) and corresponded to the coding region of the *cfaR* gene. The oligonucleotide pairs were incubated at 95°C for 5 min and then slowly cooled to room temperature to allow annealing of the oligonucleotides. The DNA probes were either 3′ end-labeled using the Biotin 3′ End DNA Labeling Kit (Promega, Canada) or were unlabeled. The DNA-protein binding reactions were performed using the LightShift Chemiluminescent EMSA Kit (Fisher Scientific, Canada) according to the manufacturer's instructions. Reactions containing unlabeled DNA and protein were analyzed by non-denaturing PAGE and the DNA was visualized afterwards using ethidium bromide. Reactions containing biotin-labeled probe and protein were analyzed by non-denaturing PAGE, after which the DNA was transferred

to nitrocellulose membrane by contact blotting and then probed with anti-Biotin-alkaline phosphatase antibodies according to the manufacturer's instructions (Fisher Scientific, Canada). Visualization of the DNA was then performed using the Chemiluminescent Nucleic Acid Detection Module (Fisher Scientific, Canada) and the ImageQuant LAS 4000 Digital Imaging System (GE Healthcare, Canada).

2.3.9 Glutaraldehyde cross-linking of CfaR proteins

Crosslinking reactions consisted of purified CfaR protein (80 pmol) and 20 mM sodium phosphate buffer (pH 7.5) in a final volume of 20 μL. The reactions were initiated with the addition of 1 μL of a 2.3% w/v glutaraldehyde solution and were incubated for 5 minutes at 37°C. Termination of the reactions was achieved by the addition of 2 μL of a 1 M Tris-HCl solution (pH 8.0), after which the cross-linked proteins were separated by SDS-PAGE on a 12% polyacrylamide gel and were visualized by staining with Coomassie brilliant blue.

2.3.10 Construction of *cfaR*, *orf1* and *cfaR*+*orf1* overexpression plasmids

Construction of the *cfaR* overexpression vector, pRLDR51-1, was described previously (Bignell et al., 2010). DNA fragments containing *orf1* alone and *cfaR* + *orf1* were amplified by PCR using Phusion DNA Polymerase according to the manufacturer's instructions, except that DMSO (5% v/v final concentration) was included in the reactions. The resulting products were digested with XbaI (New England Biolabs, Canada) and were ligated into similarly digested pRLDB50-1a to generate the *orf1* and *cfaR* + *orf1* overexpression plasmids pRLDB81 and pRLDB891, respectively. The correct orientation

of the inserts was confirmed by digestion with BamHI for pRLDB81 and with PstI, SmaI and NcoI for pRLDB891 (New England Biolabs, Canada), after which the constructed plasmids were sequenced to confirm the fidelity of the inserts. The expression plasmids were then introduced into *E. coli* ET12567/ pUZ8002 prior to transfer into *S. scabies* 87-22 by intergeneric conjugation (Kieser et al., 2000).

2.3.11 Extraction and analysis of CFA-L-Ile production

CFA-L-Ile was extracted from SFMB cultures of *S. scabies* and was quantified by analytical HPLC as described previously (Fyans et al., 2015).

2.3.12 Bioinformatics analysis

Identification of protein domains within the CfaR and ORF1 amino acid sequences was performed using the Pfam database (http://pfam.xfam.org/) (Finn et al., 2014). The logo for the PAS-LuxR protein binding sites was generated using the WebLogo server (http://weblogo.berkeley.edu/logo.cgi) (Crooks et al., 2004). Amino acid sequence alignments of the PAS and LuxR domains from CfaR and other PAS-LuxR proteins in the database were generated using ClustalW within the Geneious version 6.1.2 software (Biomatters Ltd.). The accession numbers for the protein sequences used in the alignments are listed in Table S2.2. Phylogenetic trees were constructed from the alignments using the maximum likelihood method in the MEGA 5.2.1 program (Tamura et al., 2011). Bootstrap analyses were performed with 1000 replicates in each algorithm.

2.4 Results and Discussion

2.4.1 CfaR binds to a single site located in the *cfa1* promoter region

Previous transcriptional studies showed that CfaR is required for expression of several coronafacoyl phytotoxin biosynthetic genes (Bignell et al., 2010), and overexpression of CfaR has been demonstrated to enhance phytotoxin production (Fyans et al., 2015). Given that the biosynthetic genes are expressed as a large polycistronic transcript (Bignell et al., 2010), it was hypothesized that CfaR may control gene activation from the promoter region upstream of *cfa1*, which is the first gene in the operon (Fig. 2.1A). To investigate this further, CfaR was overexpressed and purified from *E. coli* as a C-terminal 6 × histidine tagged protein ($CfaR^{full}$-HIS_6), after which it was used in EMSAs along with six DNA fragments covering different parts of the intergenic region between *cfaR* and *cfa1* (Fig. 2.2A). As shown in Fig. 2.2B, the $CfaR^{full}$-HIS_6 could only bind to two of the DNA fragments (*a* and *e*), both of which covered a 264 bp region immediately upstream of the predicted *cfa1* start codon (Fig. 2.2A). Within this region, a 16 bp imperfect palindromic DNA sequence was identified manually (positions -94 to -79 relative to the *cfa1* translation start codon; Fig. 2.2A and 2.2C), and the sequence was found to be highly similar to the previously described PimM binding site consensus sequence CTVGGGAWWTCCCBAG (Fig. 2.3) (Santos-Aberturas et al., 2011a; Santos-Aberturas et al., 2011b). EMSAs using DNA fragments lacking this palindrome confirmed that it is essential for binding of $CfaR^{full}$-HIS_6 to DNA (Fig. 2.2B). Furthermore, $CfaR^{full}$-HIS_6 could readily bind to a 40 bp labeled oligonucleotide probe (P1) containing only the palindrome and some DNA flanking sequence (Fig. 2.2C and 2.2D) whereas it did not bind

to a control 40 bp probe (P2; Fig. 2.2D) corresponding to the *cfaR* coding region (see Materials and Methods). Finally, binding to the labeled P1 probe was abolished when an excess of unlabeled P1, but not P2, was included in the reaction mixture (Fig. 2.2D), indicating that the interaction between $CfaR^{full}$-HIS_6 and P1 is highly specific.

The location of the CfaR binding site within the *cfa1* promoter was further characterized by mapping the *cfa1* TSS. Total RNA was isolated from a *S. scabies* strain (Δ*txtA*/pRLDB51-1) that overexpresses the *cfaR* gene (Bignell et al., 2010) and produces high levels of the coronafacoyl phytotoxins (Fyans et al., 2015) and RT-PCR was performed using a single reverse primer and different forward primers (Fig. 2.4A) in order to identify the approximate location of the TSS. As shown in Fig. 2.4B, two of the forward primers (DRB253 and DRB254a) allowed for amplification of a PCR product from the cDNA template whereas the third forward primer (DRB255) did not, indicating that the TSS was most likely located somewhere between DRB254a and DRB255. This was verified using non-radioactive primer extension analysis, which identified a C residue located 40 bp upstream of the *cfa* translation start site as the TSS. A putative -10 box (TATGGT) and a -35 box (TCGACC) separated by 18 nt is situated upstream of the C residue (Fig. 2.4A), and these features are consistent with the previously described consensus sequence (TTGACN-$N_{16\text{-}18}$-TASVKT) for streptomycete *E. coli* σ^{70}-like promoters (Bourn & Babb, 1995). Interestingly, the palindromic sequence required for CfaR binding is located immediately upstream of the putative -35 box (Fig. 2.4A), an arrangement that is similar to what has been described for promoters activated by PimM (the binding site of which typically overlaps the -35 box) (Santos-Aberturas et al., 2011b).

Most likely, this arrangement allows for direct contact between the transcriptional activator and RNA polymerase in order to recruit RNA polymerase to the target promoter (Lee et al., 2012).

It is noteworthy that the $CfaR^{full}$-HIS_6 protein did not bind to the DNA fragments *d* and *f*, which cover the promoter region for the *cfaR* gene (Fig. 2.2A and B). This suggests that CfaR does not regulate its own expression, a finding that is consistent with previous transcriptional data from *S. scabies* (Bignell et al., 2010) and is also consistent with the observation that PimM does not regulate its own expression (Anton et al., 2007). In addition, the entire sequence of the coronafacoyl phytotoxin biosynthetic gene cluster was screened for other potential CfaR binding sites, and although a possible binding sequence was found within the *cfa6* gene, the $CfaR^{full}$-HIS_6 protein did not bind to this site in EMSAs (data not shown). Therefore, it appears that CfaR regulates coronafacoyl phytotoxin production using a single DNA binding site within the entire gene cluster.

2.4.2 The CfaR PAS domain is required for DNA binding and protein dimerization *in vitro*

In vitro studies on the *S. natalensis* PimM protein have shown that the DNA binding activity of the protein requires the LuxR DNA binding domain but not the PAS domain, and that removal of the PAS domain actually enhances the DNA binding activity of the protein (Santos-Aberturas et al., 2011b). To investigate the role of the PAS and LuxR domains in the binding of CfaR to DNA, EMSAs were performed using two different truncated forms of the protein, $CfaR^{\Delta LuxR}$-HIS_6 and $CfaR^{\Delta PAS}$-HIS_6, which lack the LuxR and the PAS domain, respectively (Fig. 2.1B). Fig. 2.5A shows that while the $CfaR^{full}$-

HIS_6 protein could bind to the target DNA, neither of the truncated forms showed any DNA binding activity in the assay. This was expected in the case of $CfaR^{\Delta LuxR}$-HIS_6 since the protein lacks the HTH DNA binding motif; however, the lack of binding by $CfaR^{\Delta PAS}$-HIS_6 was surprising based on the previously described results for PimM. Given that transcriptional regulators that bind palindromic sequences normally do so as dimers, we next looked at whether deletion of the PAS domain affected the ability of CfaR to form homodimers using glutaraldehyde crosslinking and SDS-PAGE. As shown in Fig. 2.5B, homodimeric forms of both $CfaR^{full}$-HIS_6 (59.2 kDa) and $CfaR^{\Delta LuxR}$-HIS_6 (32.8 kDa) could be detected upon treatment with glutaraldehyde whereas only the monomeric form of $CfaR^{\Delta PAS}$-HIS_6 (20.0 kDa) could be observed under similar conditions, suggesting that the lack of DNA binding observed with $CfaR^{\Delta PAS}$-HIS_6 is most likely due to the inability of the protein to dimerize. It is noteworthy that a role for the CfaR PAS domain in protein dimerization is consistent with previous studies on the *Drosophila* circadian rhythm regulator (PER), the mouse aryl hydrocarbon receptor (AHR) and AHR nuclear translocator (ARNT), and the *Drosophila* single minded (SIM) transcription factor, where it was shown that the PAS domain in the respective proteins functions as a mediator of homo- and/or heterodimerization (Huang et al., 1993; Lindebro et al., 1995; Pongratz et al., 1998; Reisz-Porszasz et al., 1994).

It is currently unclear as to why deletion of the PAS domain had such a drastically different effect on the DNA binding activity of CfaR as compared to PimM. Possibly, it is related to differences in the type and location of the fusion tag used for purifying each protein. In the case of PimM, the full length protein and its truncated versions were purified

using an N-terminal GST tag (Santos-Aberturas et al., 2011b), whereas a C-terminal HIS_6 tag was used in the current study for purifying CfaR and its truncated versions. Given that GST fusion proteins have been reported to form dimers most likely due to GST-GST interactions (Maru et al., 1996; Niedziela-Majka et al., 1998; Terpe, 2003), it is possible that the presence of the GST protein tag on the N-terminus of the PimM DNA binding domain allowed for dimerization of the protein in the absence of the PAS domain, thereby preserving the DNA binding activity of the truncated protein.

2.4.3 Activation of coronafacoyl phytotoxin production by CfaR is enhanced by ORF1

The *cfaR* gene has been shown to be co-transcribed with a downstream gene, *scab79581* (herein referred to as *orf1*) (Fig. 2.1A), which encodes a protein of unknown function (Bignell et al., 2010). Given that co-transcribed genes are often involved in similar processes, we hypothesized that the ORF1 protein might also play a role in activating coronafacoyl phytotoxin production in *S. scabies*. To investigate this further, the *cfaR* and *orf1* genes were overexpressed individually and together in wild-type *S. scabies* 87-22, which normally produces undetectable or trace levels of CFA-L-Ile under laboratory conditions (Fyans et al., 2015). As shown in Fig. 2.6, overexpression of *cfaR* significantly enhanced CFA-L-Ile production when compared to the vector control, a result that is consistent with previous *cfaR* overexpression studies in the Δ*txtA* thaxtomin A mutant background (Fyans et al., 2015). Interestingly, overexpression of *cfaR* + *orf1* led to an even greater increase (~10 fold) in CFA-L-Ile production when compared to overexpression of *cfaR* alone, while overexpression of *orf1* alone had no significant effect on CFA-L-Ile

production when compared to the vector control (Fig. 2.6). This suggests that ORF1 somehow augments the activation of CFA-L-Ile production by CfaR, though it is currently unclear as to how this would occur. Analysis of the ORF1 protein sequence using the Pfam database revealed the presence of a ThiF family domain (PF00899.16) and a nitroreductase domain (PF00881.19) situated at the N-terminus and central region of the protein, respectively. Interestingly, the ThiF family domain is found in enzymes such as the eukaryotic ubiquitin activating enzyme E1, the *E. coli* thiamine biosynthetic enzyme ThiF and the *E. coli* molybdenum cofactor biosynthetic enzyme MoeB, all of which are known to catalyze the adenylation of a target polypeptide at the C-terminal end (Burroughs et al., 2009). In the case of ThiF and MoeB, the adenylated C-terminus of the target polypeptide is modified further to a thiocarboxylate, which then serves as a sulfur donor for cofactor biosynthesis (Burroughs et al., 2009). Possibly, ORF1 is involved in some sort of post-translational modification of CfaR in order to enhance the ability of CfaR to elicit transcriptional activation. It is interesting to note that the co-transcription of genes encoding a PAS-LuxR homologue and an ORF1 homologue has not been found in other *Streptomyces* spp., suggesting that the regulation of coronafacoyl phytotoxin production may involve a novel mechanism.

2.4.4 Phylogenetic analysis indicates that CfaR is a novel member of the PAS-LuxR protein family

Previously, it was proposed that CfaR may represent a novel member of the PAS-LuxR protein family based on phylogenetic analysis using the complete amino acid sequence of CfaR and other PAS-LuxR proteins from the database (Bignell et al., 2014b).

This analysis demonstrated that CfaR forms a distinct lineage among the different PAS-LuxR family members, though it was unclear as to whether this is mainly due to sequence differences within the PAS domain and/or the LuxR domain, or due to differences within the inter-domain regions only. To examine this further, phylogenetic trees were constructed using only the amino acid sequences of the PAS domain (Fig. 2.7A) or the LuxR DNA binding domain (Fig. 2.7B) from CfaR and other PAS-LuxR family members. Interestingly, the resulting trees showed that both the PAS and LuxR domains from CfaR form a distinct clade among the corresponding domain sequences from other family members (Fig. 2.7A and B), indicating that the uniqueness of the CfaR amino acid sequence can be extended into these conserved domains. It is also interesting to note that regardless of the sequence used to generate the tree (PAS domain, LuxR domain or full length protein), the phylogenetic analyses showed that CfaR is distantly related to the previously characterized PAS-LuxR family members PimM, PteF, AmphRIV and NysRIV (Fig. 2.7A and B) (Bignell et al., 2014b), all of which are associated with polyene antifungal antibiotic biosynthetic gene clusters (Santos-Aberturas et al., 2011a). It has been shown that PteF, AmphRIV and NysRIV are able to complement a *S. natalensis* Δ*pimM* mutant (Santos-Aberturas et al., 2011a) and that PimM is able to complement a *S. avermitis* Δ*pteF* mutant (Vicente et al., 2014). In addition, the purified PimM protein can bind to the predicted target sites for PteF, AmphRIV and NysRIV *in vitro* (Santos-Aberturas et al., 2011a). Together, these results imply that there is functional conservation among these members of the PAS-LuxR family of transcriptional regulators. Whether CfaR is also functionally interchangeable with PimM and similar PAS-LuxR proteins will require further

investigation; however, the analyses performed here suggest that CfaR may be functionally distinct from these other family members.

2.5 Concluding Remarks

This study has established that CfaR is a novel PAS-LuxR family protein that directly activates the expression of the *S. scabies* coronafacoyl phytotoxin biosynthetic genes by binding to a single site within the *cfa1* promoter region. This is the first report detailing the mechanism of coronafacoyl phytotoxin biosynthetic gene regulation by CfaR, and other than studies focused on PimM, it is the only other study to date that describes the biochemical characterization of a member of the PAS-LuxR protein family, which is highly represented among *Streptomyces* spp. and other actinomycetes. We also demonstrated that the PAS domain of CfaR is required for DNA binding and protein dimerization, a function that has not been previously described for this domain within the PAS-LuxR protein family. Given that PAS domains are known to function as sensory domains for controlling the regulatory activity of the corresponding protein (Taylor & Zhulin, 1999), we suspect that the PAS domain in CfaR has the additional role of controlling the DNA binding activity of the protein in response to a ligand or some other stimulus, and we are currently examining this further. It has been shown that the coronafacoyl phytotoxin biosynthetic genes are expressed during colonization of plant tissues (Bignell et al., 2010) and that production of CFA-L-Ile only occurs in media containing plant-derived components (Fyans et al., 2015), and so it is possible that one or more plant-derived compounds serves as a signal for activating gene expression by CfaR. A particularly interesting finding from this study was the demonstration that the co-transcribed *orf1* gene is also involved in activating

phytotoxin production, and we are currently investigating the precise role of the corresponding protein. Furthermore, the involvement of other regulators of *cfaR* via transcriptional or translational control is an area of interest given that a number of the *bld* (bald) gene global regulators that control morphological differentiation and secondary metabolism in *Streptomyces* spp. are known to modulate transcription of the *cfaR* gene or translation of *cfaR* mRNA in *S. scabies* (Bignell et al., 2010; Bignell et al., 2014a).

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2.7 Figures and Tables

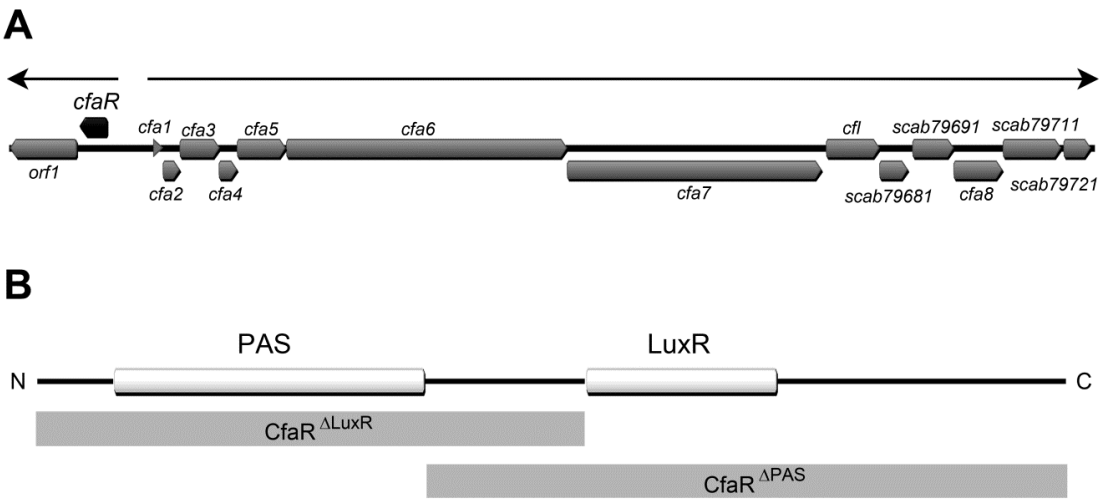

Figure 2.1. Organization of the *Streptomyces scabies* 87-22 coronafacoyl phytotoxin biosynthetic gene cluster and domain structure of the CfaR protein. (A) The block arrows represent the coding sequences within the gene cluster, and the direction of each arrow indicates the direction of transcription. The *cfaR* gene is indicated in black while all other genes are shown in gray. The thin arrows at the top of the image indicate the two transcription units that have been identified (Bignell et al., 2010). (B) The CfaR protein consists of an N-terminal PAS sensory domain (PF00989) and a C-terminal LuxR-type DNA binding domain (PF00196). The gray shaded boxes below the image show the domain composition of the truncated forms of CfaR (CfaR$^{\Delta LuxR}$ and CfaR$^{\Delta PAS}$) that were constructed and used in this study.

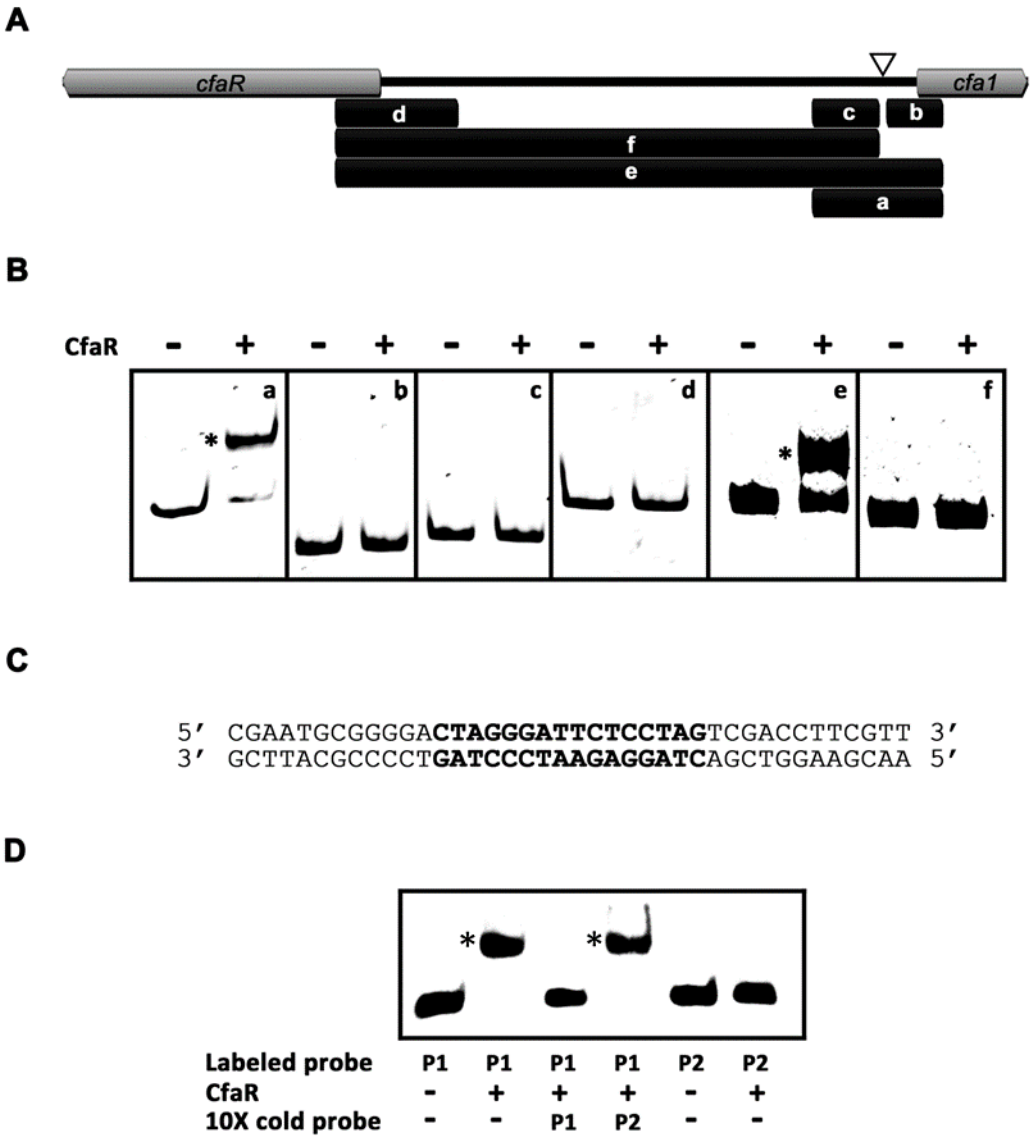

Figure 2.2. CfaR^{full}-HIS$_6$ binds to a single site within the *cfaR-cfa1* intergenic region. (A) Map of the *cfaR-cfa1* intergenic region showing the location of the DNA fragments (indicated by the black bars and labeled *a-f*) used for EMSAs. The position of the 16 bp palindrome identified upstream of *cfa1* is indicated with the white triangle. (B) EMSA results for CfaR^{full}-HIS$_6$ with the DNA fragments *a-f*. Reactions contained 50 ng of DNA

with (+) and without (-) $CfaR^{full}$-HIS_6 protein (3.7 pmol). DNA-protein complexes observed are indicated with *. (C) Sequence of the 40 bp oligonucleotide P1 probe used for EMSAs. The 16 bp palindromic sequence identified upstream of *cfa1* is shown in bold. (D) EMSA results for $CfaR^{full}$-HIS_6 with the P1 oligonucleotide probe. Reactions contained 0.1 pmol of biotin-labeled probe with (+) and without (-) $CfaR^{full}$-HIS_6 protein (2 pmol). Negative control reactions contained the 40 bp biotin-labeled oligonucleotide P2 probe in place of P1. In addition, competition assays were performed in which an excess (10×) of unlabelled (cold) probe (P1 or P2) was included in the reaction. DNA-protein complexes observed are indicated with *.

Promoter	Strand	Sequence (5`-3`)
cfa1	+	CTAGGGATTCTCCTAG
cfa1	–	CTAGGAGAATCCCTAG
pimA	+	ACCGGAAAACCCCTAG
pimA	–	CTAGGGGTTTTCCGGT
pimD	+	GTAGGGATTTCCTGGT
pimD	–	ACCAGGAAATCCCTAC
pimE	+	CTGGCATTTCCCCCAG
pimE	–	GGGGGAAATGCCAGAG
pimI	+	CTAGGTGTTTTCCGGC
pimI	–	GCCGGAAAACACCTAG
pimJ	+	CCCGGAATTTCTTGAC
pimJ	–	CTAGGGAATTCCCGAG
pimK	+	CTAAGGATTTCCCGAC
pimK	–	GTCGGGAAATCCTTAG
pimS1	+	CTGCGGGTTTCCCTAG
pimS1	–	CTAGGGAAACCCGCAG
pimS2	+	TCATGGAATTCCCTAG
pimS2	–	CTAGGGAATTCCATGA

Figure 2.3. Sequence logo of PAS-LuxR protein binding sites. The logo was constructed using WebLogo (Crooks et al., 2004) with the PimM and CfaR binding sites shown below. The overall height of the stack reflects the sequence conservation at that position, and the height of the letters within the stack designates the relative frequency of the corresponding base at that position (Schneider & Stephens, 1990).

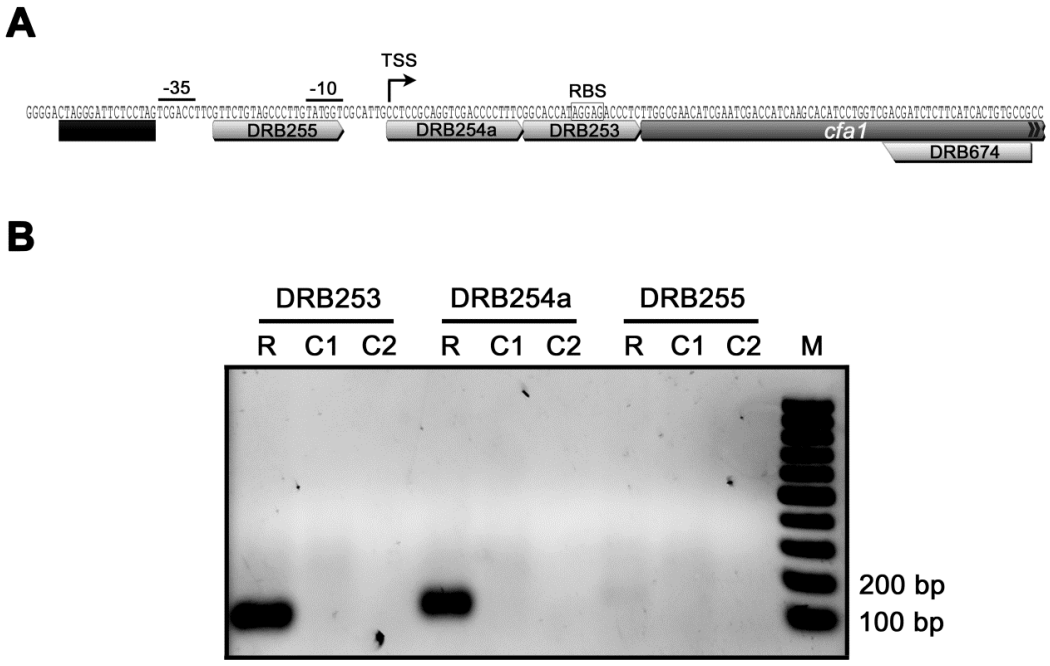

Figure 2.4. Mapping the transcription start site of *cfa1*. (A) Organization of the *cfa1* promoter region. The putative -10 and -35 hexanucleotide sequence boxes and the putative ribosome binding site (RBS) are shown along with the predicted CfaR binding site, which is indicated by the black bar. Also shown are the binding sites for the primers DRB253, DRB254a, DRB255 and DRB674, which were used for low resolution transcript mapping by RT-PCR. The transcription start site (TSS) as determined by non-radioactive primer extension analysis is also indicated. (B) Results of the low resolution transcription start site mapping by RT-PCR. Reverse transcription was performed using *S. scabies* Δ*txtA*/pRLDB51-1 total RNA and using the gene-specific primer DRB674. This was followed by PCR using the reverse primer DRB674 and the forward primers DRB253, DRB254a or DRB255. The resulting products were then analyzed by agarose gel

electrophoresis. R, PCR reactions using cDNA as template; C1, control PCR reactions using RNA (without reverse transcription) as template; C2, control PCR reactions using water as template.

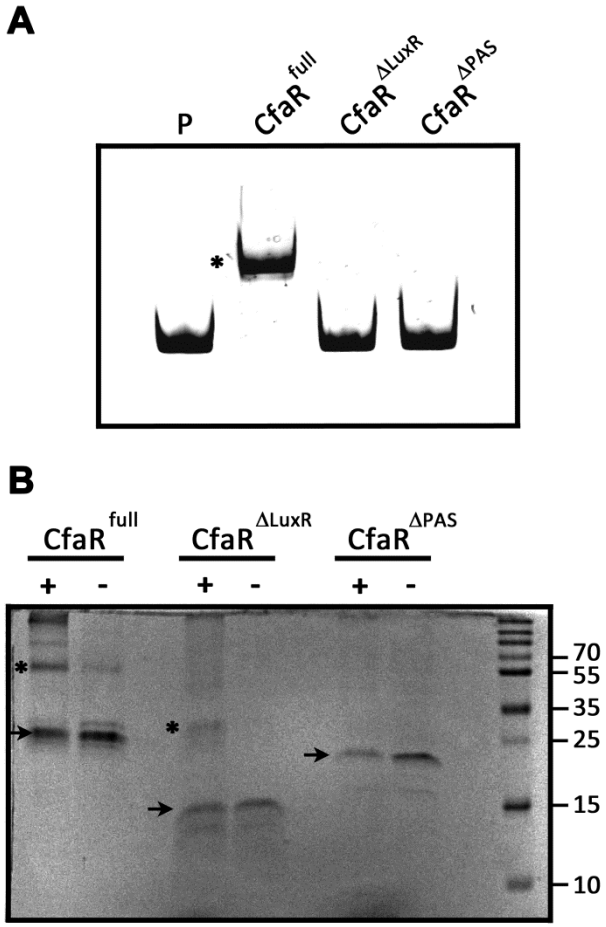

Figure 2.5. The CfaR PAS domain is required for DNA binding and protein dimerization. (A) EMSA results using CfaR^{full}-HIS$_6$, CfaR$^{\Delta LuxR}$-HIS$_6$ and CfaR$^{\Delta PAS}$-HIS$_6$ with DNA fragment *a* (Fig. 2.2A). A control reaction (P) in which no protein was added was also included. The DNA-protein complex observed is indicated with *. (B) Analysis of protein dimerization using chemical crosslinking and SDS-PAGE. CfaR^{full}-HIS$_6$, CfaR$^{\Delta LuxR}$-HIS$_6$ and CfaR$^{\Delta PAS}$-HIS$_6$ were either treated with glutaraldehyde (+) or with solvent alone (-), after which the proteins were separated by SDS-PAGE and were

visualized by staining with Coomassie brilliant blue. Protein monomers are indicated with black arrows and dimers with *. The sizes (in kDa) of the protein molecular weight marker (M) bands used for size estimation are also shown.

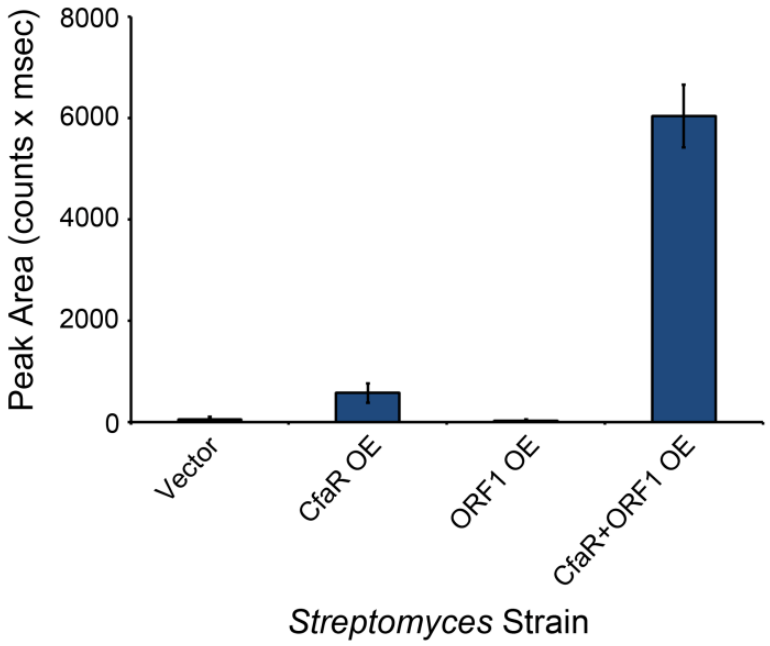

Figure 2.6. Coronafacoyl phytotoxin production is greatly enhanced by overexpression of both *cfaR* and *orf1*. *S. scabies* strains overexpressing *cfaR* alone, *orf1* alone or *cfaR* + *orf1* were cultured in SFMB medium for 7 days at 25°C, after which the culture supernatants were harvested and extracted with organic solvent. The resulting organic extracts were then analysed for CFA-L-Ile production by HPLC. Shown is the mean area of the CFA-L-Ile peak from six cultures of each strain, with error bars indicating the standard deviation. A *S. scabies* strain containing only the overexpression vector was used as a control in this experiment.

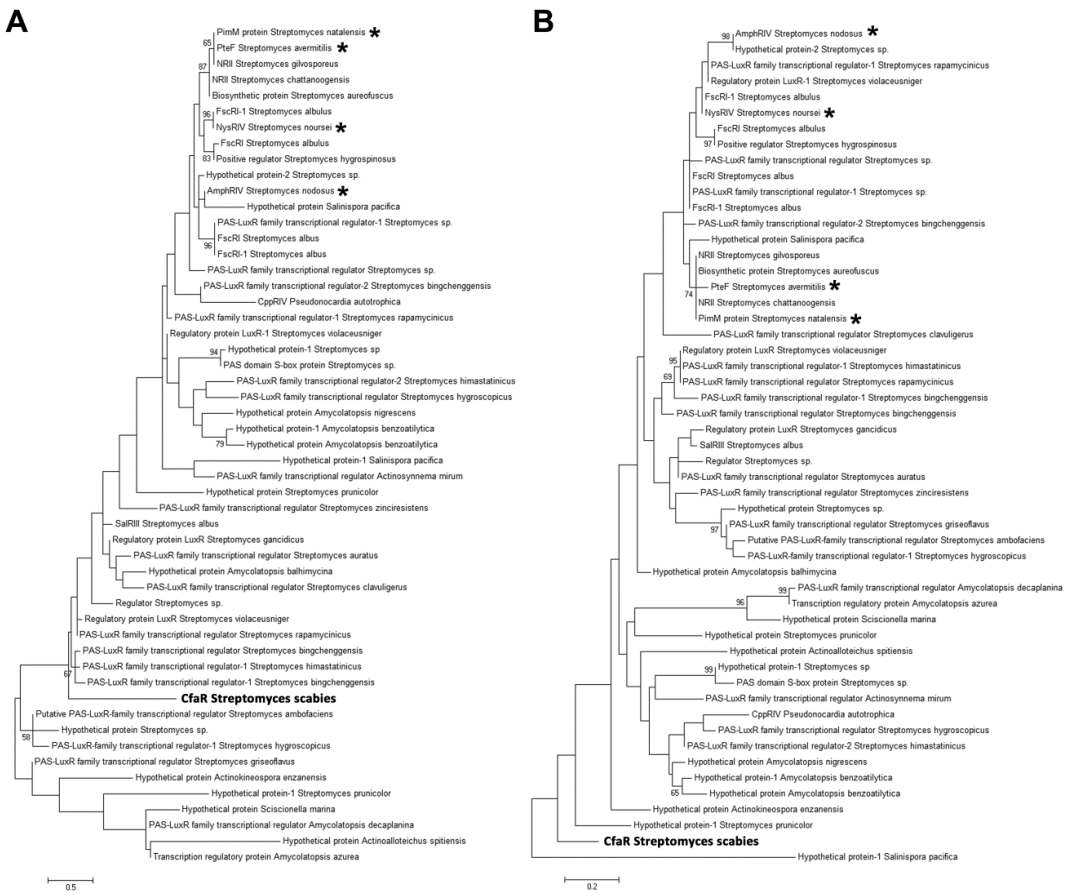

Figure 2.7. Phylogenetic analysis of PAS-LuxR family proteins from *Streptomyces* and other actinomycetes. The phylogenetic trees were constructed based on the amino acid sequences of the PAS domain (A) and the LuxR DNA binding domain (B). The trees were constructed using the maximum likelihood algorithm, and bootstrap values ≥50% for 1000 repetitions are shown. The scale bar indicates the number of amino acid substitutions per site. Family members that have been shown experimentally to be functionally interchangeable are indicated with *.

Table 2.1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Resistance†	Reference or source
***Streptomyces scabies* strains**			
87-22	Wild-type strain	n/a	(Loria et al., 1995)
Δ*txtA*	*S. scabies* 87-22 containing a deletion of the *txtA* thaxtomin biosynthetic gene	$Apra^R$	(Johnson et al., 2009)
***Escherichia coli* strains**			
DH5α	General cloning host	n/a	Gibco-BRL
NEB 5-α	DH5α derivative, high efficiency competent cells	n/a	New England Biolabs
BL21(DE3)	Protein expression strain	n/a	New England Biolabs
ET12567/pUZ8002	*dam*⁻, *dcm*⁻, *hsdS*⁻; nonmethylating conjugation host	Kan^R, Cml^R	(MacNeil et al., 1992)
Plasmids			
pET-30b	N- or C- terminal 6 × histidine fusion tag protein expression vector with T7 promoter and *lac* operator	Kan^R	Novagen
pET-30b/CfaR	pET-30b derivative carrying a DNA fragment for expression of the $CfaR^{full}$-HIS_6 protein	Kan^R	This study
pET-30b/CfaR1.1	pET-30b derivative carrying a DNA fragment for expression of the $CfaR^{\Delta LuxR}$-HIS_6 protein	Kan^R	This study
pET-30b/CfaR1.3	pET-30b derivative carrying a DNA fragment for expression of the $CfaR^{\Delta PAS}$-HIS_6 protein	Kan^R	This study
pRLDB50-1a	*Streptomyces* expression plasmid; carries the strong, constitutive promoter *ermE*p*and	$Apra^R$, $Thio^R$	(Bignell et al., 2010)

	integrates into theφC31 *attB* site		
pRLDB51-1	*scab79591* (*cfaR*) overexpression plasmid derived from pRLDB50-1a	$Apra^R$, $Thio^R$	(Bignell et al., 2010)
pRLDB81	*scab79581* (*orf1*) overexpression plasmid derived from pRLDB50-1a	$Apra^R$, $Thio^R$	This study
pRLDB891	*cfaR* + *orf1* overexpression plasmid derived from pRLDB50-1a	$Apra^R$, $Thio^R$	This study

† $Apra^R$, $Thio^R$, Kan^R and Cml^R = apramycin, thiostrepton, kanamycin and chloramphenicol resistance, respectively. n/a = not applicable.

2.8 Supplementary Materials

Table S2.1. Oligonucleotides used in this study

Primer	Sequence (5′→3′) †	Use
DRB252	GCACAGGAAGGAGGAGAACC	Forward primer for amplification of EMSA probes *a* and *c*
DRB253	GGCACCATAGGAGACCCTCT	Forward primer for *cfa1* TSS mapping by RT-PCR
BRD254a	CCTCCGCAGGTCGACCCCTTTC	Forward primer for *cfa1* TSS mapping by RT-PCR
DRB255	GTTCTGTAGCCCTTGTATGG	Forward primer for *cfa1* TSS mapping by RT-PCR
DRB283	CGGCACAGTGATGAAGAGAT	Reverse primer for amplification of EMSA probes *a*, *b* and *e*
DRB674	CGGCACAGTGATGAAGAGATCG TC	Reverse primer for *cfa1* TSS mapping by RT-PCR and primer extension analysis
LC1	GCG**CATATG**GCGAAATCAGGAG ACCCGTC	Forward primer for construction of the overexpression plasmids pET30b/CfaR and pET30b/CfaR1.1
LC3	GCG**AAGCTT**TGCGCTCTCCAGC TCGGGGT	Reverse primer for construction of the overexpression plasmids pET30b/CfaR and pET30b/CfaR1.3
LC4	GCGC**TCTAGA**CTCTGTACCACC TGACGGAAG	Reverse primer for construction of the overexpression plasmid pRLDB891
LC5	GCGC**TCTAGA**TCCTGCCGGCCC TTCGGTAA	Forward primer for construction of the overexpression plasmids pRLDB81 and pRLDB891
LC6	GCGC**TCTAGA**CCGAACACGCTC ATCCCGTC	Reverse primer for construction of the overexpression plasmid pRLDB81
LC9	GAACGCGTCGTTCGCCTCCA	Forward primer for amplification of EMSA probes *d*, *e* and *f*
LC10	GCACGACCGGCTTTCCCACA	Reverse primer for amplification of EMSA probe *d*
LC12	CGAATGCGGGGACTAGGGATTC TCCTAGTCGACCTTCGTT	Oligonucleotide EMSA probe containing the CfaR binding site; complementary with LC13

LC13	AACGAAGGTCGACTAGGAGAATCCCTAGTCCCCGCATTCG	Oligonucleotide EMSA probe containing the CfaR binding site; complementary with LC12
LC14	TGTGGTTCGCGTCCATGGGTGTCAGGCGGATGCTTAAGTC	Negative control oligonucleotide EMSA probe; complementary with LC15
LC15	GACTTAAGCATCCGCCTGACACCCATGGACGCGAACCACA	Negative control oligonucleotide EMSA probe; complementary with LC14
LC17	GCGC**CATATG**CACACGGACAGGGACTGCCT	Forward primer for construction of the overexpression plasmid pET30b/CfaR1.3
LC20	CCCGCATTCGGGTTGGCGTC	Reverse primer for amplification of EMSA probes *c* and *f*
LC21	GACCTTCGTTCTGTAGCCCT	Forward primer for amplification of EMSA probe *b*
LC45-1	GCGC**AAGCTT**CACCTCGGCGAGTGTCGTGA	Reverse primer for construction of the overexpression plasmid pET30b/CfaR1.1

† Non-homologous extensions are underlined, while engineered restriction sites are indicated in bold.

Table S2.2. Accession numbers of PAS-LuxR homologues used for phylogenetic trees

Name	Accession number
AmphRIV_Streptomyces nodosus	AAV37062.1
Biosynthetic protein_Streptomyces aureofuscus	ACD75765.1
CfaR_Streptomyces scabiei	YP_003493449.1
CppRIV_Pseudonocardia autotrophica	ABV83236.1
FscRI-1_Streptomyces albulus	WP_016577550.1
FscRI-1_Streptomyces albus	WP_003946517.1
FscRI_Streptomyces albulus	WP_016570421.1
FscRI_Streptomyces albus	YP_007749177.1
Hypothetical protein-1_Amycolatopsis benzoatilytica	WP_020659271.1
Hypothetical protein-1_Salinispora pacifica	WP_018725106.1
Hypothetical protein-1_Streptomyces prunicolor	WP_019062029.1
Hypothetical protein-1_Streptomyces sp	WP_018554963.1
Hypothetical protein-2_Streptomyces sp.	WP_017946556.1
Hypothetical protein_Actinoalloteichus spitiensis	WP_016700531.1
Hypothetical protein_Actinokineospora enzanensis	WP_018680905.1
Hypothetical protein_Amycolatopsis balhimycina	WP_020638021.1
Hypothetical protein_Amycolatopsis benzoatilytica	WP_020659273.1
Hypothetical protein_Amycolatopsis nigrescens	WP_020673261.1
Hypothetical protein_Salinispora pacifica	WP_018827366.1
Hypothetical protein_Sciscionella marina	WP_020494548.1
Hypothetical protein_Streptomyces prunicolor	WP_019062028.1
Hypothetical protein_Streptomyces sp.	WP_019328950.1
NRII_Streptomyces chattanoogensis	ACM45445.1
NRII_Streptomyces gilvosporeus	ACM45446.1
NysRIV_Streptomyces noursei	AAF71781.1
PAS domain S-box protein_Streptomyces sp.	WP_016467294.1
PAS-LuxR family transcriptional regulator-1 _Streptomyces bingchenggensis	YP_004958910.1
PAS-LuxR family transcriptional regulator-1_Streptomyces himastatinicus	WP_009712487.1
PAS-LuxR family transcriptional regulator-1_Streptomyces rapamycinicus	WP_020866044.1
PAS-LuxR family transcriptional regulator-1_Streptomyces sp.	WP_018469600.1

PAS-LuxR family transcriptional regulator-2_Streptomyces bingchenggensis	YP_004967281.1
PAS-LuxR family transcriptional regulator-2_Streptomyces himastatinicus	WP_009712640.1
PAS-LuxR-family transcriptional regulator-1_Streptomyces hygroscopicus	YP_006242418.1
PAS-LuxR family transcriptional regulator_Actinosynnema mirum	YP_003102220.1
PAS-LuxR family transcriptional regulator_Amycolatopsis decaplanina	WP_007029767.1
PAS-LuxR family transcriptional regulator_Streptomyces auratus	WP_006607812.1
PAS-LuxR family transcriptional regulator_Streptomyces avermitilis	NP_821584.1
PAS-LuxR family transcriptional regulator_Streptomyces bingchenggensis	YP_004967325.1
PAS-LuxR family transcriptional regulator_Streptomyces clavuligerus	WP_003959350.1
PAS-LuxR family transcriptional regulator_Streptomyces griseoflavus	WP_004936272.1
PAS-LuxR family transcriptional regulator_Streptomyces hygroscopicus	YP_006242550.1
PAS-LuxR family transcriptional regulator_Streptomyces rapamycinicus	WP_020866268.1
PAS-LuxR family transcriptional regulator_Streptomyces sp.	WP_019328946.1
PAS-LuxR family transcriptional regulator_Streptomyces zinciresistens	WP_007491099.1
PimM protein_Streptomyces natalensis	CAM35468.1
Positive regulator_Streptomyces hygrospinosus	AFQ68276.1
Putative PAS-LuxR-family transcriptional regulator_Streptomyces ambofaciens	CAJ89366.1
Regulator_Streptomyces sp.	ABB88526.1
Regulatory protein LuxR-1_Streptomyces violaceusniger	YP_004814000.1
Regulatory protein LuxR_Streptomyces gancidicus	WP_006129792.1
Regulatory protein LuxR_Streptomyces violaceusniger	YP_004813791.1
SalRIII_Streptomyces albus	ABG02265.1
Transcription regulatory protein_Amycolatopsis azurea	WP_005165576.1

Chapter 3: ORF1 is a ThiF-family protein that promotes coronafacoyl phytotoxin production and pathogenicity in the common scab pathogen *Streptomyces scabies*

Zhenlong Cheng, Luke Bown, Brandon Piercey and Dawn R.D. Bignell

3.1 Abstract

The potato common scab (CS) pathogen *Streptomyces scabies* produces coronafacoyl-L-isoleucine (CFA-L-Ile), which is a member of the coronafacoyl family of phytotoxins that are synthesized by several different plant pathogenic bacteria. The biosynthetic gene cluster for producing CFA-L-Ile consists of 13 biosynthetic genes and one regulatory gene, *cfaR*, which directly controls the expression of the biosynthetic genes. An additional gene, *orf1*, is located downstream of *cfaR* and encodes a protein showing similarity to ThiF family proteins from other actinomycetes. Previously it was shown that *orf1* is co-transcribed with *cfaR* and that overexpression of both *cfaR* and *orf1* in *S. scabies* results in significantly higher CFA-L-Ile production levels compared to the overexpression of *cfaR* alone, suggesting that *orf1* may also function in the regulation of CFA-L-Ile production. In this study, we showed that deletion of the *orf1* gene results in a significant reduction in CFA-L-Ile production, and that the loss of *orf1* can be compensated by the overexpression of *cfaR* only. Semi-quantitative RT-PCR confirmed that the overexpression of both *cfaR* and *orf1* leads to higher expression of the CFA-L-Ile biosynthetic genes as compared to both the control and the overexpression of *cfaR* alone, and virulence bioassays demonstrated that the CfaR+ORF1 overproducing strain causes greater necrosis and pitting of potato tuber tissue as compared to strains that produce less CFA-L-Ile. Bioinformatics analysis of the ORF1 amino acid sequence suggested that it may function as an AMPylator,

which is an enzyme that catalyzes the AMPylation (also known as adenylylation or adenylation) of a substrate. Though it was predicted that ORF1 may post-translationally modify CfaR by AMPylation, analysis of purified HIS_6-CfaR from *S. scabies* using mass spectrometry did not detect any post-translational modifications of the protein in the presence of ORF1. The results of our study show that ORF1 is important but not essential for CFA-L-Ile production in *S. scabies*, and they provide further evidence supporting a role for CFA-L-Ile as a virulence factor in *S. scabies* plant pathogenicity.

3.2 Introduction

CS is a very important disease of potato (*Solanum tuberosum*) caused by several different plant pathogenic *Streptomyces* spp. (Wanner & Kirk, 2015). The symptoms of the disease vary from superficial to deep-pitted or raised lesions that form on the surface of potato tubers, and while these lesions do not affect the nutritional quality of the potatoes, they have a negative impact on the tuber market value. It has been estimated that CS caused economic losses between 15.3 and 17.3 million Canadian dollars in 2002 in Canada (Hill & Lazarovits, 2005), and in Tasmania, Australia, losses attributed to CS have been estimated at $3.66 million Australian dollars per acre, representing about 4% of the industry value (Wilson, 2004). CS may also affect potato yields by causing delayed emergence of the tubers and decreased tuber sizes (Hiltunen et al., 2005).

The main pathogenicity factor produced by scab-causing *Streptomyces* spp. is a nitrated 2,5-diketopiperazine phytotoxin called thaxtomin A (King & Calhoun, 2009). Pure thaxtomin A can induce necrosis on excised potato tuber tissue and can cause scab-like

lesions on immature potato tubers (Bignell et al., 2014). It has been noted that there is a positive correlation between thaxtomin A production and the pathogenicity of scab-causing *Streptomyces* spp. (Goyer et al., 1998; King et al., 1991; Kinkel et al., 1998; Loria et al., 1995) and thaxtomin A - deficient mutant strains do not cause typical disease symptoms (Healy et al., 2000; Joshi et al., 2007). Although the exact function of thaxtomin A in plant pathogenicity is not clear, several lines of evidence suggest that it targets cellulose synthesis in the plant host (Bischoff et al., 2009; Duval & Beaudoin, 2009; Fry & Loria, 2002; Scheible et al., 2003) and may be required for the penetration of plant tissue during host colonization (Loria et al., 2008).

Recently, *S. scabies* was reported to be able to produce CFA-L-Ile, which is a member of the coronafacoyl family of phytotoxins (Fyans et al., 2015). Coronafacoyl phytotoxins are known or suspected to be produced by several different plant pathogenic bacteria, and while they are not essential for the pathogenicity of the producing organisms, they typically enhance the severity of disease symptoms induced by these organisms (Bignell et al., 2018). The best characterized coronafacoyl phytotoxin is coronatine (COR), which is an important virulence determinant produced by the Gram-negative plant pathogen *Pseudomonas syringae*. COR contributes to pathogen invasion and persistence within plant tissues, and to disease symptom development (Xin & He, 2013). It functions as a molecular mimic of the plant hormone jasmonoyl-L-isoleucine (JA-L-Ile), and production of COR within the plant host leads to both activation of JA-mediated signalling and suppression of salicylic acid (SA)-mediated signalling (Xin & He, 2013). This, in turn, results in suppression of the plant immune responses that are important for combating

P. syringae infection (Katsir et al., 2008). CFA-L-Ile and other coronafacoyl phytotoxins exhibit similar bioactivities against plant tissues as COR, suggesting that they may also function as molecular mimics of JA-L-Ile (Bignell et al., 2018). A mutant of *S. scabies* unable to produce CFA-L-Ile was previously shown to cause less severe disease symptoms in a tobacco seedling bioassay, though the organism was still pathogenic (Bignell et al., 2010). While this suggests that CFA-L-Ile production contributes to the virulence phenotype of *S. scabies*, it is currently unclear whether the development and/or severity of CS disease symptoms caused by *S. scabies* is affected by production of this phytotoxin.

The biosynthetic gene cluster for producing CFA-L-Ile in *S. scabies* is composed of at least 15 genes (Fig. 3.1), of which 13 are co-transcribed as a single polycistronic mRNA and encode the enzymes required for coronafacoyl phytotoxin biosynthesis (Bignell et al., 2010). Another gene called *cfaR* is divergently transcribed from the biosynthetic operon and encodes a cluster-situated regulator (CSR) that controls the expression of the CFA-L-Ile biosynthetic genes (Cheng et al., 2015). CfaR is a 265 amino acid protein belonging to the PAS-LuxR family of transcriptional regulators, which are widely distributed among actinomycete bacteria (Bignell et al., 2010). Expression of the CFA-L-Ile biosynthetic genes and production of CFA-L-Ile is induced by overexpression of CfaR, suggesting that CfaR is a positive activator of the CFA-L-Ile biosynthetic pathway (Bignell et al., 2010; Cheng et al., 2015). CfaR binds to a single site within the promoter region that drives expression of the biosynthetic operon, and this binding activity requires both the LuxR DNA binding domain and the PAS domain, the latter of which is involved in protein homodimer formation (Cheng et al., 2015). Downstream of *cfaR* is a second gene

called *orf1* (also known as *SCAB79581*), which encodes a 636 amino acid protein. *orf1* is co-transcribed with *cfaR* (Bignell et al., 2010) and overexpression of both CfaR and ORF1 causes significantly greater CFA-L-Ile production in *S. scabies* as compared to overexpression of CfaR alone (Cheng et al., 2015). These results suggest that ORF1 may also function in the regulation of CFA-L-Ile production in *S. scabies.*

In this study, we employed genetic and biochemical strategies to further investigate the function of the *orf1* gene in CFA-L-Ile production. Our results show that ORF1 is important but not essential for metabolite production, and it may serve as a "helper" protein in assisting the function of CfaR in activating CFA-L-Ile biosynthesis. Furthermore, we demonstrate that enhanced CFA-L-Ile production by overexpression of both CfaR and ORF1 augments the severity of disease symptoms induced by *S. scabies* during infection of potato tuber tissue. Our study provides further evidence that CFA-L-Ile is an important virulence factor in *S. scabies*, and that its production in this organism is controlled by multiple CSRs.

3.3 Materials and Methods

3.3.1 Bacterial strains, cultivation and maintenance

Bacterial strains used in this study are listed in Table 3.1. *Escherichia coli* strains were routinely cultivated in Luria-Bertani (LB) Lennox medium (Fisher Scientific, Canada) at 37°C unless otherwise stated. Where required, the LB medium was supplemented with kanamycin, apramycin or chloramphenicol as described previously (Cheng et al., 2015). *E. coli* strains were maintained at -80°C in 20% v/v glycerol (Sambrook & Russell, 2001).

Streptomyces strains were routinely cultured at 28°C on potato mash agar (PMA) (Fyans et al., 2015) for sporulation and maintenance, or in trypticase soy broth (TSB) (BD Biosciences, Canada) for protein purification and fluorescence intensity measurements. When necessary, the growth medium was supplemented with apramycin, kanamycin, hygromycin B or thiostrepton at 50, 50, 50 or 25 μg/mL final concentration, respectively. For analysis of thaxtomin A or CFA-L-Ile production, seed cultures of *S. scabies* strains were prepared by inoculating 50 μL of a frozen spore stock, 1 mL of a mycelial stock, or fresh spores from a PMA plate into 5 mL of TSB in a 50 mL spring flask. The seed cultures were incubated at 28°C for 24-48 hr with shaking (200 rpm) until dense mycelial growth was obtained, after which they were subcultured (1-2% v/v inoculum) into 3 × 5 mL of oat bran broth (OBB) (Johnson et al., 2007) or SFMB (Kieser et al., 2000) in 6-well tissue culture plates for production of thaxtomin A or CFA-L-Ile, respectively. The cultures were incubated at 25°C with shaking (125 rpm) for 7 days prior to processing for metabolite analysis. For gene expression analysis, *S. scabies* TSB seed cultures (0.5 mL) were sub-cultured into 25 mL of SFMB in a 125 mL spring flask. The cultures were then incubated at 25°C and 200 rpm for 2 days, after which the mycelia were harvested for RNA isolation. For total protein isolation from *Streptomyces*, TSB seed cultures (25 mL) were sub-cultured into 1 L of TSB, after which the cultures were incubated at 25°C and 200 rpm for 48 hours. *S. scabies* and *Streptomyces coelicolor* strains were maintained at -80°C as spore suspensions in 20% v/v glycerol (Kieser et al., 2000) or as mycelial suspensions in TSB containing 25% glycerol (Shepherd et al., 2010).

3.3.2 Plasmids, primers and DNA manipulation

Plasmids used in this study are listed in Table 3.1. Plasmids were manipulated in *E. coli* using standard procedures (Sambrook & Russell, 2001). Standard desalted oligonucleotides used in this study were purchased from Integrated DNA Technologies (USA) and are listed in Table S3.1. All PCR-amplified genes in this study were verified by sequencing. DNA sequencing was performed at The Centre for Applied Genomics (TCAG, Toronto, Canada).

3.3.3 Bioinformatics analyses

BlastP (https://blast.ncbi.nlm.nih.gov) was performed using the ORF1 amino acid sequence to identify homologous proteins in the database. Alignment of the ORF1 amino acid sequence with that of other ThiF family proteins was performed using ClustalW within the Geneious version 6.1.2 software (Biomatters Ltd.). The accession numbers for the protein sequences used in the alignment are listed in Table S3.2. A phylogenetic tree was constructed from the alignment using the maximum likelihood method in the MEGA program version 7.0.14 (Tamura et al., 2011). Bootstrap analysis was performed with 1000 replicates. Pfam (http://pfam.xfam.org) and BlastP were used to search for conserved domains in ORF1.

The monomeric 3-dimensional model of ORF1 was produced using the default parameters of I-TASSER v5.1 (Roy et al., 2012; Zhang, 2008). The resultant model was then visualized in PyMOL (The PyMOL Molecular Graphics System, Version v2.0.1, Schrödinger, LLC.). The structural model was analyzed using COFACTOR and COACH

to identify putative ligands that might interact with ORF1 (Roy et al., 2012; Yang et al., 2013; Zhang et al., 2017) .

Codons within the *orf1* coding sequence that are rare in *E. coli* were calculated according to the method described by (Daniel et al., 2015)

3.3.4 RNA isolation and analysis of gene expression

RNA isolation from *S. scabies* and RT-PCR analysis of gene expression were performed as described before (Cheng et al., 2015). *murX* gene served as the loading control (Joshi et al., 2007). The gene-specific primers used for the RT-PCR analysis are listed in Table S3.1.

3.3.5 Construction of the *S. scabies* Δ*txtA*/Δ*orf1*, Δ*txtA*/Δ*cfaR* and Δ*txtA*/Δ*cfaR*/Δ*orf1* mutant strains

Gene deletion mutants were constructed using the Redirect PCR targeting system (Gust et al., 2003). Cosmid 158 (Cos158), harbouring the *cfaR* and *orf1* genes, was introduced into the *E. coli* strain BW25113/pIJ790. The deletion cassettes were PCR-amplified using pIJ10700 as the template and using the primers DRB292 and DRB293 for deletion of *cfaR*, JS1 and JS2 for deletion of *orf1*, and JS1 and DRB293 for deletion of *cfaR*+*orf1* (Table S3.1). The PCR products were gel purified and then electroporated into arabinose-induced *E. coli* BW25113/pIJ790/Cos158 that was cultured at 28°C. The resulting mutant cosmids were verified by PCR and were then introduced into the *S. scabies* Δ*txtA* mutant (Table 3.1) by conjugation with *E. coli* as described previously (Kieser et al.,

2000). Exconjugants were selected for resistance to hygromycin B and sensitivity to kanamycin, and deletion of the target gene(s) in the mutant isolates was confirmed by PCR.

3.3.6 Genetic complementation of the *orf1* deletion mutant

The *orf1* overexpression plasmid pRLDB81, the *cfaR* overexpression plasmid pRLDB51-1, and the *cfaR*+*orf1* overexpression plasmid pRLDB891 (Table 3.1) were each introduced into the Δ*txtA*/Δ*orf1* mutant strain by conjugation with *E. coli*. As a control, the empty vector pRLDB50-1a (Table 3.1) was also introduced into the strain. The resulting exconjugants were selected by culturing on media containing thiostrepton.

3.3.7 Construction of *Streptomyces* gene overexpression plasmids harbouring the *ermE*p*, *kasO*p* and SP44 promoters

Synthetic DNA fragments (Eurofin MWG Operon LLC, USA) containing the SP44 and *kasO*p* promoters (Bai et al., 2015) were A-tailed using Taq DNA polymerase (New England Biolabs) and were cloned into the pGEM-T vector (Promega Corporation), according to manufacturer's instructions to generate pGEM/SP44 and pGEM/*kasO*p*, respectively (Fig. S3.1, Table 3.1).

A fragment containing the kanamycin resistant gene (*neo3*) and promoter was PCR-amplified using the LC78 and LC79 primers and the pET30(b) plasmid as template, and was cloned into the NheI site of pIJ8660-*ermE*p* to make pOEEN. The SP44 and *kasO*p* fragments were released from pGEM/SP44 and pGEM/*kasO*p*, respectively, by digestion with EcoRV and NdeI, and were cloned into EcoRV and NdeI - digested pOEEN, to replace the *ermE*p* promoter. This produced pOSEN and pOKEN, the plasmids expressing *egfp*

under control of the SP44 and *kasO*p* promoters, respectively. pOEEN, pOSEN and pOKEN were each introduced into the *S. scabies* Δ*txtA*/Δ*cfaR*/Δ*orf1* and *S. coelicolor* M1154 strains by conjugation with *E. coli,* and exconjugants were selected using media containing kanamycin.

cfaR was PCR-amplified using plasmid pRLDB891 as template and using the LC52 and LC53 primers, and the resulting product was used to replace the *egfp* gene of pIJ8660-*ermE*p* by cloning into the NdeI and NotI sites to make pOEC. A fragment containing the *neo3* gene and promoter was then PCR-amplified using the LC78 and LC79 primers and the pET30(b) plasmid as template, and was cloned into the NheI site of pOEC to generate pOECN. The SP44 and *kasO*p* fragments were released from pGEM/SP44 and pGEM/*kasO*p*, respectively, by digestion with EcoRV and NdeI. Following gel extraction, the fragments were used to replace the *ermE*p* promoter of pOECN by cloning into the EcoRV and NdeI sites to produce pOSCN and pOKCN. pOECN, pOSCN and pOKCN, which express *cfaR* from the *ermE*p*, SP44 and *kasO*p* promoters, respectively, were then introduced into the *S. scabies* mutant strain Δ*txtA*/Δ*cfaR*/Δ*orf1* by conjugation with *E. coli,* and exconjugants were selected using media containing kanamycin.

A fragment containing both *cfaR* and *orf1* was PCR amplified using the LC52 and LC71 primers and plasmid pRLDB891 as template, and was ligated into the pGEM-T easy vector. After sequencing, a clone in which the *cfaR + orf1* insert was flanked by NdeI (on LC52 primer) and NotI (on pGEM-T vector) sites was selected and designated as pGEM/891N-N. The *cfaR + orf1* fragment was released from pGEM/891N-N by digestion

with NdeI and NotI and was ligated into NdeI and NotI - digested pOSCN to generate pOSCON. pOSCON was introduced into *S. scabies* Δ*txtA*/Δ*cfaR*/Δ*orf1* by conjugation with *E. coli,* and exconjugants were selected using media containing kanamycin.

All plasmids are listed in Table 3.1 and primers are listed in Table S3.1. A schematic diagram of the overexpression plasmids and the construction strategy used is shown in Fig. S3.2.

3.3.8 eGFP fluorescence assay

Fresh spores of *S. scabies* Δ*txtA*/Δ*cfaR*/Δ*orf1* and *S. coelicolor* M1154, containing plasmids expressing *egfp* under control of the SP44 (pOSEN), *kasO*p* (pOKEN) or *ermE*p* (pOEEN) promoters, were inoculated into 5 mL of TSB and were cultured for 48 hours with shaking. Detection of eGFP fluorescence was performed according to the protocol described previously (Moore, 2015) with the following modifications. The TSB seed cultures were standardized as described by Brana (Brana et al., 1985), and then a 0.59 mg equivalent Dry cell weights (DCWs) of each was added to 25 mL of TSB and the cultures were incubated as triplicates at 25°C and 200 rpm. From each culture, 1.5 mL was removed at 24, 48 and 72 hours and the mycelia were pelleted by centrifugation. The mycelial pellets were washed twice with 1 mL of PBS (20 mM sodium phosphate and 300mM sodium chloride, pH 7.4) and were re-suspended in 200 µL of PBS. The suspensions were transferred into a 96 well plate, and fluorescence due to eGFP production was measured using a Synergy H1MG plate reader (BTH1MG, Biotek, Winooski, VT,

USA) with excitation at 488nm, emission at 510nm, gain (manual) at 50 and probe height at 7.5 mm.

3.3.9 Purification of HIS_6-CfaR from *S. scabies*

The promoter fragments used in this study carry sequences encoding HIS tags (HIS_8 for *ermE*p* and HIS_6 for SP44 and *kasO*p*), therefore allowing for overproduction of N-terminal HIS-tagged proteins in *Streptomyces.* The *S. scabies* strain Δ*txtA*/Δ*cfaR*/Δ*orf1,* containing pOSCN or pOSCON, was used to purify HIS_6-CfaR that was overproduced together or without the ORF1 protein. Cultures used for purification of HIS_6-CfaR were prepared as described in Section 3.3.1. The mycelia were harvested from 1 L cultures by centrifugation and were resuspended in 30 mL of Binding buffer [10 mM Tris-HCl, 300 mM NaCl and 20 mM imidazole (pH 7.8)] supplemented with 1× cOmplete™ Protease Inhibitor Cocktail (Roche). Cell lysis was achieved using a Q125 ultrasonic processor sonicator at 80% power and 5 sec on / 5 sec off cycle for 3 min (Qsonica L.L.C, USA). The insoluble cell debris was removed by centrifugation, and the supernatant containing soluble proteins was stored on ice. To bind the HIS-tagged protein, 0.5 mL of Ni-NTA agarose resin (QIAgen Inc, Canada) was centrifuged, washed with 1 mL of Binding buffer, and mixed with the lysate supernatant. The mixture was gently agitated for 1 h on ice. The resin slurry was transferred to an Econo-Column® Chromatography Column [1.5 × 10 cm (diameter × length)] (Bio-Rad Laboratories, Inc. USA) and the supernatant was removed by gravity flow. The resin was washed twice with 25 mL of Binding buffer and three times with 25 mL of Washing buffer [10 mM Tris-HCl, 300 mM NaCl and 50 mM imidazole (pH 7.8)]. The bound HIS_6-CfaR protein was eluted with 0.5 mL of Elution buffer [10 mM

Tris-HCl, 300 mM NaCl and 300 mM imidazole (pH 7.8)] per fraction for a total of 15 fractions. The eluted protein fractions (20 μL each) were analyzed by SDS-PAGE on a 12% (w/v) gel and were visualized by staining the gel with Coomassie brilliant blue stain [50% (v/v) methanol; 10% (v/v) glacial acetic acid; 0.1% (w/v) Coomassie Brilliant Blue] or were analyzed by Western blot analysis as described below.

3.3.10 Western blot analysis

Western blot analysis was performed by transferring proteins from SDS-PAGE to Amersham™ Hybond™-ECL membranes (GE Healthcare Canada) using a Mini-PROTEAN® Tetra Cell and Mini Trans-Blot® Module (Bio-Rad Laboratories USA) and following the manufacturer's instructions. The primary antibody was a 6×His Tag Monoclonal Antibody (Thermo Fisher Scientific Canada), used at a 1:1000 dilution. The secondary antibody was an anti-Mouse IgG2b Secondary Antibody HRP (Thermo Fisher Scientific Canada) used at a 1:2000 dilution. Signals were visualized using Amersham™ ECL™ Western Blotting Detection Reagents (GE Healthcare Canada) and an ImageQuant™ LAS 4000 Digital Imaging System (GE Healthcare Canada).

3.3.11 Mass spectrometry (MS) analysis of the purified HIS_6-CfaR

HIS_6-CfaR protein samples were separated by SDS-PAGE, and the bands were excised from the Coomassie-stained gel with a clean razor and were stored in 1% v/v acetic acid. Mass spectrometry analysis of the protein samples was performed at the SPARC Biocentre at the Toronto Hospital for Sick Children (Toronto, ON, Canada), and the results were viewed using the Scaffold 4 software (Proteome Software, Inc).

3.3.12 Overproduction of HIS_6-CfaR and ORF1-HIS_6 in *E. coli*

The pACYCDuet-1/*cfaR* plasmid, which allows for overexpression of N-terminal 6 × histidine tagged CfaR protein (HIS_6-CfaR) in *E. coli*, was constructed by cloning the *cfaR* gene, which was PCR-amplified using the primers LC72 and LC73 and plasmid pRLDB891 as template, into the EcoRI and HindIII sites of the pACYCDuet-1 vector. The *orf1* gene was PCR-amplified using the LC65 and LC77 primers and pRLDB891 as template and was cloned into the NdeI and HindIII sites of pET30b to produce pET-30(b)/*orf1*, which allows for overexpression of C-terminal 6 × histidine tagged ORF1 (ORF1-HIS_6) (Table 3.1). The plasmids were introduced into the *E. coli* expression hosts BL21(DE3), BL21(DE3)/pLysS, or Rosetta™(DE3) by chemical transformation. Proteins was expressed and purified as described before (Cheng et al., 2015) except that the overexpression cultures were incubated at 16°C for 24 h following induction with IPTG. Proteins were purified as described in Section 3.3.9 with the following modification: sonication was performed at 50% power and 5 sec on / 5 sec off cycle for 5 times.

3.3.13 Detection of CFA-L-Ile production by *S. scabies* strains

Small-scale extraction and HPLC detection of CFA-L-Ile from *S. scabies* culture supernatants was as described before (Fyans et al., 2015). The area of the CFA-L-Ile peak detected in each sample was obtained using the ChemStation software (B.04.03; Agilent Technologies Canada Inc.). DCWs from metabolite production cultures were determined by harvesting the mycelia by centrifugation, drying the mycelial pellets for 24 h at 50°C,

and then weighing the pellets using an analytical balance. The normalized CFA-L-Ile production levels were reported as peak area (counts × msec) per mg DCW.

3.3.14 Detection of thaxtomin A production by *S. scabies* strains

Small-scale extraction and HPLC detection of thaxtomin A from *S. scabies* culture supernatants was as described before (Fyans et al., 2016). The ChemStation software was used to determine the peak area of the thaxtomin A detected in each sample, and DCWs were measured for the metabolite production cultures as described above. The normalized thaxtomin A production levels were reported as the peak area (counts × msec) per mg DCW.

3.3.15 Potato tuber bioassays

The pathogenicity of *S. scabies* strains was assessed using a potato tuber bioassay as described previously (Loria et al., 1995) with some modifications. Strains were plated onto PMA plates and were incubated at 28°C for 7 days until the organisms were well sporulated. Potatoes were purchased from the grocery store and were peeled and surface sterilized for 10 min in 15% v/v bleach (Chlorox) before being washed in sterile water. The tubers were aseptically cut horizontally into 1 cm thick slices, which were then placed onto sterile Whatman filter paper (moistened with sterile water) in sterile glass Petri dishes. Agar plugs (~ 7 mm diameter) from the PMA plates were obtained using the blunt end of 1 ml sterile pipette tips and were placed spore-side down onto the potato tuber slices. Uninoculated PMA medium served as a negative control. All tested *S. scabies* strains were placed onto the same tuber slice to eliminate variation among potatoes. To test the biological activity of pure CFA-L-Ile and thaxtomin A, sterile paper disks (0.6 cm in

diameter) were placed onto potato tuber slices, and 100 nmoles of CFA-L-Ile and 1 nmole of thaxtomin A were added onto each paper disk. The Petri dishes with the potato tuber slices were sealed with parafilm and were incubated at room temperature in the dark. Photos were taken at days 2, 4, 7 and 10 post-inoculation. The bioassays were performed three times.

3.3.16 Statistical analyses

All HPLC analyses were performed using triplicate samples originating from three independent cultures. Statistical analysis of the metabolite production levels was conducted using a one-way ANOVA, and *a posteriori* multiple comparisons of least squared means were performed using the Tukey test. In the case of the data presented in Fig. 3.6, a Student's t-test was performed instead of a one-way ANOVA. P values ≤ 0.05 were considered statistically significant in all analyses.

3.4 Results

3.4.1 ORF1 is a member of the ThiF protein family

The *orf1* gene is located downstream of *cfaR* and encodes a predicted 636 amino acid protein. A search of the Pfam database generated two matches to known protein domains within the amino acid sequence. The region between amino acids 26 and 286 aligned with the ThiF family domain (*THIF*, PF00899) (Vander Horn et al., 1993), which is found in enzymes such as the eukaryotic ubiquitin activating enzyme E1, the *E. coli* thiamine biosynthetic enzyme ThiF and the *E. coli* molybdenum cofactor biosynthetic enzyme MoeB (Leimkühler et al., 2001). A putative nitroreductase domain

(*TM1568_NiRdase,* PF14512) (Bryant & DeLuca, 1991) was detected in the region between amino acids 319 and 372. BlastP analysis using the ORF1 amino acid sequence identified multiple homologues within the database and defined the ThiF domain between amino acids 55 and 177 within the ORF1 sequence. We chose 10 non-redundant ORF1 homologues which, when compared with ORF1, have ≥ 96% coverage and ≥ 59% amino acid identity, for phylogenetic analysis (Table S3.2). Four of the ORF1 homologues are encoded by genes that were very recently shown to be situated next to genes encoding homologues of CfaR in the respective genome sequences (Bown et al., 2017). *cfaR* homologues were also found in three additional genome sequences that harbour an *orf1* homologue; however, there is currently not enough information to confirm if the genes are located next to each other in these genomes (Table S3.2). Phylogenetic analysis revealed that the ORF1 homologues form two distinct clades, of which clade I includes ORF1 itself as well as the homologues that are encoded next to *cfaR* homologues (Fig. 3.2). Notably, the ORF1 homologues identified in other plant pathogenic *Streptomyces* spp. (*S. europaeiscabiei*, *S. turgidiscabies* and *S. reticuliscabiei*) are located in clade II and thus do not cluster with ORF1 (Fig. 3.2).

Members of the ThiF protein family are known to exhibit AMPylation (also known as adenylylation or adenylation) activity (Hershko & Ciechanover, 1998; Lehmann & Ealick, 2006; Leimkühler et al., 2001; Taylor et al., 1998; Vander Horn et al., 1993; Xi et al., 2001), which is the covalent attachment of AMP to a protein or other molecule through ATP hydrolysis (Itzen et al., 2011). A study by Regni and colleagues characterized the ATP binding site of the ThiF family protein MccB from *E. coli*, and they found that ATP

binds within a cleft centered around a ATP binding motif (GCGGIG) (Regni et al., 2009). GCGGIG in MccB matches a well-known nucleotide binding motif, GXGXXG (Spitaler et al., 2000), and this binding motif along with other amino acids shown to interact with ATP is conserved in other ThiF family proteins, including ORF1 (Fig. 3.3A). We built a 3D model of the ORF1 protein based on the entire amino acid sequence, and the conserved amino acids associated with the ATP binding site form a "pocket" within the predicted ORF1 structure (Fig. 3.3B). Mg^{2+} is required for AMPylation catalysed by MccB (Regni et al., 2009; Roush et al., 2008), and an amino acid residue (D214) involved in Mg^{2+} binding is also conserved in other ThiF family proteins, including ORF1 (Fig. 3.3A).

Together, our analyses suggest that, like other ThiF family members, ORF1 may bind ATP and $Mg^{2+}$and exhibit AMPylation activity towards a substrate molecule.

3.4.2 ORF1 is a positive regulator of coronafacoyl phytotoxin production in *S. scabies*

Work described previously (Cheng et al., 2015) demonstrated that the production of CFA-L-Ile is greatly increased in *S. scabies* when the *cfaR* regulator gene is overexpressed together with *orf1* as compared to when *cfaR* is overexpressed on its own. To confirm that the observed increase in metabolite production is due to an increase in expression of the corresponding biosynthetic genes, semi-quantitative RT-PCR was performed in order to examine the expression of the *cfa1* gene, which is the first gene in the coronafacoyl phytotoxin biosynthetic operon (Bignell et al., 2010). As shown in Fig. 3.4, the transcription level of *cfa1* was increased when *cfaR* was overexpressed alone, and the expression level was even greater when *cfaR* and *orf1* were both overexpressed. In

contrast, the expression level of *cfa1* as well as that of *cfaR* and *orf1* was very low in the wild-type *S. scabies* and vector control strains (Fig. 3.4). We also noted that the expression level of *cfaR* appeared to be increased when both *cfaR* and *orf1* were overexpressed as compared to when *cfaR* alone was overexpressed (Fig. 3.4). Thus, the results confirm that overexpression of *orf1* has a positive effect on the transcription of the CFA-L-Ile biosynthetic operon. Moreover, the results verify that the expression of the CFA-L-Ile biosynthetic and regulatory genes is very low in wild-type *S. scabies*, accounting for the low level production of the metabolite by this strain under laboratory conditions (Fyans et al., 2015).

To further study the role of the *orf1* gene in CFA-L-Ile biosynthesis, a deletion mutant was constructed in the *S. scabies* Δ*txtA* strain, which is deficient in thaxtomin A production (Table 3.1). This strain has been used as it was previously shown to produce elevated levels of CFA-L-Ile as compared to *S. scabies* 87-22 (Fyans et al., 2015), and RT-PCR analysis confirmed that expression of the biosynthetic genes is higher in the Δ*txtA* strain than in strain 87-22 (Fig. S3.3). The Δ*txtA*/Δ*orf1* mutant was constructed and five mutant isolates were tested for CFA-L-Ile production. As shown in Figure 3.5A, the mutant isolates produced only 17.8-35.9% of the metabolite production level observed in the parental Δ*txtA* strain, though production could still occur (Fig. 3.5A). Furthermore, while deletion of *orf1* decreased CFA-L-Ile production, deletion of *cfaR* or both *cfaR* and *orf1* abolished metabolite production completely (Fig. 3.5B). Heterologous overexpression of the *orf1* gene in the Δ*txtA*/Δ*orf1* mutant isolate partially restored CFA-L-Ile production to 59.6% of the level observed in the Δ*txtA* parental strain, while overexpression of *cfaR* in

the same strain resulted in higher CFA-L-Ile production than in the parental Δ*txtA* strain (Fig. 3.6), suggesting that high expression of *cfaR* is sufficient to compensate for the loss of *orf1* in *S. scabies.* Overexpression of both *cfaR*+*orf1* in the Δ*txtA*/Δ*orf1* isolate also allowed for higher CFA-L-Ile production as compared to the parental strain (Fig. 3.6).

Together, our results suggest that CfaR is the main CSR controlling CFA-L-Ile biosynthesis, while ORF1 augments metabolite production but is not essential for production.

3.4.3 Development of plasmids for protein overexpression and purification in *Streptomyces*

We next wanted to investigate how ORF1 functions as a positive co-regulator of coronafacoyl phytotoxin biosynthetic gene expression and metabolite production in *S. scabies*. Given that CfaR functions as a DNA binding transcriptional activator of the biosynthetic genes and that ORF1 is predicted to exhibit AMPylation activity, one possibility is that ORF1 regulates CfaR activity by post-translationally modifying the CfaR protein. To explore this possibility, we first tried to overexpress and purify N-terminal HIS-tagged CfaR protein from *S. scabies* by expressing the gene from the strong, constitutive *ermE*p* promoter, which was previously used for the CfaR ± ORF1 overproduction studies (Cheng et al., 2015). The HIS_8-CfaR overproduction plasmid pOECN was constructed by replacing the *egfp* gene of pIJ8660-*ermE*p* with the *cfaR* gene and then incorporating a kanamycin resistance gene into the plasmid backbone (Table 3.1; see Section 3.3.7). The plasmid was introduced into the *S. scabies* Δ*txtA*/Δ*cfaR*/Δ*orf1* strain, and numerous

attempts were made to detect the HIS_8-CfaR protein in soluble protein preparations by Western analysis; however, all attempts were unsuccessful (data not shown).

It was next decided to develop plasmids that use stronger *Streptomyces* promoters for overproducing the CfaR protein in *S. scabies*. The *kasO*p* (Wang et al., 2013) and SP44 (Bai et al., 2015) promoters were chosen as both are stronger than the *ermE*p* promoter, with SP44 being the strongest of the three (Bai et al., 2015). Two plasmids, pOKEN and pOSEN, were constructed in which the *egfp* gene was expressed from the *kasO*p* and SP44 promoter, respectively (Fig. S3.2; Table 3.1). The *egfp* gene served as a useful reporter for assessing the activity of each promoter. In addition, all the cloned promoter fragments were designed to include sequence that would allow for the overproduction of a protein with an N-terminal 6 × HIS tag following replacement of the *egfp* gene with a target gene (Fig. S3.1 and S3.2A).

To test the *kasO*p* and SP44 promoters, the pOKEN and pOSEN plasmids along with pOEEN, in which *egfp* gene is under the control of *ermE*p* promoter, were each introduced into the *S. scabies* Δ*txtA*/Δ*cfaR*/Δ*orf1* mutant as well as *S. coelicolor* M1154. *S. coelicolor* M1154 is an engineered strain commonly used for the heterologous expression of *Streptomyces* secondary metabolite gene clusters (Gomez-Escribano & Bibb, 2011). The relative activity of each promoter was then measured at different time points using an eGFP fluorescence assay as described in Section 3.3.8. As shown in Fig. S3.4A, at all three time points, *kasO*p* and SP44 produced greater fluorescence levels compared to *ermE*p*, with SP44 producing the highest levels. The highest level of fluorescence was detected at 24 h

for *kasO*p* and SP44 in *S. scabies*, while lower levels were detected 48 and 72 h. (Fig. S3.4A). Similar results were obtained when *S. coelicolor* M1154 was used (Fig. S3.4B).

Then we tested the CFA-L-Ile production levels in *S. scabies* strains overproducing HIS-tagged CfaR protein. Three plasmids, pOSCN, pOKCN and pOECN, which overproduce HIS_6-CfaR using the SP44, *kasO*p* and *ermE*p* promoters, respectively, (Table 3.1) were each introduced into *S. scabies* Δ*txtA*/Δ*cfaR*/Δ*orf1*, and the production of CFA-L-Ile was examined after culturing the strains for 7 days in SFMB medium. As shown in Fig. 3.7A, the CFA-L-Ile production level was significantly higher when HIS_6-CfaR was overproduced using SP44 versus *kasO*p*, and both promoters resulted in significantly higher production levels as compared to the *ermE*p* promoter. The production of related peaks that are thought to represent minor coronafacoyl phytotoxins in *S. scabies* (Fyans et al., 2015) was also found to be higher in the strains containing the SP44 and *kasO*p* expression plasmids (Fig. 3.7B). Thus, the results confirm that the *kasO*p* and SP44 promoters can be used for gene overexpression in *S. scabies*, with SP44 resulting in the highest level of gene expression. Moreover, the results demonstrate that the HIS_6-CfaR protein is functional in *S. scabies* since it was able to induce CFA-L-Ile production in the Δ*txtA*/Δ*cfaR*/Δ*orf1* mutant background.

3.4.4 Purification and analysis of CfaR expressed in the presence and absence of ORF1

In order to investigate whether CfaR is post-translationally modified by ORF1, the HIS_6-CfaR protein was overproduced by itself or together with ORF1 using the SP44

promoter. Two plasmids, pOSCN (overproduction of HIS_6-CfaR alone) and pOSCON (overproduction of HIS_6-CfaR+ORF1) (Table 3.1) were each introduced into *S. scabies* Δ*txtA*/Δ*cfaR*/Δ*orf1*, and HIS_6-CfaR protein was purified from each strain after culturing in TSB for 48 hr. SDS-PAGE analysis of the purified HIS_6-CfaR protein samples revealed a single band with the expected molecular weight for HIS_6-CfaR (~30kDa), and Western analysis using anti-HIS antibodies confirmed that the protein band is HIS_6-CfaR (Fig. 3.8).

MS analysis was performed to identify possible post-translational modifications of CfaR, including acetylation, ammonia loss, deamidation, glutamic acid or glutamine to pyroglutamate conversion, oxidation, AMPylation and carbamidomethylation. Although ORF1 is predicted to exhibit AMPylation activity based on the presence of the ThiF-family domain, the MS analysis did not detect any AMP-modified HIS_6-CfaR when produced in the presence or absence of ORF1 (Fig. 3.9). Only two types of modifications, oxidation and deamination, were observed in the HIS_6-CfaR protein, and these modifications were observed both in the presence and absence of ORF1. Most likely, the deamination observed is an artefact caused by the fragmentation in the mass spectrometer (J. Krieger, personal communication).

3.4.5 Overproduction of CfaR and ORF1 in *E. coli*

As we were unable to detect any differences in post-translational modification of CfaR in the presence and absence of ORF1, we next attempted to purify both CfaR and ORF1 from *E. coli* in order to further analyze the relationship between the two proteins. The *cfaR* and *orf1* genes were cloned into the overexpression vectors pACYCDuet-1 and

pET-30(b), respectively (Table 3.1), which allows for production of HIS_6-CfaR and ORF1-HIS_6 in *E. coli*. pACYCDuet-1/*cfaR* was transformed into *E. coli* strain BL21(DE3), with or without pET-30(b)/*orf1*.

Western blot analysis using Anti-HIS antibodies revealed the presence of a ~30 kDa band corresponding to the expected size of HIS_6-CfaR in all of the soluble and insoluble fractions. In contrast, no specific band corresponding to ORF1 was detected (Fig. 3.10, samples 1 and 2). We then tried using the *E. coli* BL21(DE3)/pLysS expression strain for production of ORF1-HIS_6 in order to eliminate any possible toxic effects of ORF1 overproduction on the *E. coli* cells (Table 3.1). The pET-30(b)/*orf1* plasmid was transformed into this strain, and soluble and insoluble protein fractions from IPTG-induced cultures were collected and analyzed using anti-HIS antibodies. When compared to protein extract from the BL21(DE3)/pLysS strain lacking the expression plasmid, there was no obvious band corresponding to ORF1-HIS_6 in the extract from the pET-30(b)/*orf1*-containing strain, and thus the production of ORF1-HIS_6 could not be detected (Fig. 3.10, sample 3).

Another possible explanation for our inability to successful overexpress ORF1 in *E. coli* is that the ORF1 coding sequence contains codons that are rare in *E. coli*. The codons in *orf1* that are rarely used in *E. coli* are listed in Table S3.3. We therefore decided to try using the *E. coli* Rosetta™(DE3) expression strain (Table 3.1) as it supplies tRNAs for the rare codons AGG, AGA, AUA, CUA, CCC and GGA. ORF1-HIS_6 production was induced in the Rosetta™(DE3) strain carrying pET-30(b)/*orf1*; however, Western blot analysis

showed no specific signal for ORF1-HIS_6 in the total protein extracted from the cell culture (Fig. 3.10, sample 4). This may be due to the fact that the most frequent rare codon, CGG, is not supplied by the Rosetta™(DE3) strain (Table S3.3).

There are six cysteine residues in ORF1, of which one is located in the ThiF domain, one which is located within the C-terminal region, and four which are located in the N-terminal region ahead of the ThiF domain (data not shown). Cysteine residues are involved in disulfide bond formation, which contributes to the stability of proteins (Betz, 1993; Liu et al., 2016; Zavodszky et al., 2001). The *E. coli* strains we used did not support disulfide bond formation and, therefore, this may have accounted for our inability to detect the ORF1 protein.

3.4.6 Increased production of CFA-L-Ile enhances the virulence of *S. scabies* during infection of potato tuber tissue

Thaxtomin A is the main pathogenicity determinant of *S. scabies* and is essential for CS disease development. It can cause necrosis on excised potato tuber tissue and can induce scab-like lesions on minitubers cultured in the lab (Bignell et al., 2014). CFA-L-Ile and other coronafacoyl phytotoxins have been shown to cause hypertrophy of potato tuber tissue (Fyans et al., 2015); however, there is currently no direct evidence that CFA-L-Ile production by *S. scabies* contributes to CS disease development or disease symptom severity. In a tobacco seedling bioassay, a *S. scabies* Δ*cfa6* mutant that was unable produce CFA-L-Ile caused reduced stunting and necrosis of the tobacco seedling roots as compared to the wild-type strain. In contrast, no differences in the virulence phenotype of the wild-

type and mutant strains was observed in a potato tuber bioassay (Bignell et al., 2010). As wild-type *S. scabies* 87-22 produces only trace levels of CFA-L-Ile in liquid culture, it is possible that the lack of observable differences in virulence between strain 87-22 and the Δ*cfa6* mutant may be due to the low level production of CFA-L-Ile by strain 87-22. The *S. scabies* overexpression strains with increased CFA-L-Ile production generated in this study provided an opportunity to test whether enhanced production of CFA-L-Ile leads to enhanced virulence. To test this hypothesis, we performed a potato tuber slice bioassay using four different *S. scabies* strains: (1) wild-type 87-22, (2) wild-type vector control (87-22/pRLDB50-1a), (3) wild-type with *cfaR* overexpression plasmid (87-22/pRLDB51-1) and (4) wild-type with *cfaR*+*orf1* overexpression plasmid (87-22/pRLDB891). These strains were previously shown to produce varying amounts of CFA-L-Ile in liquid culture (wild type ≈ vector control < *cfaR* overexpression < *cfaR*+*orf1* overexpression; (Cheng et al., 2015). As shown in Fig. 3.11, the *cfaR* and *cfaR*+*orf1* overexpression strains caused greater necrosis and pitting of the potato tuber tissue as compared to the wild-type and vector control strains at all time points examined, with the *cfaR*+*orf1* overexpression strain causing the most severe disease symptoms. All of the strains were shown to produce equivalent amounts of the essential virulence factor thaxtomin A in liquid culture, suggesting that the observed differences in disease severity are not simply due to differences in thaxtomin A production (Fig. S3.5).

To test the function of CFA-L-Ile without thaxtomin A present, bioassays were performed using the following thaxtomin A deficient strains: (1) Δ*txtA*, (2) Δ*txtA* vector control (Δ*txtA*/pRLDB50-1a), (3) Δ*txtA* with *cfaR* overexpression plasmid

(Δ*txtA*/pRLDB51-1) and (4) Δ*txtA* with *cfaR*+*orf1* (Δ*txtA*/pRLDB891). These strains produce varying amounts of CFA-L-Ile in liquid culture (Δ*txtA* ≈ vector control < *cfaR* overexpression < *cfaR*+*orf1* overexpression; data not shown). As shown in Fig. 3.12, the *cfaR* and *cfaR*+*orf1* overexpression strains were the only ones that caused significant hypertrophy of the potato tuber tissue, an effect attributed to CFA-L-Ile phytotoxin production (Fyans et al., 2015). This confirmed that the overexpression of *cfaR* and *cfaR*+*orf1* enhances the production of CFA-L-Ile during colonization of potato tuber tissue by *S. scabies*. It is noteworthy that the amount of necrosis and pitting of the potato tuber tissue was less in the absence of thaxtomin A (Fig. 3.12), suggesting that both CFA-L-Ile and thaxtomin A are required for severe disease symptom development by *S. scabies.* However, when potato tuber tissue was treated with pure thaxtomin A and/or CFA-L-Ile, the observed disease symptoms were not enhanced by the presence of both toxins versus thaxtomin A alone, indicating that the effect of CFA-L-Ile is not mediated exclusively through synergistic interactions with thaxtomin A (Fig. 3.13).

3.5 Discussion

Work presented in this study confirms that ORF1 is a positive co-regulator of CFA-L-Ile production in *S. scabies*. Overexpression of the *orf1* gene together with the *cfaR* CSR led to enhanced expression of the CFA-L-Ile biosynthetic genes as compared to overexpression of *cfaR* alone (Fig. 3.4), while deletion of *orf1* in the Δ*txtA* mutant background caused decreased CFA-L-Ile production, though some production could still occur (Fig. 3.5A). Overexpression of *orf1* was able to partially compensate for the loss of

ORF1 in the Δ*txtA*/Δ*orf1* mutant (Fig. 3.6). It is noteworthy that a Δ*txtA*/Δ*cfaR* mutant was completely abolished in CFA-L-Ile production (Fig. 3.5B) and overexpression of *cfaR* alone was able to fully compensate for the loss of ORF1 in the Δ*txtA*/Δ*orf1* mutant (Fig. 3.6). Taken together, our results suggest that CfaR is the primary CSR controlling CFA-L-Ile biosynthetic gene expression and metabolite production, while ORF1 functions as a positive "helper" protein by augmenting CFA-L-Ile production in a CfaR-dependent manner. As *cfaR* and *orf1* gene pairs are conserved in other *Streptomyces* spp. (Bown et al., 2017), it is likely that this mechanism of gene regulation is not limited to *S. scabies.*

ORF1 shares similarity with members of the ThiF protein family, members of which are known to function as AMPylators. For example, the founding member of the family is the *E. coli* ThiF protein, which is involved in the biosynthesis of thiamine by catalyzing the AMPylation of the carboxy terminus of the intermediate sulfur carrier ThiS (Lehmann & Ealick, 2006; Vander Horn et al., 1993; Xi et al., 2001). MoeB is another ThiF family member that functions in molybdopterin biosynthesis by catalyzing the AMPylation of the MoaD subunit of molybdopterin synthase (Leimkühler et al., 2001). In *E. coli*, MccB catalyses adenylation of MccA during the biosynthesis of microcin C7 (MccC7) (Regni et al., 2009). ORF1 also shows similarity to ubiquitin activating E1 family proteins, which catalyze the formation of ubiquitin adenylate during the activation of ubiquitin in eukaryotes (Hershko & Ciechanover, 1998). The conservation of amino acids shown to be involved in binding to ATP and Mg^{2+} in other AMPylators was also seen in ORF1; therefore, we predicted that ORF1 may function as an AMPylator (Fig. 3.3).

Protein AMPylation is recognized as an important post-translational modification that regulates the activity of proteins (Woolery et al., 2010). For example, Fic-1 catalyzes the AMPylation of DNA gyrase subunit B (GyrB), thus inhibiting bacterial DNA replication in *Pseudomonas fluorescens* (Lu et al., 2016). The Legionnaires' disease pathogen, *Legionella pneumophila*, produces bacterial protein DrrA, which AMPylates the human Rab1 protein. This AMPylation recruits Rab1 to the cytosolic face and affects the intracellular vesicular trafficking (Müller et al., 2010). We hypothesized that ORF1 may also exhibit AMPylation activity and that it might regulate the activity of CfaR by catalyzing the transfer of an AMP molecule to CfaR. However, MS analysis was unable to detect AMPylation or other modifications of HIS_6-CfaR produced in the presence of ORF1. It is worth mentioning that one peptide fragment (VRPQQMTRFPVGR), located within the C-terminal region of CfaR and 14 amino acids from the LuxR DNA binding domain, could not be detected in either of the HIS_6-CfaR protein samples analyzed (Fig. 3.9). Therefore, the modification status of this region of CfaR is unknown. It has been reported that AMPylation-type post-translational modifications often target tyrosine, threonine and serine residues in the target protein (Broncel et al., 2012; Casey & Orth, 2018; Grammel et al., 2011; Hedberg & Itzen, 2015). As there is a threonine (T) within the undetected peptide, we cannot rule out the possibility of AMPylation or other forms of modification in this region.

The potential modification of CfaR by ORF1 could be tested *in vitro* if we are able to express and purify both proteins. We tried to express and purify both CfaR and ORF1 in *E. coli* without success, possibly due to the high rate of the rare CGG codon in the ORF1

coding sequence. The Rosetta™ 2(DE3) strain (EMD Millipore Corporation), which supplies tRNAs for 7 rare codons (AGA, AGG, AUA, CUA, GGA, CCC, and CGG), should be tested. Another possible reason for the failure of ORF1 expression is that the *E. coli* strains we used do not support disulfide bond formation, which contributes to protein stability (Betz, 1993; Liu et al., 2016; Zavodszky et al., 2001). There are six cysteine residues in ORF1, suggesting potential disulfide bond formation in this protein. The *E. coli* Origami™ 2 strain (EMD Millipore Corporation) carries mutations in the thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes and would be an option to express ORF1 with enhanced disulfide bond formation.

Another important finding from this study was the revelation that strains of *S. scabies* producing higher levels of CFA-L-Ile cause greater necrosis and pitting of potato tuber tissue comparted to strains producing lower metabolite levels (Fig. 3.11), suggesting that there is a positive correlation between CFA-L-Ile production and disease symptom severity during infection of potato tubers. These results provide further supporting evidence that CFA-L-Ile is an important virulence factor that, together with thaxtomin A and other virulence factors, likely contributes to the severity of CS disease symptoms induced by *S. scabies* during infection. Our results are consistent with other studies that demonstrated that COR and other coronafacoyl phytotoxins enhance the severity of disease symptoms induced by plant pathogenic microbes during infection (Bignell et al., 2018). COR is known to promote pathogen invasion and persistence within plant tissues, and it contributes directly to disease symptom development (Bignell et al., 2018). The primary function of COR is believed to be related to its ability to function as a molecular mimic of

JA-L-Ile and to suppress SA-mediated plant defense responses through stimulation of JA signaling and consequent antagonistic crosstalk (Brooks et al., 2005; Geng et al., 2014). In addition, COR has been reported to suppress pathogen defense mechanism in a SA independent manner in *Arabidopsis* (Geng et al., 2012). Given the similarities in structure and bioactivity between CFA-L-Ile and COR, it is possible that CFA-L-Ile has similar functions as COR in mediating JA signaling, though this remains to be determined.

A final contribution of this study was the construction of plasmids that allow for high level gene expression in *S. scabies* and other *Streptomyces* spp. using the SP44 and *kasO*p* promoters. We showed using both an eGFP fluorescence assay and a metabolite production assay that the SF44 and *kasO*p* promoters are both significantly stronger than the *ermE*p* promoter in *S. scabies* (Fig. 3.7A and S3.4), and the SP44 promoter allowed for the successful purification of HIS_6-CfaR protein from *S. scabies* cultures (Fig. 3.8), a feat that was unsuccessful using the *ermE*p* promoter. We also observed increased production of what are believed to be minor coronafacoyl phytotoxins produced by *S. scabies* following overexpression of *cfaR* using both SP44 and *kasO*p* (Fig. 3.7B). Previous attempts to characterize these minor compounds were unsuccessful due to the inability to recover sufficient amounts for structural analysis, and thus the SP44 promoter in particular may enable the purification of these compounds for further studies.

3.6 References

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3.7 Figures and Tables

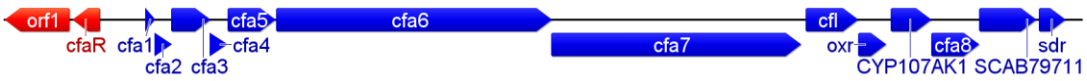


Figure 3.1. Coronafacoyl phytotoxin biosynthetic gene cluster in *S. scabies*. The CSR gene *cfaR* and the downstream *orf1* gene are shown in red. Genes encoding biosynthetic enzymes are in blue.

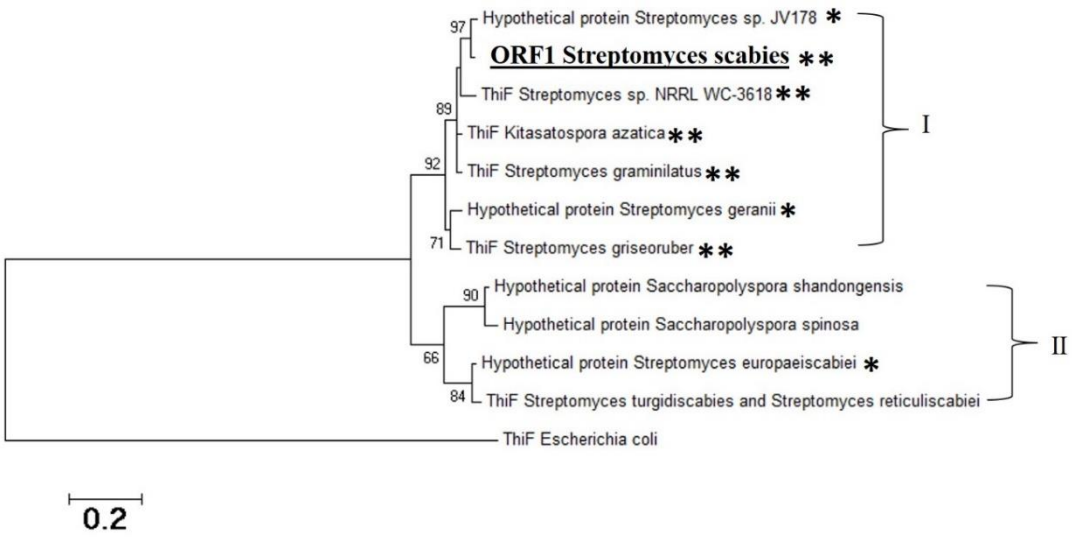
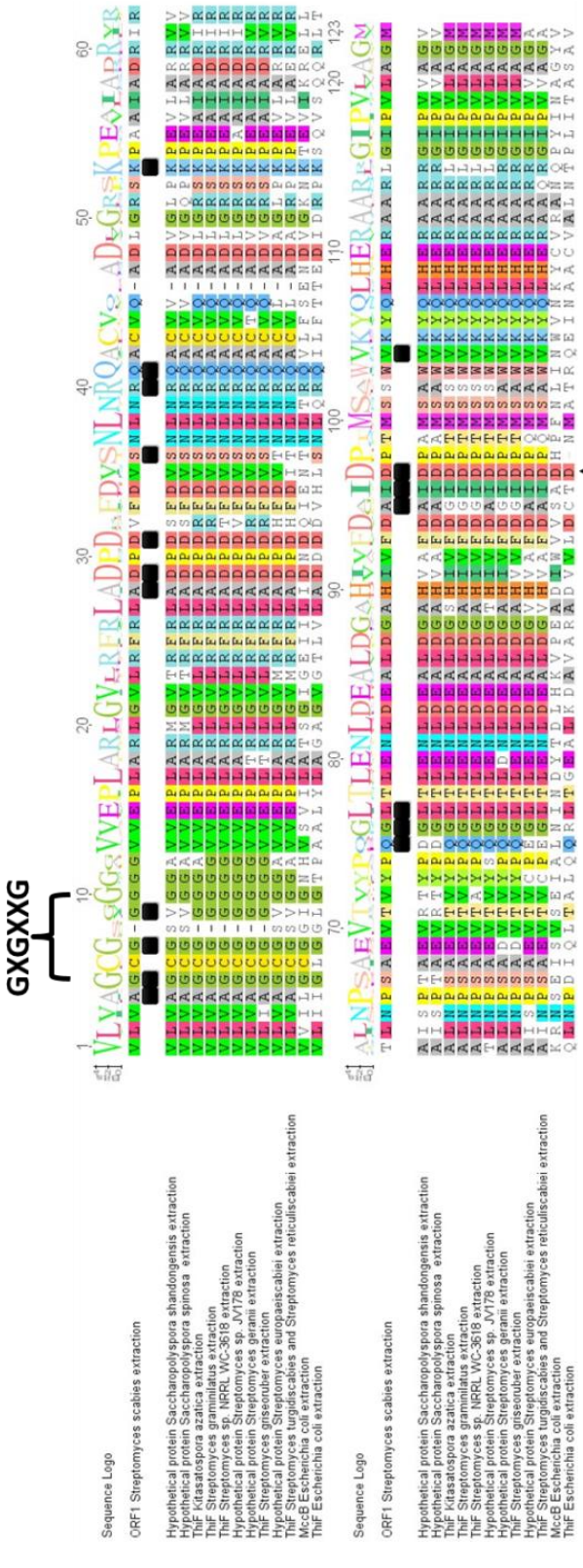


Figure 3.2. Phylogenetic analysis of ORF1 and other ThiF family proteins from actinomycetes. The phylogenetic tree was constructed using the maximum likelihood algorithm, and bootstrap values ≥50% for 1000 repetitions are shown. The scale bar indicates the number of amino acid substitutions per site. The ThiF protein from *E. coli* served as an outgroup. Family members that are encoded next to *cfaR* homologues within the respective genomes are indicated with **, and members that may or may not be encoded next to *cfaR* homologues are indicated with *. The two primary clades identified are indicated with I and II.

(A)


GXGXXG
Mg2+ binding
Sequence Logo
ORF1 Streptomyces scabies extraction
Hypothetical protein Saccharopolyspora shandongensis extraction
Hypothetical protein Saccharopolyspora spinosa extraction
ThiF Kitasatospora azatica extraction
ThiF Streptomyces graminilatus extraction
ThiF Streptomyces sp. NRRL WC-3618 extraction
Hypothetical protein Streptomyces sp. JV178 extraction
Hypothetical protein Streptomyces geranii extraction
ThiF Streptomyces griseoruber extraction
Hypothetical protein Streptomyces europaeiscabiei extraction
ThiF Streptomyces turgidiscabies and Streptomyces reticuliscabiei extraction
MccB Escherichia coli extraction
ThiF Escherichia coli extraction

(B)

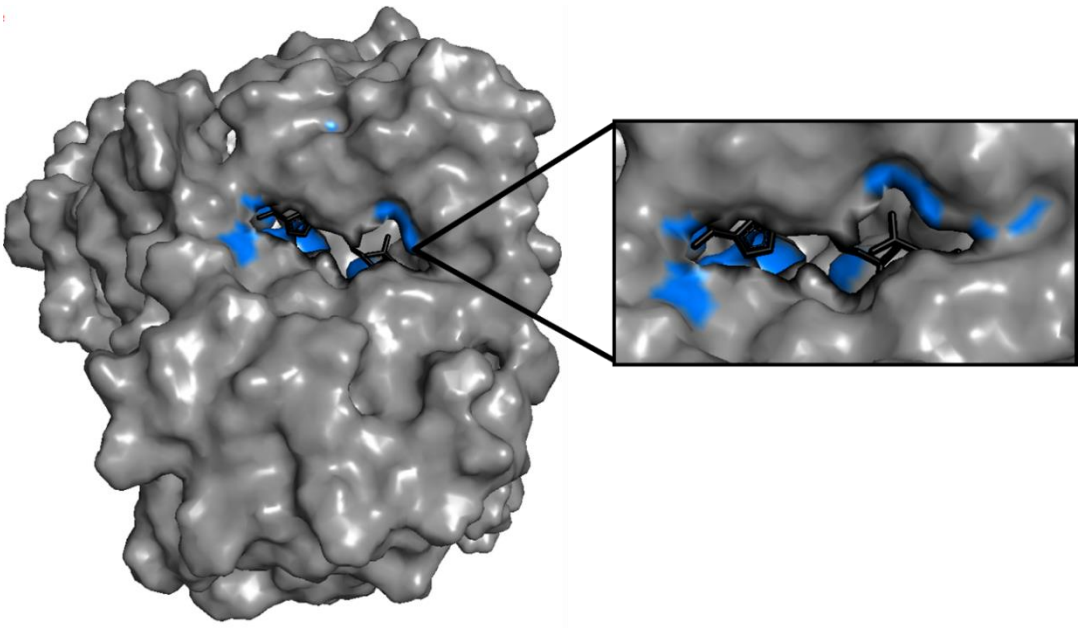


Figure 3.3. ORF1 contains a potential ATP binding site. (A) Partial amino acid alignment of ORF1 and other ThiF family proteins. Sequences shown are truncated according to the ThiF domain of ORF1. The conserved GXGXXG nucleotide binding motif and the amino acid for Mg^{2+} binding (D214 in MccB) are labeled. Amino acids involved in ATP binding in ThiF family proteins are indicated by the black boxes in ORF1, which are A43, G44, G46, G47, A66, D67, D69, S74, R78, Q79, K90, Q112, G113, L114, A132, I133, D134 and V141. Conserved amino acids from the ThiF proteins are shown in color shadings. (B) Predicted 3D model of the ORF1 protein based on the entire amino acid sequence and showing the potential ATP binding pocket. The amino acids predicted to be involved in ATP binding are indicated in blue. A molecule of ATP (indicated by the black stick model) is shown bound to the protein in the proposed binding pocket.

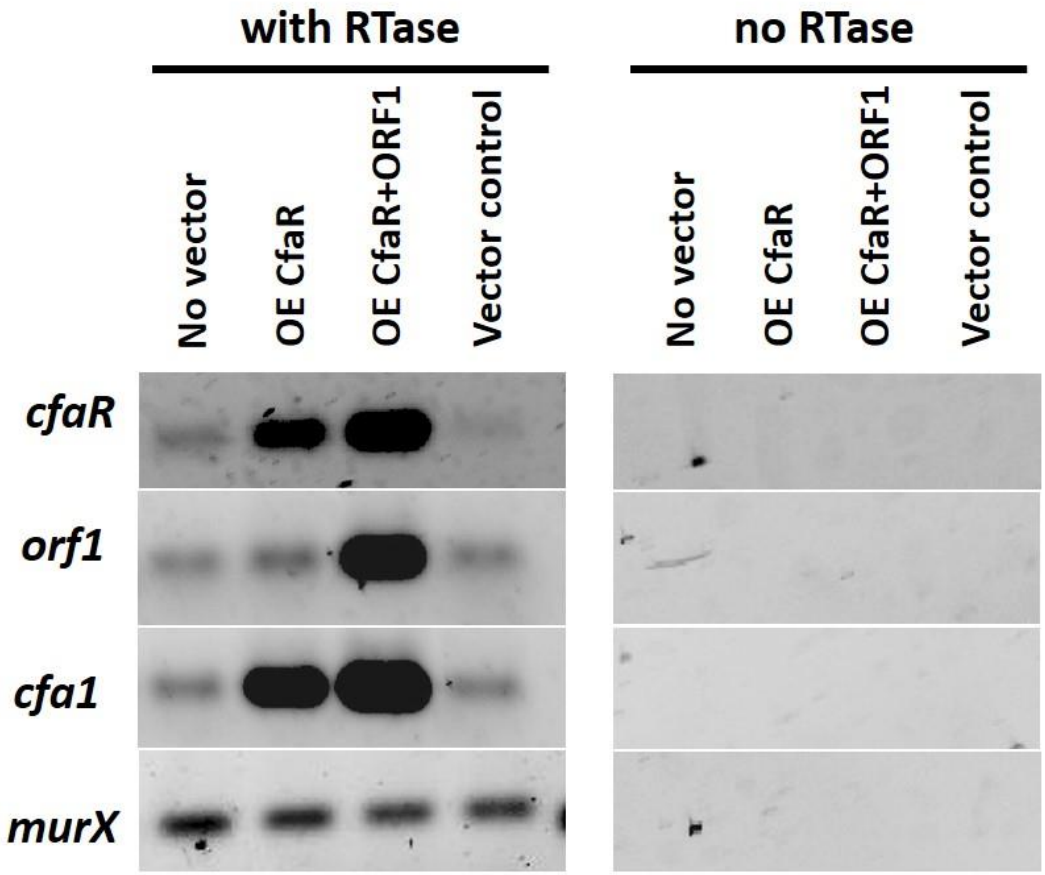
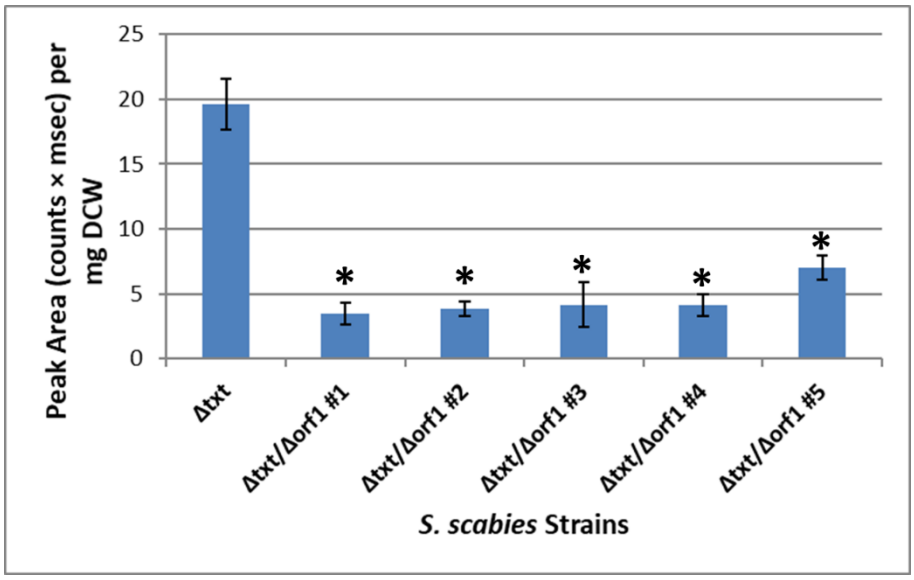


Figure 3.4. Semi-quantitative RT-PCR analysis showing enhanced gene transcription by overexpression of CfaR and CfaR+ORF1 in *S. scabies* 87-22. Gene expression was analyzed in *S. scabies* 87-22 containing the *cfaR* overexpression plasmid pRLDB51-1 (OE CfaR) and the *cfaR*+*orf1* overexpression plasmid pRLDB891 (OE CfaR+ORF1). *S. scabies* 87-22 with plasmid pRLDB50-1a (Vector control) and without (No vector) were used as controls. Reactions were conducted with cDNA template that was prepared using reverse transcriptase (with RTase), and no reverse transcription (no RTase) control templates were included. Primers targeting the *cfaR*, *orf1*, *cfa1* and *murX* genes were used in PCR reactions that were performed with 25 (*cfaR*), 30 (*orf1* and *cfa1*) and 28 (*murX*)

cycles. The amplified products were analyzed with 1.2% agarose gel electrophoresis. The *murX* gene served as a loading control.

(A)


Peak Area (counts × msec) per mg DCW
25
20
15
10
5
0
*
*
*
*
*
Δtxt
Δtxt/Δorf1 #1
Δtxt/Δorf1 #2
Δtxt/Δorf1 #3
Δtxt/Δorf1 #4
Δtxt/Δorf1 #5
S. scabies Strains

(B)

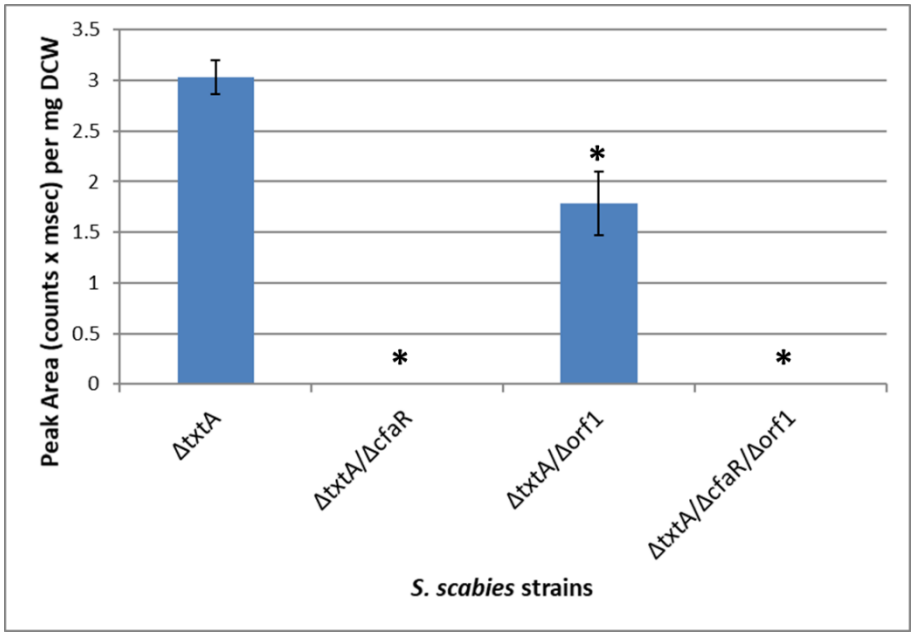

Peak Area (counts x msec) per mg DCW
3.5
3
2.5
2
1.5
1
0.5
0
*
*
*
ΔtxtA
ΔtxtA/ΔcfaR
ΔtxtA/Δorf1
ΔtxtA/ΔcfaR/Δorf1
S. scabies strains

Figure 3.5. Analysis of CFA-L-Ile production in different Δ*txtA*/Δ*orf1* mutant isolates (A) and in the Δ*txtA*/Δ*cfaR*, Δ*txtA*/Δ*orf1* and Δ*txtA*/Δ*cfaR*/Δ*orf1* mutant strains (B). *S. scabies* strains were cultured in SFMB medium for 7 days at 25°C, after which the culture supernatants were harvested and extracted with organic solvent. The resulting organic extracts were analyzed for CFA-L-Ile production by HPLC, and the CFA-L-Ile peak area in each extract was normalized using the corresponding dry cell weight (DCW) measurement. The bars in each figure represent the average normalized CFA-L-Ile peak area from triplicate cultures of each strain, and the error bars represent the standard deviation from the mean. Metabolite production levels that were determined to be significantly different from that of the control strain (Δ*txtA*) are indicated by * ($p \leq 0.05$).

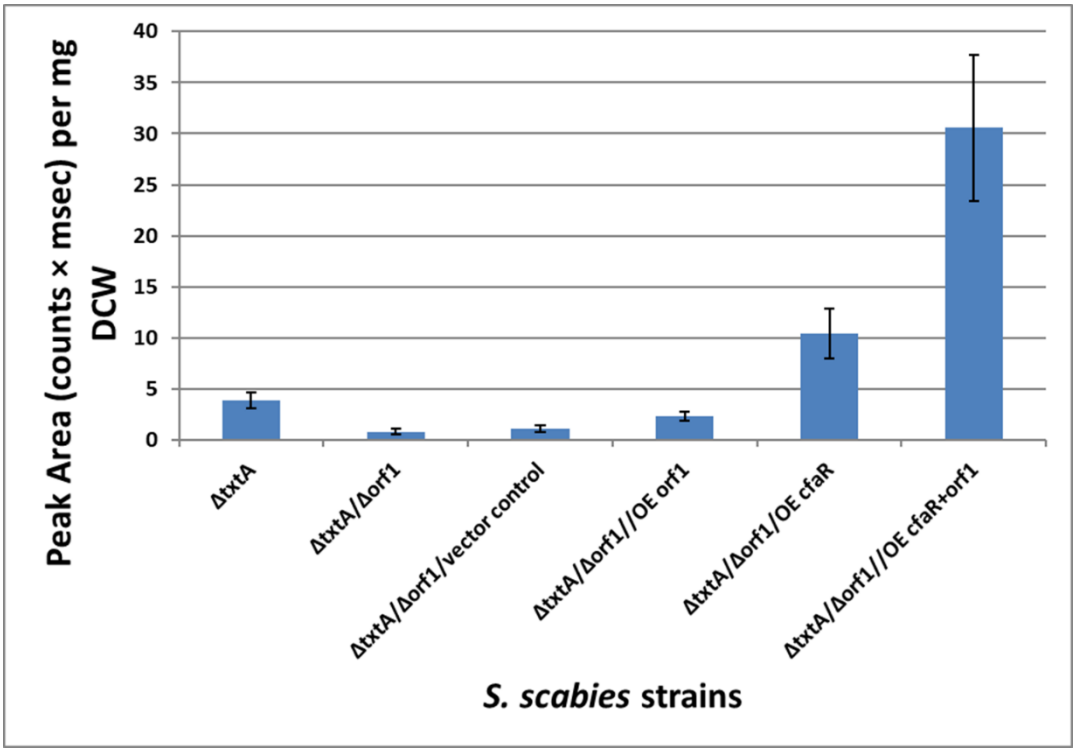
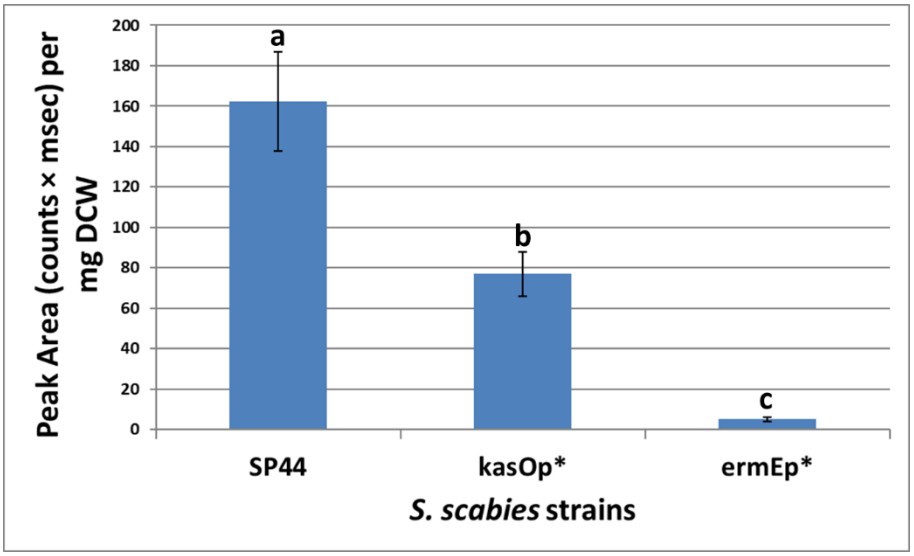


Figure 3.6. Complementation of the *orf1* deletion mutant. *S. scabies* strains were cultured in SFMB medium for 7 days at 25°C, after which the culture supernatants were harvested and extracted with organic solvent. The resulting organic extracts were then analysed for CFA-L-Ile production by HPLC. The resulting CFA-L-Ile peak areas were then normalized using dry cell weights (DCWs). The bars represent the average normalized CFA-L-Ile peak area from triplicate cultures of each strain, and the error bars represent the standard deviation from the mean. Deletion of *orf1* reduced CFA-L-Ile production (Δ*txtA* vs. Δ*txtA*/Δ*orf1*, $p \leq 0.05$). Complementation with *orf1* recovered the metabolite production (Δ*txtA*/Δ*orf1* vs. Δ*txtA*/Δ*orf1*/OE *orf1*, $p \leq 0.05$) though production was still

lower than that observed in the parental Δ*txtA* strain (Δ*txtA* vs. Δ*txtA*/Δ*orf1*/OE *orf1*, $p \leq 0.05$). Complementation with *cfaR* or *cfaR*+*orf1* fully recovered CFA-L-Ile production (Δ*txtA*/Δ*orf1* vs. Δ*txtA*/Δ*orf1*/OE *cfaR* or Δ*txtA*/Δ*orf1* vs. Δ*txtA*/Δ*orf1*/OE *cfaR*+*orf1*, $p \leq 0.05$), which was also higher than in the Δ*txtA* strain (Δ*txtA* vs. Δ*txtA*/Δ*orf1*/OE *cfaR* or Δ*txtA* vs. Δ*txtA*/Δ*orf1*/OE *cfaR*+*orf1*, $p \leq 0.05$). The control vector had no effect on CFA-L-Ile production (Δ*txtA*/Δ*orf1* vs. Δ*txtA*/Δ*orf1*/vector control, $p > 0.05$; Δ*txtA* vs. Δ*txtA*/Δ*orf1*/vector control, $p \leq 0.05$).

(A)


Peak Area (counts × msec) per mg DCW
200
180
160
140
120
100
80
60
40
20
0
a
b
c
SP44
kasOp*
ermEp*
S. scabies strains

(B)

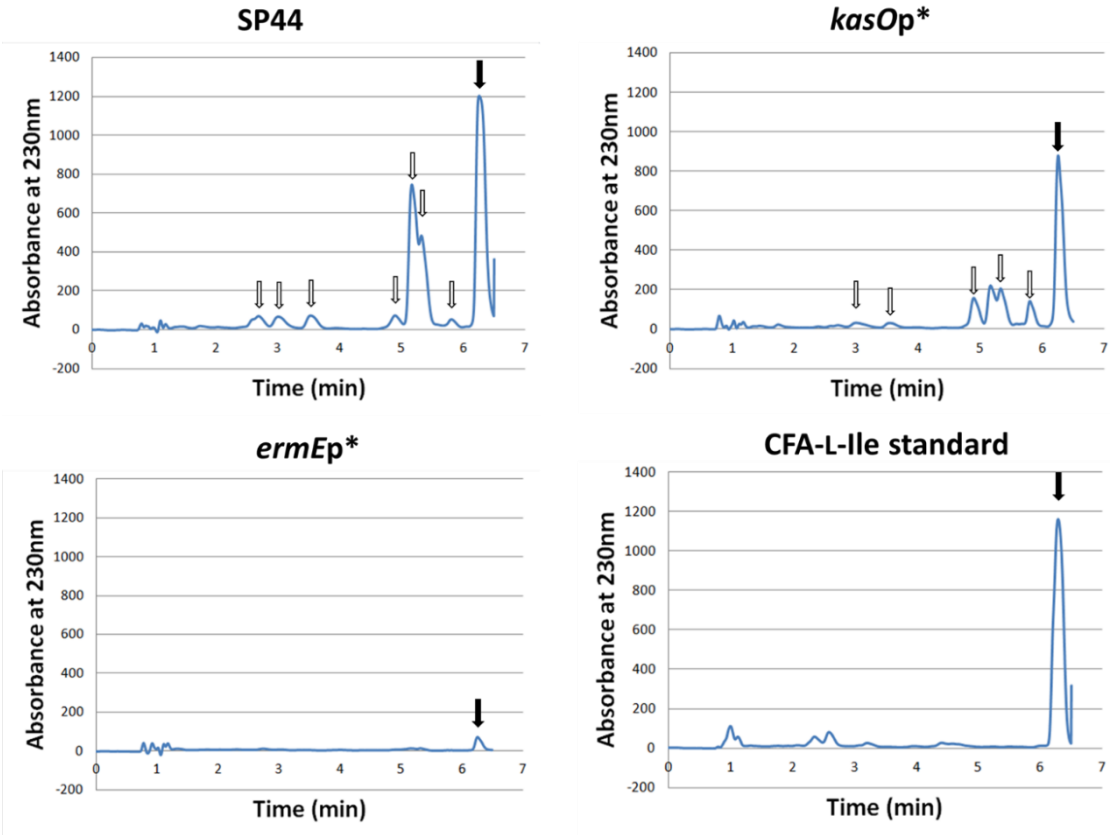

SP44
kasOp*
ermEp*
CFA-L-Ile standard
Absorbance at 230nm
Time (min)

Figure 3.7. Coronafacoyl phytotoxin production is enhanced by overexpressing *cfaR* from the SP44 and *kasO*p* promoters. The *cfaR* gene was cloned into three plasmids and was expressed in *S. scabies* strain, Δ*txtA*/Δ*cfaR*/Δ*orf1*, from the SP44 (in pOSCN plasmid), *kasO*p* (pOKCN) and *ermE*p* (pOECN) promoters. After culture and extraction, the resulting organic extracts were diluted 4 times in methanol before being analysed for CFA-L-Ile production by HPLC. **(A)** Shown is the mean area per dry cell weight (DCW) of the CFA-L-Ile peak from triplicate cultures of each strain, with error bars indicating the standard deviation from the mean. Means of groups indicated with no letter in common are significantly different ($p \leqslant 0.05$). **(B)** Absorbance chromatograms obtained for the culture extracts of three overexpression strains and the CFA-L-Ile standard. The CFA-L-Ile peak in each is indicated by solid arrows. Other minor peaks that are potentially related to CFA-L-Ile are indicated with hollow arrows.

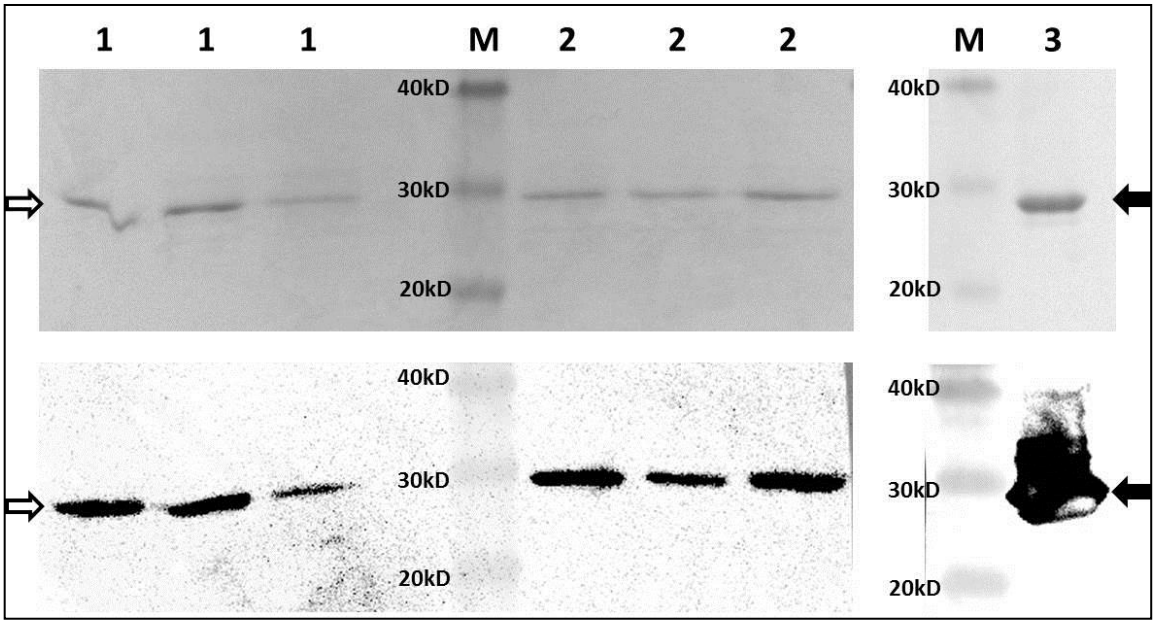
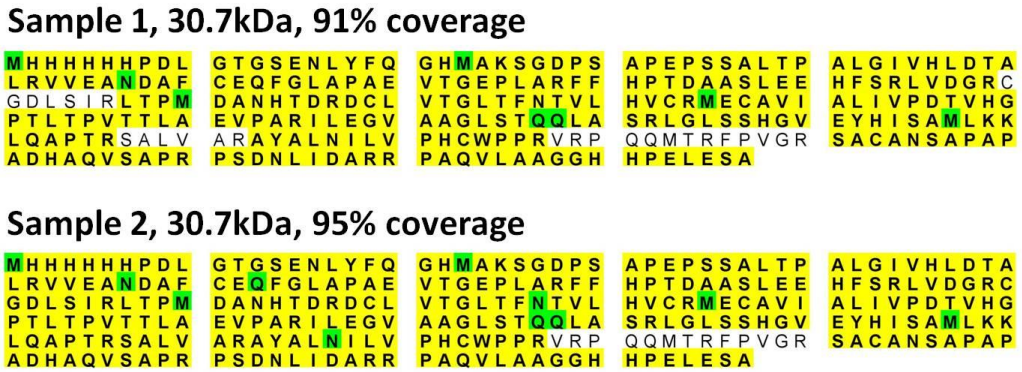
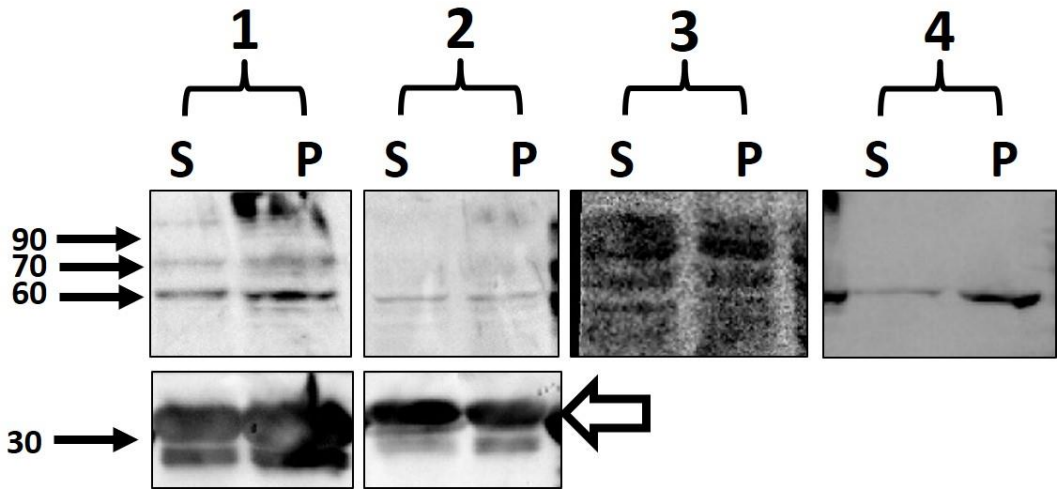


Figure 3.8. Overproduction and purification of HIS_6-CfaR from *S. scabies* strains expressing (lane 1) or lacking (lane 2) ORF1. HIS_6-CfaR was overproduced in the *S. scabies* Δ*txtA*/Δ*cfaR*/Δ*orf1* mutant together with or without ORF1 using the SP44 promoter. The HIS_6-CfaR protein was purified from 48h TSB cultures and was analyzed by SDS-PAGE and Coomassie Brilliant Blue staining (upper panel) and by Western blot analysis (lower panel). The bands corresponding to HIS_6-CfaR are indicated with hollow arrows. Purified CfaR-HIS_6 (25pmol) from *E. coli* (lane 3, indicated with solid arrows) was included for comparison. M: PiNK Plus Prestained Protein Ladder (GeneDireX, Inc.).



Amino acids	Positions	Modification	Sample 1	Sample 2
M	1	oxidation	+	+
M	23	oxidation	+	+
N	57	deamidated	+	+
Q	63	deamidated	N	+
M	110	Oxidation	+	+
N	127	deamidated	N	+
M	135	oxidation	+	+
Q	177	Deamidated	+	+
Q	178	Deamidated	+	+
M	197	oxidation	+	+
N	217	deamidated	N	+

Figure 3.9. Mass spectrometry analysis of HIS$_6$-CfaR purified from *S. scabies*. Sample 1 is the HIS$_6$-CfaR protein purified from an ORF1-expressing strain, while Sample 2 is the protein purified from an ORF1-deficient strain. The areas shaded in yellow indicate the amino acid sequences detected in the analysis, and the % coverage for each sample is indicated. Amino acids for which modifications were detected are highlighted in green, and the modifications that were detected are listed in the table below.



Samples	Overexpression plasmids		Host strains
	CfaR	ORF1	
1	pACYCDuet-1/*cfaR*	N/A	BL21 (DE3)
2	pACYCDuet-1/*cfaR*	pET-30(b)/*orf1*	BL21 (DE3)
3	N/A	pET-30(b)/*orf1*	BL21 (DE3)/pLysS
4	N/A	pET-30(b)/*orf1*	Rosetta™(DE3)

Figure 3.10. Overproduction of HIS-tagged CfaR and ORF1 proteins in different *E. coli* host strains. Overexpression plasmids for HIS_6-CfaR (pACYCDuet-1/*cfaR*) and ORF1-HIS_6 (pET30(b)/*orf1*) were transformed into *E. coli* BL21(DE3), BL21(DE3)/pLysS and/or Rosetta™(DE3), as shown in the table. For all experiments, the *E. coli* cells were cultured in LB at 16°C following induction with 1mM IPTG. Cells were

harvested after 24h incubation and were lysed by sonication. Total soluble and insoluble proteins (20 µg) from the same volume of bacterial culture was analyzed by Western blot analysis using anti-HIS antibodies. The exposure time for visualizing the Western blot was 30 sec when visualizing HIS_6-CfaR (indicated with hollow arrows) and from 1 to 5 min when visualizing ORF1-HIS_6 (no specific ORF1 signal was detected).

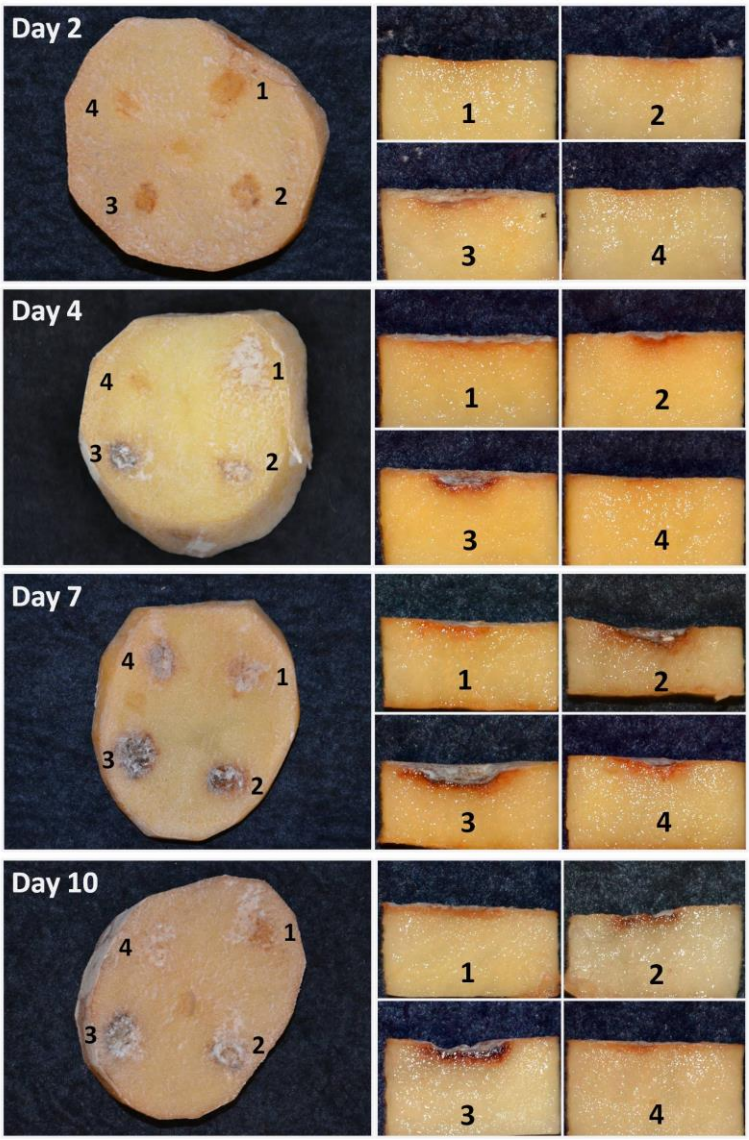


Figure 3.11. Overexpression of *cfaR* and *orf1* enhances the virulence phenotype of *S. scabies* 87-22. Potato tuber disk assay was performed using wild-type *S. scabies* 87-22, carrying (1) no vector, (2) *cfaR* overexpression plasmid, pRLDB51-1, (3) *cfaR+orf1* overexpression plsmid, pRLDB891 and (4) control vector, pRLDB50-1a. Photos were taken at 2, 4, 7 and 10 days post-inoculation. The bioassay was performed three times in total, and representative results are shown.

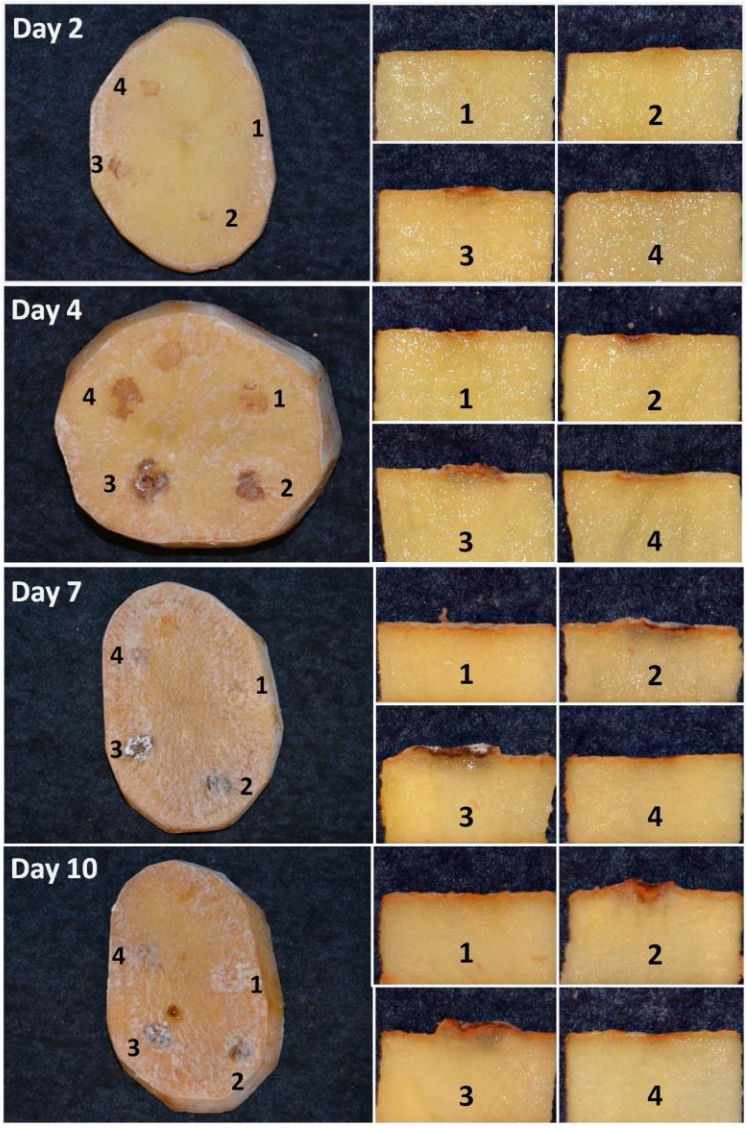


Figure 3.12. Overexpression of *cfaR* and *orf1* causes enhanced potato tissue hypertrophy by *S. scabies* Δ*txtA*. Potato tuber disk assay was performed using *S. scabies* Δ*txtA* strain, carrying (1) no vector, (2) *cfaR* overexpression plasmid, pRLDB51-1, (3) *cfaR+orf1* overexpression plasmid, pRLDB891 and (4) control vector, pRLDB50-1a. Photos were taken at 2, 4, 7 and 10 days post-inoculation. The bioassay was performed three times in total, and representative results are shown.

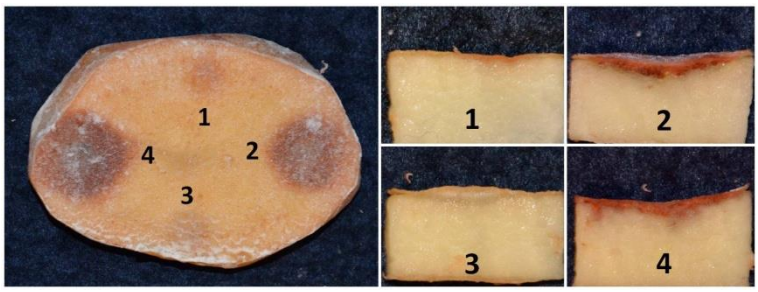


Figure 3.13. Effects of pure thaxtomin A ± CFA-L-Ile on potato tuber tissue. Potato tuber disks were treated with (1) 100% MeOH + 0.1% formic acid (solvent control), (2) thaxtomin A (1 nmol), (3) CFA-L-Ile (100 nmol) and (4) thaxtomin A (1 nmol) + CFA-L-Ile (100 nmol). Photos were taken at 10 days post-inoculation. The bioassay was performed three times in total, and representative results are shown.

Table 3.1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Resistance †	Reference or source
***S. scabies* strains**			
87-22	Wild-type strain	n/a	(Loria et al., 1995)
Δ*txtA*	*S. scabies* 87-22 containing a deletion of the *txtA* thaxtomin biosynthetic gene	$Apra^R$	(Johnson et al., 2009)
Δ*txtA*/Δ*orf1*	Δ*txtA* containing a deletion of *orf1* gene	$Apra^R$, Hyg^R	This study
Δ*txtA*/Δ*cfaR*	Δ*txtA* strain containing a deletion of the *cfaR* gene	$Apra^R$, Hyg^R	This study
Δ*txtA*/Δ*cfaR*/Δ*orf1*	Δ*txtA* strain containing a deletion of the *cfaR* and *orf1* genes	$Apra^R$, Hyg^R	This study
***S. coelicolor* strains**			
M1154	*S. coelicolor* M145 derivative, Δ*act* Δ*red* Δ*cpk* Δ*cda PS I&PS II::aac(3)IV*		(Gomez-Escribano & Bibb, 2011)
***E. coli* strains**			
DH5α	General cloning host	n/a	Gibco-BRL
NEB 5-α	DH5α derivative, high efficiency competent cells	n/a	New England Biolabs
ET12567/pUZ8002	*dam*$^-$, *dcm*$^-$, *hsdS*$^-$; nonmethylating conjugation host	Kan^R, Cam^R	(MacNeil et al., 1992)
BW25113/pIJ790	Host strain for Redirect PCR targeting system	Cam^R	(Gust et al., 2003)
BL21(DE3)	Protein expression strain	n/a	New England Biolabs
BL21(DE3)/pLysS	Protein expression strain BL21(DE3) with pLysS plasmid, exhibits lower background expression of target genes	Cam^R	Promega Corporation
Rosetta™(DE3)	BL21(DE3) derivative, supplies tRNAs for expression of genes containing the rare codons	Cam^R	EMD Millipore Corporation

	AGG, AGA, AUA, CUA, CCC and GGA		
Plasmids or cosmids			
pIJ10700	PCR template for hygromycin resistance cassette used for Redirect PCR targeting	Hyg^R	(Gust et al., 2003)
pRLDB50-1a	*Streptomyces* expression plasmid derived from pSET152; carries the strong, constitutive *ermE*p* promoter and integrates into the φC31 *attB* site	$Apra^R$, $Thio^R$	(Bignell et al., 2010)
pRLDB51-1	*cfaR* overexpression plasmid derived from pRLDB50-1a	$Apra^R$, $Thio^R$	(Bignell et al., 2010)
pRLDB891	*cfaR* + *orf1* overexpression plasmid derived from pRLDB50-1a	$Apra^R$, $Thio^R$	(Cheng et al., 2015)
pRLDB81	*orf1* overexpression plasmid derived from pRLDB50-1a	$Apra^R$, $Thio^R$	(Cheng et al., 2015)
pIJ8660-*ermE*p*	*ermE*p* promoter + 8 × HIS + *tev* inserted upstream of the *egfp* gene in pIJ8660	$Apra^R$	(Moore, 2015)
pGEM-T	Vector for PCR product cloning	Amp^R	Promega Corporation
pGEM/SP44	DNA fragment consisting of the SP44 promoter + 6 × HIS + *tev* cloned into the pGEM-T vector	Amp^R	This study
pGEM/*kasO*p*	DNA fragment consisting of the *kasO*p* promoter + 6 × HIS + *tev* cloned into the pGEM-T vector	Amp^R	This study
pGEM/891N-N	PCR-amplified fragment, containing both *cfaR* and *orf1*, cloned into the pGEM-T vector	Amp^R	This study
pOEEN	pIJ8660-*ermE*p* derivative containing the kanamycin resistance gene inserted into the NheI site	$Apra^R$, Kan^R	This study
pOEC	pIJ8660-*ermE*p* derivative in which *egfp* is replaced by *cfaR*	$Apra^R$	This study

pOECN	pOEC derivative in which the kanamycin resistance gene is inserted into the NheI site	Apra^R, Kan^R	This study
pOSCN	pOECN derivative in which the [*ermE*p* promoter + 8 × HIS + *tev*] fragment is replaced with the [SP44 promoter + 6 × HIS + *tev*] fragment from pGEM/SP44	Apra^R, Kan^R	This study
pOSCON	pOSCN derivative in which the *cfaR* gene is replaced with a *cfaR*+*orf1* DNA fragment	Apra^R, Kan^R	This study
pOSEN	SP44 replaced *ermE*p* in pOEEN	Apra^R, Kan^R	This study
pOKCN	pOECN derivative in which the [*ermE*p* promoter + 8 × HIS + *tev*] fragment is replaced with the [*kasO*p* promoter + 6 × HIS + *tev*] DNA fragment from pGEM/*kasO*p*	Apra^R, Kan^R	This study
pOKEN	*kasO*p* replaced *ermE*p* in pOEEN	Apra^R, Kan^R	This study
pACYCDuet-1/*cfaR*	pACYCDuet-1 derivative carrying a DNA fragment for expression of HIS_6-CfaR protein	Cam^R	This study
pET-30(b)/ORF1	pET-30b derivative carrying a DNA fragment for expression of ORF1-HIS_6 protein	Kan^R	This study
Cosmid 158	SuperCos1 derivative containing the *S. scabies* 87-22 CFA-L-Ile biosynthetic gene cluster	Amp^R, Kan^R	(Bignell et al., 2010)
Cosmid 158/Δ*orf1*	Cosmid 158 derivative containing a deletion of the *orf1* gene	Amp^R, Kan^R, Hyg^R	This study

Cosmid 158/Δ*cfaR*	Cosmid 158 derivative containing a deletion of the *cfaR* gene	Amp^R, Kan^R, Hyg^R	This study
Cosmid 158/Δ*cfaR*/Δ*orf1*	Cosmid 158 derivative containing a deletion of the *cfaR* and *orf1* genes	Amp^R, Kan^R, Hyg^R	This study

† $Apra^R$, $Thio^R$, Kan^R and Cam^R = apramycin, thiostrepton, kanamycin and chloramphenicol resistance, respectively. n/a = not applicable.

3.8 Supplementary Materials

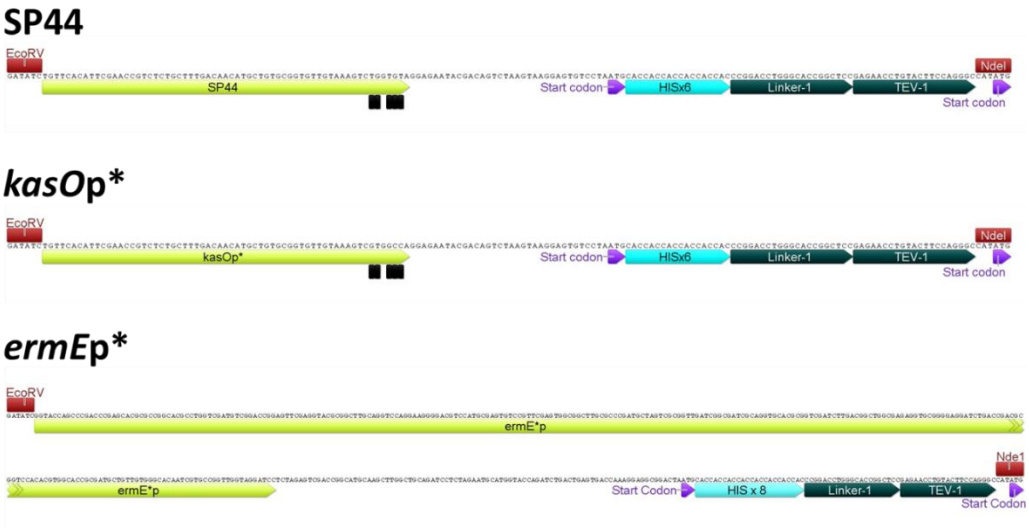
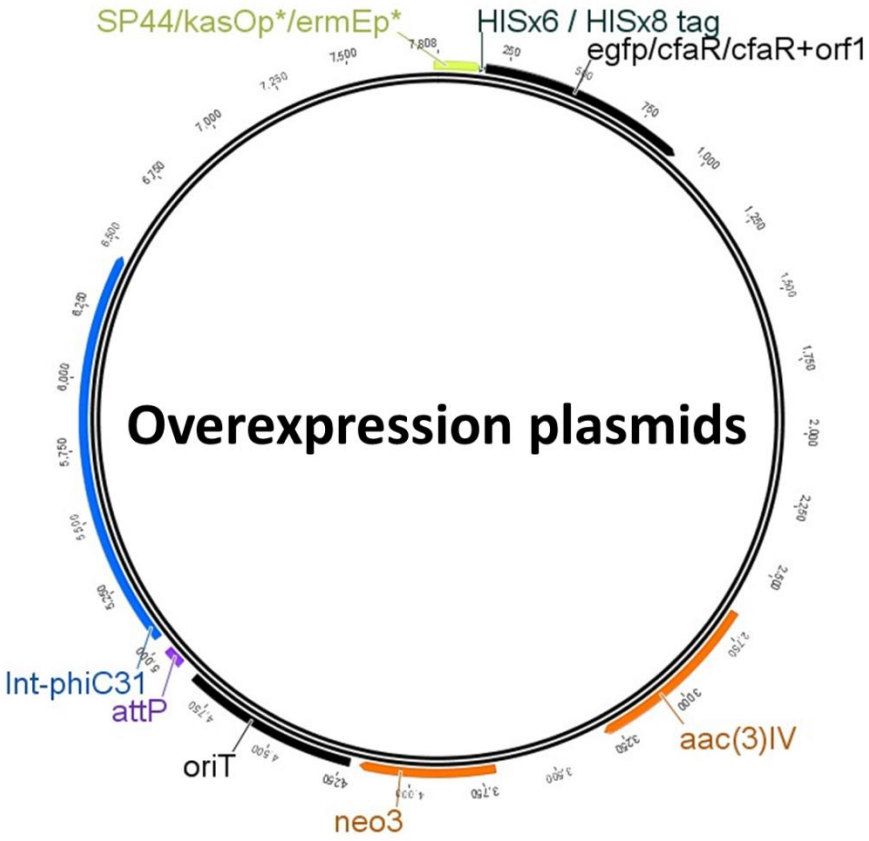


Figure S3.1. Structure of the SP44, *kasO*p* and *ermE*p* promoter fragments used in this study. The sequence differences between SP44 and *kasO*p* are highlighted with black boxes. Promoter sequences of SP44, *kasO*p* and *ermE*p* are shown in light green. HIS×$_6$ and HIS×$_8$: two types of HIS tags. TEV-1, the cleavage site for tobacco etch virus (TEV) protease to remove HIS-tags. Linker-1: linker sequence between HIS-tag and TEV-1. EcoRV and NdeI: two restriction sites designed to incorporate the promoters into pIJ8660 plasmid and its derivatives. The start codons/first amino acid residues from the expressed genes are included in NdeI sites.

(A)


SP44/kasOp*/ermEp*
HISx6 / HISx8 tag
egfp/cfaR/cfaR+orf1
Overexpression plasmids
Int-phiC31
attP
oriT
neo3
aac(3)IV

(B)

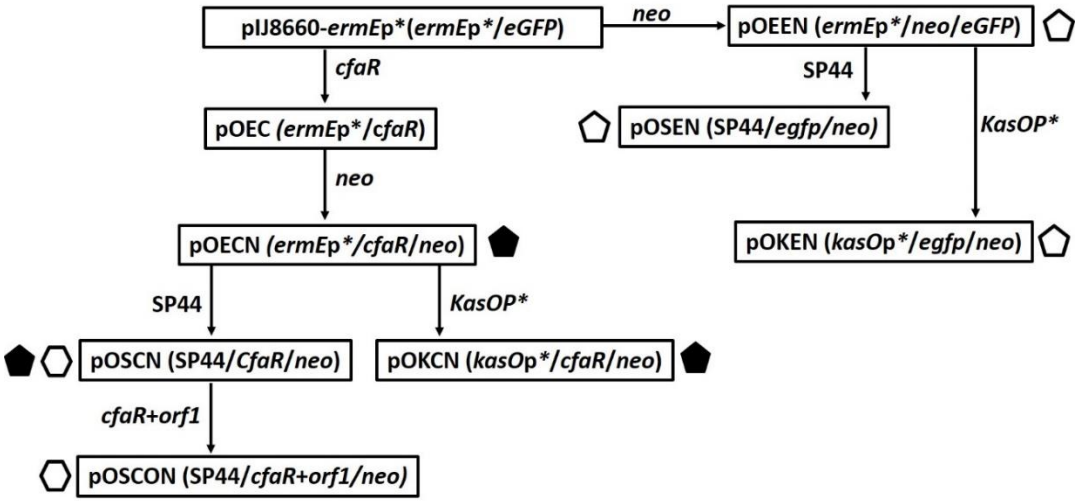

pIJ8660-ermEp*(ermEp*/eGFP)
neo
pOEEN (ermEp*/neo/eGFP)
cfaR
SP44
pOEC (ermEp*/cfaR)
pOSEN (SP44/egfp/neo)
KasOP*
neo
pOECN (ermEp*/cfaR/neo)
pOKEN (kasOp*/egfp/neo)
SP44
KasOP*
pOSCN (SP44/CfaR/neo)
pOKCN (kasOp*/cfaR/neo)
cfaR+orf1
pOSCON (SP44/cfaR+orf1/neo)

Figure S3.2. Construction of *Streptomyces* overexpression plasmids used in this study. (A) Schematic diagram of the plasmids that were constructed or used in the study. The plasmids are all derivatives of the pIJ8660 vector (Sun et al., 1999) and allow for overproduction of N-terminal HIS-tagged proteins in *Streptomyces*. (B) Flow diagram showing the sequence of cloning steps used to construct the plasmids. Within the brackets are the promoter (*ermE*p*, *kasO*p* or SP44) and overexpression gene(s) (*egfp*, *cfaR* or *cfaR+orf1*) that are present in each plasmid, and *neo* indicates the presence of the kanamycin resistance gene in some plasmids. Plasmids used for the eGFP fluorescence assay (pOSEN, pOKEN and pOEEN) are indicated by hollow pentagons; those used for testing CFA-L-Ile production (pOSCN, pOKCN and pOECN) are indicated by solid pentagons; and those used for HIS_6-CfaR overproduction and purification in *S. scabies* (pOSCN and pOSCON) are indicated by hollow hexagons.

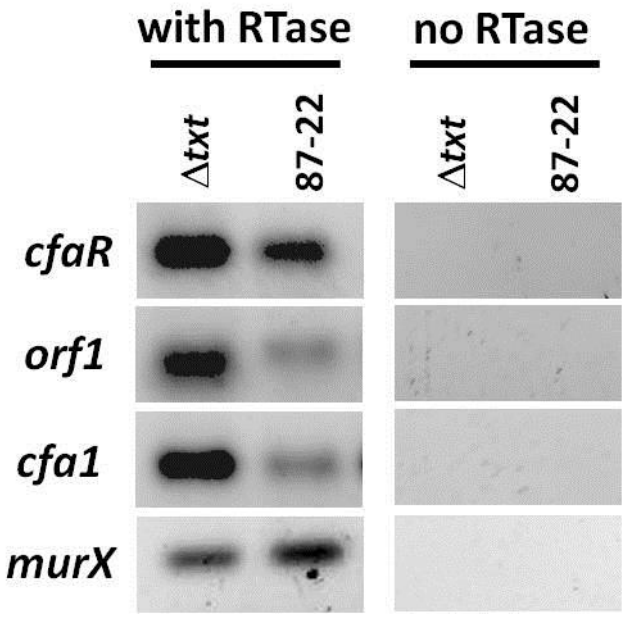
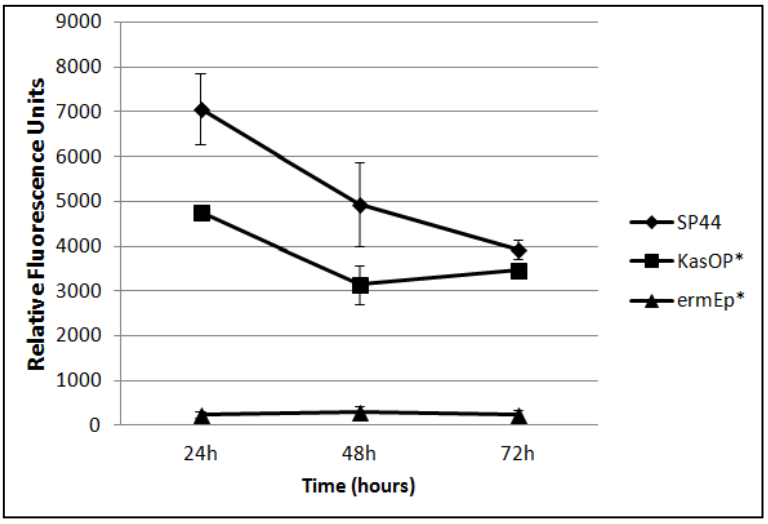


Figure S3.3. Semi-quantitative reverse-transcription PCR analysis showing gene expression in *S. scabies* wild type (87-22) and the Δ*txtA* mutant strain. Cultures were grown for 48 h in SFMB medium, after which total RNA was extracted and was used as a template for reverse transcription reactions containing (with RTase) or lacking (no RTase) reverse transcriptase enzyme. The resulting cDNA was used in PCR reactions with primers targeting *cfaR*, *orf1*, *cfa1* and *murX*. Amplification was performed using 27 (*cfaR*), 30 (*orf1* and *cfa1*) and 28 (*murX*) cycles. The amplified products were analyzed by gel electrophoresis using a 1.2% w/v agarose gel. The *murX* gene served as a loading control, while reactions performed using the no RTase template served as a negative control.

(A)



(B)

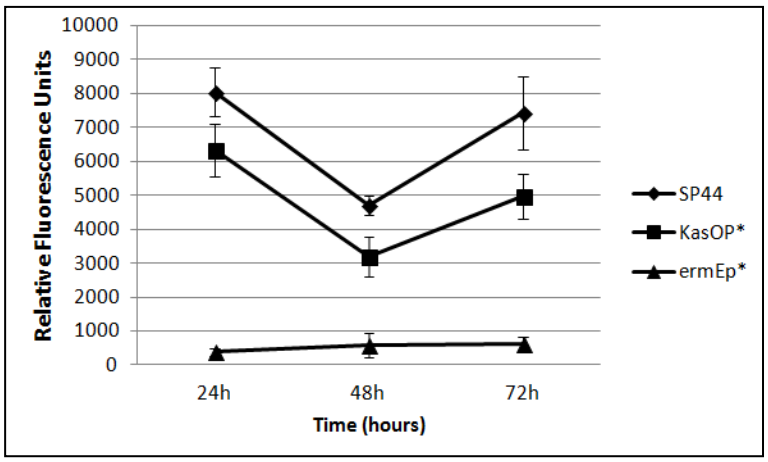


Figure S3.4. Fluorescence assay for detecting promoter activities in *Streptomyces*. Shown is the mean fluorescence density from triplicate cultures of each strain, with error bars indicating the standard deviation from the mean. The *egfp* gene was expressed under the control of three promoters, SP44 (in pOKEN plasmid), *kasO*p* (in pOKEN plasmid) and *ermE*p* (in pOEEN plasmid), in (A) *S. scabies* Δ*txtA*/Δ*cfaR*/Δ*orf1* and (B) *S. coelicolor* M1154.

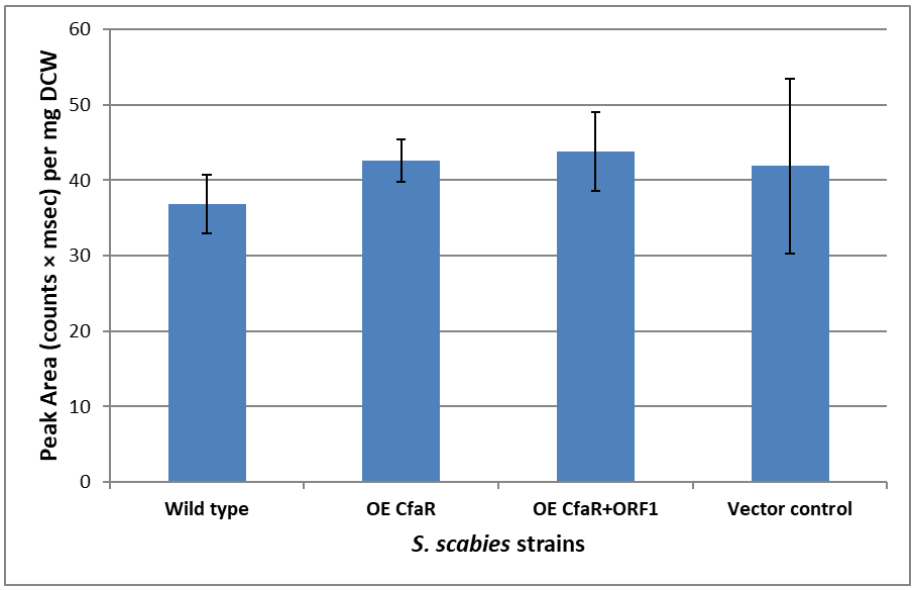


Figure S3.5: Thaxtomin A production in OBB medium by *Streptomyces* strains. Thaxtomin A production was analyzed by HPLC following organic extraction of culture supernatants. Shown is the mean area of the thaxtomin A peak per mg dry cell weight (DCW) from three cultures of each strains, and error bars indicate the standard deviation from the mean. No statistically significant differences in production were observed among the different test strains ($p > 0.05$).

Table S3.1. Oligonucleotides used in this study.

Primer	Sequence (5'→3') †	Use
DRB299	GGACGGTGTTGAACGTGAG	Forward primer for *cfaR* expression by RT-PCR
DRB300	GAACACTTCTCCCGACTCGT	Reverse primer for *cfaR* expression by RT-PCR
DRB484	CGGTAGTCGAAGACGTACAGG	Forward primer for *orf1* expression by RT-PCR
DRB485	GATGTCGTCCTGGGTCAAGT	Reverse primer for *orf1* expression by RT-PCR
LC93	CGACGATCTCTTCATCACTG	Forward primer for *cfa1* expression by RT-PCR
LC94	GAGATGGTGATACCGAAGAG	Reverse primer for *cfa1* expression by RT-PCR
DRB21	GTCTGGCAGTTCCAGGAGTC	Forward primer for *murX* expression by RT-PCR
DRB22	AGGTGTTCCACCACAGGAAG	Reverse primer for *murX* expression by RT-PCR
JS1	CTATCGGGCCCACCGGACGGAGTC CTCGATCCGGCAGCG TGTAGGCTGGAGCTGCTT	Forward primer for *orf1* or *cfa+orf1* deletion
JS2	ATGGCCACTGACACCCACCGGGCG GACATCGACATGATGATTCCGGGG ATCCGTCGACC	Reverse primer for *orf1* deletion
DRB292	TCATGCGCTCTCCAGCTCGGGGTG GTGGCCGCCTGCGGCTGTAGGCTG GAGCTGCTTC	Forward primer for *cfaR* deletion
DRB293	GTGGCGAAATCAGGAGACCCGTCG GCGCCGGAGCCCTCGATTCCGGGG ATCCGTCGACC	Reverse primer for *cfaR* and *cfa+orf1* deletion
LC78	GCGC**GCTAGC**CCGCCTCGGCCTCT GAGCTA	Cloning of the kanamycin resistance gene in the NheI site of pIJ8660-*erm*E*p**
LC79	GCGC**GCTAGC**ATCCCCGCGCTGGA GGATCA	Cloning of the kanamycin resistance gene in the NheI site of pIJ8660-*erm*E*p**
LC52	GT**CATATG**GCGAAATCAGGAGACC CGTC	Cloning of *cfaR* or *cfaR+orf1* to replace *egfp* in pIJ8660-*erm*E*p**
LC53	CA**GCGGCC**GCTCATGCGCTCTCCA GCTCGG	Cloning of *cfaR* to replace *egfp* in pIJ8660-*erm*E*p**
LC71	GCGC**GATATC**CTATCGGGCCCACC GGACGG	Cloning of *cfaR+orf1* to replace *egfp* in pIJ8660-*erm*E*p**

LC72	GACC**GAATTC**GATGGCGAAATCAG GAGACCCGTC	Cloning of *cfaR* into pACYCDuet-1 for expression of HIS_6-CfaR
LC73	GCGC**AAGCTT**TCATGCGCTCTCCA GCTCGGGGT	Cloning of *cfaR* into pACYCDuet-1 for expression of HIS_6-CfaR
LC77	GCGC**AAGCTT**TCGGGCCCACCGGA CGGAGT	Cloning of *orf1* into pET30(b) for expression of ORF1-HIS_6 protein
LC65	GCGC**CATATG**GCCACTGACACCCA CCG	Cloning of *orf1* into pET30(b) for expression of ORF1-HIS_6 protein

† Non-homologous extensions are underlined, while engineered restriction sites are indicated in bold.

Table S3.2. Accession numbers of ORF1 homologues used for the phylogenetic analysis.

Name	Accession number of ORF1 homologue	Accession number of CfaR homologue
Hypothetical protein Streptomyces geranii	WP_105967716.1	WP_105968181.1†
Hypothetical protein Saccharopolyspora spinosa	WP_101376574.1	n/a*
Hypothetical protein Streptomyces sp. JV178	WP_099965306.1	WP_099965395.1†
Hypothetical protein Saccharopolyspora shandongensis	WP_093278035.1	n/a*
Hypothetical protein Streptomyces europaeiscabiei	WP_079104625.1	WP_046709612.1†
ThiF Streptomyces griseoruber	WP_055634617.1	WP_055634616.1‡
ThiF Streptomyces graminilatus	WP_055532744.1	WP_055532742.1‡
ThiF Streptomyces sp. NRRL WC-3618	WP_053745352.1	WP_053745353.1‡
ThiF Kitasatospora azatica	WP_051969274.1	WP_051969273.1‡
ORF1 Streptomyces scabies	WP_013005368.1	CBG74919.1‡
ThiF Streptomyces turgidiscabies and Streptomyces reticuliscabiei	WP_006378371.1	n/a*
ThiF Escherichia coli	WP_097458193.1•	n/a*

‡ CfaR homologues that are encoded next to the corresponding ORF1 homologue.

* n/a = No CfaR homologues identified in the same genome as the ORF1 homologue.

† CfaR homologues that are encoded within the same genome as the corresponding ORF1 homologue, but may not be encoded immediately next to the ORF1 homologue

• The *E. coli* ThiF protein was used as an outgroup for the phylogenetic analysis

Table S3.3. Rare codon frequency in the *orf1* gene

Amino Acid	Rare Codon	Number of Occurrence
Arginine	CGA	1
	CGG	40
	AGG	3
	AGA	2
Glycine	GGA	6
	GGG	10
Leucine	CUA	2
Proline	CCC	17
Threonine	ACG	12

Chapter 4: Effect of microbial and plant-derived molecules on the production of coronafacoyl phytotoxins in the common scab pathogen *Streptomyces scabies*

Zhenlong Cheng, Luke Bown, Brandon Piercey and Dawn R. D. Bignell

4.1 Abstract

Streptomyces scabies is one of the principal causative agents of common scab disease, which is an economically important disease affecting potato production worldwide. This organism produces the phytotoxic specialized metabolite thaxtomin A, which is essential for disease development, as well as coronafacoyl-L-isoleucine, a member of the coronafacoyl family of phytotoxins that contribute to the virulence phenotype of several plant pathogenic bacteria. The goal of this study was to identify microbial or plant-derived molecules that modulate the production of CFA-L-Ile in *S. scabies.* Cellobiose and suberin, which both function as inducers of thaxtomin A production, were tested to determine whether they can also activate the production of CFA-L-Ile. Our results showed that neither cellobiose nor suberin is able to induce CFA-L-Ile production in wild-type *S. scabies* 87-22 in liquid culture, whereas both cellobiose and suberin exhibit inhibitory effects on metabolite production in other *S. scabies* strains. In addition, we showed that CFA-L-Ile production is not stimulated by the addition of potato tuber peels or extracts to the culture medium. The DNA binding activity of CfaR, the cluster-situated activator for CFA-L-Ile biosynthesis, was shown to be antagonized by CFA-L-Ile itself but not by other related metabolites. Our results suggest that CFA-L-Ile production is under strict control in *S. scabies* and is regulated by a negative feedback mechanism involving the end product of the biosynthetic pathway.

4.2 Introduction

Streptomyces is a genus of Gram-positive filamentous *Actinobacteria*. They are well recognized for their ability to produce a wide variety of bioactive specialized metabolites (also known as secondary metabolites) with useful applications in medicine and in agriculture (Berdy, 2005). Among the hundreds of species described to date, only a small number (< 3%) are known to function as plant pathogens and cause important crop diseases such as potato common scab (CS). CS is characterized by the formation of superficial, erumpent or pitted lesions on the potato tuber surface, and these lesions result in economic losses by reducing the quality and market value of the potato crop. Current control practices for managing CS have been summarized (Dees & Wanner, 2012) and include physical (irrigation), chemical (low pH, fungicides, sulphur fertilizer) and biological (resistant potato cultivars) strategies; however, none have been shown to effectively manage the disease in a reliable manner.

Streptomyces scabies (syn. *S. scabiei*) is the best characterized and most widely distributed causal agent of CS disease (Dees & Wanner, 2012). *S. scabies* produces a phytotoxic specialized metabolite, thaxtomin A, which functions as a cellulose synthesis inhibitor and is considered the key virulence factor leading to CS disease (Bischoff et al., 2009; Duval & Beaudoin, 2009; Fry & Loria, 2002; King & Calhoun, 2009; Scheible et al., 2003). Numerous studies have demonstrated a positive correlation between the production of thaxtomin A and the pathogenicity of *S. scabies* and other scab-causing organisms (Goyer et al., 1998; Healy et al., 2000; King et al., 1991; Kinkel et al., 1998; Loria et al., 1995). Though the exact role of thaxtomin A in the infection process is not fully understood,

it has been proposed that the production of this phytotoxin together with the filamentous nature of streptomycetes may allow the pathogen to more easily penetrate expanding plant tissues during host colonization (Loria et al., 2008).

S. scabies has also been shown to produce coronafacoyl-L-isoleucine (CFA-L-Ile), which is a member of the coronafacoyl family of phytotoxins that are also produced by other plant pathogenic bacteria (Bignell et al., 2018). The best characterized family member is coronatine (COR), which consists of the bicyclic hydrindane ring – based polyketide coronafacic acid (CFA) linked to coronamic acid (CMA), an ethylcyclopropyl amino acid derived from L-isoleucine (Bignell et al., 2018). COR contributes to the virulence phenotype of the Gram-negative plant pathogen *Pseudomonas syringae* (Uppalapati et al., 2007) by promoting the invasion and multiplication of the pathogen within plant tissues and by contributing directly to disease symptom development (Xin & He, 2013). Recent work from our lab has shown that elevated CFA-L-Ile production by *S. scabies* increases the severity of disease symptoms induced by the pathogen on potato tuber tissue, suggesting that CFA-L-Ile also contributes to the virulence phenotype of *S. scabies* during host colonization and infection (see Chapter 3).

The production of CFA-L-Ile is controlled by CfaR, a cluster-situated regulator (CSR) encoded within the coronafacoyl phytotoxin biosynthetic gene cluster in *S. scabies.* CfaR is a novel member of the PAS-LuxR family of transcriptional activators that control specialized metabolite production in various *Streptomyces* spp. (Anton et al., 2007; Fuqua et al., 1994; Santos-Aberturas et al., 2011; Vicente et al., 2014; Wei et al., 2011; Wu et al.,

2013). CfaR activates the expression of the CFA-L-Ile biosynthetic genes (Bignell et al., 2010) by binding directly to the promoter region that drives expression of the genes, and this DNA binding activity requires the N-terminal PAS domain, which is involved in protein homodimer formation (Cheng et al., 2015). PAS domains within proteins often function as sensory domains that regulate protein activity by sensing various signals or binding small molecule ligands (Taylor & Zhulin, 1999). Whether the PAS domain within CfaR plays a similar role is currently unknown.

A second regulatory gene, *orf1*, which is co-transcribed with *cfaR*, encodes a ThiF family protein. Genetic studies conducted in Chapters 2 and 3 showed that ORF1 augments the production of CFA-L-Ile in a CfaR-dependent manner, suggesting that ORF1 is a "helper" protein that assists CfaR in activating metabolite production. The exact function of ORF1, however, has yet to be determined.

The production of thaxtomin A in *S. scabies* has been shown to be induced by the plant oligosaccharides cellobiose and cellotriose as well as the lipid plant polymer suberin (Francis et al., 2015; Johnson et al., 2007; Jourdan et al., 2016; Lerat et al., 2010). Cellobiose and cellotriose can both serve as a ligand for the cellulose utilization regulator CebR, which functions as a repressor of the thaxtomin A biosynthetic genes. Binding of cellobiose or cellotriose inhibits the DNA binding activity of CebR, and this allows for increased expression of the thaxtomin biosynthetic genes (Francis et al., 2015). Suberin is thought to promote the onset of secondary metabolism in *Streptomyces* spp., though the exact mechanism by which it does so is currently unknown (Lerat et al., 2010). Although

the study by Lerat and colleagues showed that cellobiose or suberin alone stimulated only trace levels of thaxtomin A production by *S. scabies* in a minimal starch medium, production was strongly stimulated when both compounds were present in the culture medium, suggesting that the compounds exhibit a synergistic effect on thaxtomin A production in this organism (Lerat et al., 2010).

In order to better understand the factors controlling coronafacoyl phytotoxin production in *S. scabies*, we set out to identify microbial or plant-derived molecules that influence CFA-L-Ile production in this organism. Specifically, the study aimed to determine whether the thaxtomin A inducers suberin and cellobiose can also serve as inducers of CFA-L-Ile biosynthesis in *S. scabies*, and whether other compounds present in potato tuber flesh or peel can stimulate metabolite production in liquid culture. In addition, we investigated whether the DNA binding activity of the CfaR CSR is influenced by CFA-L-Ile and other related compounds.

4.3 Materials and Methods

4.3.1 Bacterial strains, cultivation and maintenance

Bacterial strains used in this study are listed in Table 4.1. *Escherichia coli* strains were routinely cultivated in Luria-Bertani (LB) Lennox medium (Fisher Scientific, Canada) at 37°C unless otherwise stated. Liquid cultures were grown on a rotary shaker at 200rpm. Where required, the medium was supplemented with kanamycin or apramycin (Sigma Aldrich, Canada) at 50 μg/mL final concentration. *E. coli* strains were maintained at -80°C in 20% v/v glycerol (Sambrook & Russell, 2001) .

S. scabies strains were routinely cultured at 28°C on potato mash agar (PMA) solid medium (Fyans et al., 2015). When necessary, the growth medium was supplemented with apramycin or thiostrepton (Sigma Aldrich, Canada) at 50 or 25 µg/mL final concentration, respectively. For production of CFA-L-Ile, seed cultures were prepared by inoculating 50 µL of a *S. scabies* spore stock or 1 mL of a glycerol mycelial stock into 5 mL of trypticase soy broth (TSB) liquid medium (BD Biosciences, Canada) in a 50 mL spring flask. The flasks were incubated at 28°C with shaking (200 rpm) for 24-48 hr until dense mycelial growth was obtained. The seed cultures (100 µL) were then sub-cultured into 3 × 5 mL of soy flour mannitol broth (SFMB) liquid medium (Fyans et al., 2015) in 6-well tissue culture plates (Fisher Scientific, Canada), after which the cultures were incubated at 25°C and 125 rpm for 7 days. For production of thaxtomin A, the seed cultures (100 µL) were sub-cultured into 3 × 5 mL of minimum starch medium (MSM) (Lerat et al., 2010) in 6-well tissue culture plates (Fisher Scientific, Canada), after which the cultures were incubated at 25°C and 125 rpm for 7 days.

To test the effect of cellobiose and/or suberin on phytotoxin production, SFMB or MSM culture media were supplemented with cellobiose (Sigma Aldrich, Canada) at concentrations of 0, 0.2, 0.5, 1 and 2% w/v, and/or suberin (kindly provided by Dr. Carole Beaulieu, Université de Sherbrooke) at 0, 0.05, 0.1, 0.15, 0.2 and 0.5% w/v before autoclaving. For preparation of the SFMB medium or medium with suberin, a blender was routinely used to homogenize the insoluble soy flour (± suberin) prior to sterilization of the medium. When SFMB was supplemented with potato peel/juice, potatoes were peeled, the peel was homogenized in a blender and drained, and 1 g fresh weight of the solid

homogenate was added to 25 mL of SFMB medium, after which the medium was autoclaved. The peeled potatoes were separately homogenized in a blender and the homogenate was centrifuged at room temperature at 4,500 × *g* for 15 min. The supernatant was then filter sterilized, and 1 mL was added to 25 mL of autoclaved SFMB medium prior to inoculation.

S. scabies strains were maintained at -80°C as spore suspensions in 20% v/v glycerol (Kieser et al., 2000) or as mycelial suspensions in TSB containing 25% glycerol (Shepherd et al., 2010).

4.3.2 Analysis of CFA-L-Ile production by *S. scabies*

HPLC analysis was performed as described in Chapter 3, Section 3.3.13.

4.3.3 Analysis of thaxtomin A production by *S. scabies*

Small scale extraction of thaxtomin A and metabolite detection by reverse phase HPLC was performed as described before (Fyans et al., 2016).

4.3.4 Dry cell weight measurement and HPLC result standardization

To measure dry cell weight (DCW), the mycelia from the entire liquid culture was collected and centrifuged, and the culture supernatant was removed. The pellet was then heated to 50°C and incubated at this temperature for 24 hours or until completely dried. The weight of the tube and dried cell pellet was measured using an analytical balance (Sartorius AG) and the DCW was calculated by subtracting the weight of the tube itself. For cultures in which suberin was added, the initial weight of the added suberin was

subtracted from the DCW obtained since suberin is insoluble and largely remains within the culture after incubation, an observation that was also noted by Lerat and colleagues (Lerat et al., 2010). DCW was not measured for cultures with potato peels and/or juice. The peak area of CFA-L-Ile or thaxtomin A obtained from the HPLC chromatogram for the corresponding culture extract was divided by the DCW (in mg) for the corresponding culture to give the normalized peak area (counts × msec) per mg DCW.

4.3.5 Identification of known and predicted CebR binding sites in the *S. scabies* 87-22 genome

CebR binding sequences in *S. scabies* (Francis et al., 2015), *Streptomyces griseus* (Marushima et al., 2009) and *Streptomyces reticuli* (Schlösser et al., 1999) have been described before. A 10-bp core sequence (GGAGCGCTCC) that is conserved in all of the sequences was used in a nucleotide Blast (BlastN) (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) search of the genome of *S. scabies* 87-22 (taxid: 680198 in GenBank) in order to identify known and putative CebR binding sites.

4.3.6 Overexpression of histidine-tagged CfaR in *E. coli* and protein purification

Overexpression of the full length CfaR protein with a C-terminal histidine tag (CfaR-HIS_6) in *E. coli* and protein purification was performed as described before (Cheng et al., 2015).

4.3.7 Electrophoretic mobility shift assay (EMSA)

DNA fragment a from Figure 2.2A was used as probe. CFA-L-Ile purified from *S. scabies* cultures was provided by Dr. Joanna Fyans (Department of Biology, Memorial University of Newfoundland). Pure COR was purchased from Sigma Aldrich Canada (Cat. No. C8115-1MG), and pure CFA was purchased from Dr. Carol Bender (Department of Plant Pathology, Oklahoma State University).

EMSAs were performed using the LightShift® Chemiluminescent EMSA Kit (Fisher Scientific, Canada) as described previously (Cheng et al., 2015) with modifications: a 20 µL reaction system contains CfaR-HIS_6 protein (0.277 µM), test compound (1.545 µM) and DNA probe (6.294 nM). Control reactions were set up that either lacked the protein or contained solvent (DMSO) in place of the test compound. For examining the concentration-dependent effect of CFA-L-Ile on CfaR DNA binding activity, different final concentrations of CFA-L-Ile (0, 6.18, 3.09, 1.545, 0.7725, 0.38625 and 0.193125 µM) were added to the binding reactions.

Following incubation at room temperature for 20 min, the reactions were subjected to electrophoresis on a 6% w/v PAGE, and DNA bands were visualized under UV light following staining with ethidium bromide (EtBr).

4.3.8 Statistical analyses

All HPLC analyses were performed using triplicate samples originating from three independent cultures. Statistical analysis of metabolite production levels was conducted using a one-way ANOVA, and *a posteriori* multiple comparisons of least squared means

were performed using the Tukey test. *P* values ≤ 0.05 were considered statistically significant in all analyses.

4.4 Results

4.4.1 Effect of cellobiose on CFA-L-Ile production in *S. scabies*

As CFA-L-Ile has been shown to enhance the virulence of *S. scabies* during host-pathogen interactions (Chapter 3), and the biosynthetic genes are known to be expressed during colonization of plant roots by *S. scabies* (Bignell et al., 2010), we were interested in determining whether production of this metabolite is induced by signalling molecules from the plant host. Cellobiose is a plant-derived disaccharide that is a known inducer of thaxtomin A production in *S. scabies* (Lerat et al., 2010), and its ability to induce thaxtomin A production in *S. scabies* 87-22 was confirmed in this study (Fig. S4.1A). Cellobiose was also found to influence the growth of *S. scabies* 82-22 in MSM medium. As a carbon and energy source, cellobiose stimulated the growth of the organism to produce more biomass (Fig. S4.1B), and the mycelia formed clumps with smaller diameters at higher concentrations of cellobiose (Fig. S4.1C).

To test if cellobiose can also induce the production of CFA-L-Ile, *S. scabies* was cultured in SFMB medium that was supplemented with cellobiose at a final concentration of 0.5% w/v. This concentration was chosen for our initial studies as it was previously reported to stimulate the production of thaxtomin A (Lerat et al., 2010). While no CFA-L-Ile production was detected from the wild-type *S. scabies* 87-22 (data not shown), inhibition of metabolite production in the presence of 0.5% w/v cellobiose was observed

with Δ*txtA*/pRLDB51-1 strain (Fig. 4.1), which produces elevated levels of CFA-L-Ile as compared to *S. scabies* 87-22 due to the presence of the *cfaR* overexpression plasmid pRLDB51-1 in the Δ*txtA* mutant background (Fyans et al., 2015). To further analyze the inhibitory effect of cellobiose, the production of CFA-L-Ile was assessed in SFMB medium supplemented with different concentrations of cellobiose (0%, 0.2%, 0.5%, 1% and 2% w/v). The wild type *S. scabies* 87-22 and two CFA-L-Ile over producing strain, 87-22/pRLDB51-1 and Δ*txtA*/pRLDB51-1, were cultured in the supplemented SFMB medium. At all concentrations tested, cellobiose failed to induce the production of detectable levels of CFA-L-Ile in the *S. scabies* 87-22 strain (data not shown). In the case of the 87-22/pRLDB51-1 and Δ*txtA*/pRLDB51-1 strains, which both overexpress *cfaR* and thus produce higher baseline levels of CFA-L-Ile, increasing concentrations of cellobiose had an overall inhibitory effect on CFA-L-Ile production, with the greatest inhibition occurring in the presence of 2% w/v cellobiose (Fig. 4.2). Thus, under the experimental conditions used, cellobiose appears to suppress CFA-L-Ile production rather than stimulating production.

4.4.2 The CFA-L-Ile biosynthetic gene cluster lacks putative CebR binding sites

CebR is a DNA binding protein and a repressor for genes involved in cellulose metabolism in *Streptomyces* (Marushima et al., 2009; Schlösser et al., 1999; Schlösser et al., 2000). The binding of CebR is relieved by the ligands cellobiose and cellotriose, resulting in activation of gene expression (Schlösser et al., 2000). CebR binding sequences have been described in several *Streptomyces* spp. (Francis et al., 2015; Marushima et al., 2009; Schlösser et al., 1999), and a comparison of all of the binding sites identified in *S.*

griseus (Marushima et al., 2009), *S. reticuli* (Schlösser et al., 1999) and *S. scabies* (Francis et al., 2015) revealed a 10-bp core (GGAGCGCTCC) that is absolutely conserved in all of the sequences. A BlastN search of the *S. scabies* genome using this 10-bp core sequence generated 64 hits (Table S4.1), including one located in between the thaxtomin synthetase A (*txtA*) gene (no. 21) and the thaxtomin CSR gene (*txtR*), and one within the thaxtomin synthetase B (*txtB*) gene (no. 20). Both of these sites have been experimentally confirmed to function as CebR binding sites *in vitro* (Francis et al., 2015). In contrast, no CebR binding site was detected within the coronafacoyl phytotoxin biosynthetic gene cluster, suggesting that production of CFA-L-Ile, unlike that of thaxtomin A, is not directly regulated by CebR. It is noteworthy that one potential CebR site (no. 56) was found in SCAB_83911, which encodes one of six polyketide synthase enzymes involved in the biosynthesis of concanamycin (Bignell et al., 2014). Concanamycin is a known phytotoxin produced by *S. scabies* (Haydock et al., 2005; Natsume et al., 1996; Natsume et al., 1998) and is another putative virulence factor (Bignell et al., 2014).

4.4.3 Effect of potato suberin on CFA-L-Ile production

Suberin, a complex polymer found of the surface of potato tubers, is another known inducer of thaxtomin A production in *S. scabies* (Lerat et al., 2010). This was confirmed in our study, though only low levels of thaxtomin A were detected when the suberin concentration was 0.5% w/v, and no thaxtomin production was detected at lower concentrations (Fig. S4.2). Most likely, this is due to the fact that *S. scabies* 87-22 grew more poorly in the MSM medium with lower suberin concentrations (data not shown), and

suberin did not promote the growth of *S. scabies* in the medium to the same extent as cellobiose (Fig. S4.1).

To test the ability of suberin to induce the production of CFA-L-Ile, *S. scabies* strains 87-22 and Δ*txtA*/pRLDB51-1 were cultured in SFMB medium supplemented with suberin at a final concentration of 0.1% w/v. This concentration has been reported to stimulate the production of thaxtomin A by *S. scabies* (Lerat et al., 2010), and thus we chose it for our initial studies. No detectable CFA-L-Ile production was observed in the presence of 0.1% w/v suberin for *S. scabies* 87-22 (data not shown), while Δ*txtA*/pRLDB51-1 produced similar metabolite level as in the untreated cultures (Fig. 4.1). To further investigate the effect of suberin, wild-type *S. scabies* 87-22 and two over producing strains, 87-22/pRLDB51-1 and Δ*txtA*/pRLDB51-1, were cultured in SFMB medium supplemented with different concentrations of suberin (0%, 0.05%, 0.1%, 0.15%, and 0.2% w/v). At all concentrations tested, suberin did not induce a detectable amount of CFA-L-Ile production in *S. scabies* 87-22 (data not shown). In the case of strain 87-22/pRLDB51-1, the production of CFA-L-Ile was significantly reduced when 0.1% or 0.15% w/v suberin was present in SFMB medium as compared to the untreated cultures, while relative production was similar to that in the untreated cultures when 0.2% w/v suberin was added (Fig. 4.3A). For the Δ*txtA*/pRLDB51-1 strain, CFA-L-Ile production was significantly reduced in the presence of 0.15% and 0.2% w/v suberin, while lower concentrations of suberin did not have a significant effect on production as compared to the untreated cultures (Fig. 4.3B).

Lerat and colleagues reported that the presence of both suberin and cellobiose stimulated higher thaxtomin A production levels by *S. scabies* than when each compound was added alone (Lerat et al., 2010). No induction of CFA-L-Ile was seen in the culture of *S. scabies* 87-22 when supplemented with both suberin and cellobiose (data not shown). When strain Δ*txtA*/pRLDB51-1 was cultured in the presence of both 0.1% w/v suberin and 0.5% w/v cellobiose, production of CFA-L-Ile was again reduced as compared to the untreated control and was similar to the metabolite production level observed in the cultures treated with 0.5% w/v cellobiose alone (Fig. 4.1). Our results therefore suggest that as with cellobiose, suberin at certain concentrations has an inhibitory effect on CFA-L-Ile production, and combining cellobiose and suberin does not enhance the production of CFA-L-Ile by *S. scabies*.

4.4.4 Effect of potato tuber tissue or juice on CFA-L-Ile production

Although suberin did not stimulate CFA-L-Ile production when *S. scabies* was cultured in SFMB medium, it is possible that other molecules derived from potato tuber tissue can stimulate metabolite biosynthesis. To test this, potato tuber peels and tuber tissue juice were prepared as described in the Materials and Methods section and were used to supplement SFMB medium. Wild-type *S. scabies* 87-22 and the Δ*txtA*/pRLDB51-1 strain were cultured in the supplemented SFMB medium, and CFA-L-Ile metabolite production levels were monitored following incubation. *Streptomyces* DCW was not measured to standardize CFA-L-Ile production as there was a significant amount of remaining potato biomass that was impossible to measure separately. *S. scabies* 82-22 did not produce detectable CFA-L-Ile levels in any of the culture media (data not shown), and the

Δ*txtA*/pRLDB51-1 strain produced CFA-L-Ile in the non-supplemented medium, but surprisingly, it produced no detectable levels of the metabolite in the medium supplemented with potato peels and/or tuber tissue juice (Fig. 4.4).

4.4.5 CFA-L-Ile inhibits the DNA binding activity of CfaR

PAS domains within proteins are known to be involved in regulating protein activity by sensing various signals, including light, oxygen, or voltage (Crosson et al., 2003), or by binding small molecule ligands (Henry & Crosson, 2011; Möglich et al., 2009; Taylor & Zhulin, 1999). The PAS domain within the CfaR regulator that controls CFA-L-Ile production was previously shown to be involved in protein homodimer formation (Cheng et al., 2015), and it was predicted that it may also play a role in controlling the DNA binding activity of the protein in response to a signal or small molecule ligand. As it is known that the end product(s) and/or intermediate(s) of a biosynthetic pathway can interact with CSRs to control specialized metabolite production in *Streptomyces* spp. (Du et al., 2011; Jiang & Hutchinson, 2006; Li et al., 2013; Li et al., 2018; Mao et al., 2013; Niu et al., 2016; Tahlan et al., 2007; Xu et al., 2010), we hypothesized that CFA-L-Ile itself may function as a small molecule ligand for CfaR. To test this, EMSAs were performed using purified CfaR-HIS_6 protein in the presence of CFA-L-Ile and two related molecules, CFA (a biosynthetic intermediate of CFA-L-Ile) and COR (a structural analog of CFA-L-Ile) (Fig. 4.5A). Among the tested molecules, CFA-L-Ile was the only one that obviously inhibited the DNA binding activity of CfaR *in vitro* (Fig. 4.5B). DNA binding activity was slightly inhibited by equimolar amounts of CFA, while COR did not cause any inhibition (Fig. 4.5B). To further investigate the effect of CFA-L-Ile, EMSAs were repeated using

different concentrations of the metabolite. The inhibition of CfaR binding activity by CFA-L-Ile occurs in a concentration dependent manner, and inhibition could be observed with as little as 0.7725 nmol of CFA-L-Ile (Figure 4.5C). Our results therefore suggest that the DNA binding activity of CfaR is negatively regulated by CFA-L-Ile, the end product of the biosynthetic pathway.

4.5 Discussion

The production of virulence factors by bacterial pathogens is often tightly controlled and involves multiple levels of regulation in order to ensure that such factors are synthesized at the appropriate level and only when they are required. In *S. scabies*, the production of the virulence-associated CFA-L-Ile phytotoxin is under control of the CSR CfaR, which acts as a transcriptional activator of the biosynthetic genes, and ORF1, which enhances phytotoxin production in a CfaR-dependent manner (Cheng et al., 2015; Chapter 3). *S. scabies* also produces the virulence-associated thaxtomin A phytotoxin, the biosynthesis of which is regulated by TxtR, a CSR encoded within the thaxtomin biosynthetic gene cluster (Joshi et al., 2007). The thaxtomin and coronafacoyl phytotoxin biosynthetic genes are additionally modulated by four global regulatory genes, *bldA*, *bldD*, *bldG* and *bldH* (Bignell et al., 2014), suggesting that there is overlap in the regulatory networks controlling these virulence factors. The production of thaxtomin A is stimulated in liquid culture by both cellobiose and suberin, and we hypothesized that these plant-derived molecules might also influence the production of other *S. scabies* virulence factors such as CFA-L-Ile. Thus, one of the aims of this study was to investigate the effect of cellobiose and suberin on CFA-L-Ile production by *S. scabies*.

Cellobiose is the smallest subunit of cellulose, and its influence on thaxtomin A biosynthesis is thought to be primarily mediated through the cellulose utilization regulator CebR, which binds to two sites within the thaxtomin biosynthetic gene cluster (Francis et al., 2015). Binding of cellobiose to CebR inhibits the DNA binding activity of CebR, thus leading to activation of thaxtomin biosynthetic gene expression (Francis et al., 2015). Although the stimulatory effect of cellobiose on thaxtomin A biosynthesis was repeated in this study (Fig. S4.1), we saw no induction of CFA-L-Ile production in the presence of cellobiose by wild-type *S. scabies* 87-22, which has been reported previously to only produce trace levels of CFA-L-Ile under laboratory conditions (Fyans et al., 2015).

We also examined the effect of cellobiose on the production of CFA-L-Ile in two other strains, 87-22/pRLDB51-1 and Δ*txtA*/pRLDB51-1, both of which produce elevated levels of CFA-L-Ile due to overexpression of the *cfaR* regulatory gene from the strong, constitutive *ermE*p* promoter (Cheng et al., 2015; Fyans et al., 2015; Chapter 3). Unexpectedly, we observed an inhibitory effect of cellobiose on CFA-L-Ile production in both strains under the culturing conditions used (Fig. 4.2). BlastN results showed that there are no CebR binding sites within or near the coronafacoyl phytotoxin biosynthetic gene cluster in *S. scabies* (Fig. S4.1), suggesting that CebR is not involved in the direct regulation of CFA-L-Ile biosynthesis. Although, it is currently unclear how cellobiose functions to suppress metabolite production in these strains, it is possible that cellobiose influences the expression of other genes in the *S. scabies* genome that subsequently inhibit CFA-L-Ile production. It is notable that several genes encoding predicted transcriptional regulators are associated with potential CebR binding sites (Table S4.1), and thus it is

possible that cellobiose-mediated regulation of one or more of these regulatory genes might account for the observed decrease in CFA-L-Ile production, though further studies would be needed to explore this idea. Overall, our results suggest that while cello-oligosaccharides can serve as direct inducers of thaxtomin A biosynthesis in *S. scabies*, they do not function to control the production of all the known virulence factors in the same manner.

Suberin is another reported inducer of thaxtomin A biosynthesis in *S. scabies*, and while the mechanism by which it functions in this manner is not fully understood, it has been shown that suberin can trigger the onset of secondary metabolism in *S. scabies* and in other *Streptomyces* spp., thereby promoting the production of specialized metabolites by these organisms (Lerat et al., 2010). A proteomic study by Lauzier and colleagues showed that suberin enhances the production of proteins involved in the biosynthesis or transport of signaling molecules linked to the onset of secondary metabolism in *S. scabies* (Lauzier et al., 2008). This suggests that in addition to thaxtomin A, other specialized metabolites such as CFA-L-Ile may also be induced by suberin in this organism. However, we were unable to demonstrate induction of CFA-L-Ile production in wild-type *S. scabies* 87-22 at any of the suberin concentrations tested (data not shown). It is noteworthy, though, that the same concentrations of suberin also failed to induce thaxtomin A production in MSM medium, and only a higher concentration (0.5% w/v) allowed for stimulation of detectable thaxtomin A biosynthesis (Fig. S4.2). Whether suberin at 0.5% w/v can induce CFA-L-Ile production in *S. scabies* 87-22 remains to be determined.

As observed with cellobiose, suberin was found to have an inhibitory effect on CFA-L-Ile production at some concentrations when strains 87-22/pRLDB51-1 and Δ*txtA*/pRLDB51-1 were used, though the exact effect of the suberin at a given concentration varied depending on the strain (Fig. 4.3). Supplementation with both suberin (0.1% w/v) and cellobiose (0.5% w/v) also had an inhibitory effect on CFA-L-Ile production by strain Δ*txtA*/pRLDB51-1, though the inhibition was similar to that observed when the culture was supplemented with cellobiose (0.5% w/v) alone (Fig. 4.1). It is noteworthy that we observed a significant amount of variability in the results among replicate experiments for the suberin studies. This may be due in part to the fact that suberin is not soluble in the culture medium, and a blender was needed to break down the suberin into smaller pieces. Although attempts were made to ensure that the insoluble materials in the culture media were evenly distributed among the replicate culture wells, it is possible that different wells received different amounts of suberin, thus resulting in differences in metabolite production levels. Also, the blending of the suberin within the media may have resulted in particles of varying sizes, and this might have influenced the efficiency of suberin metabolism by the *S. scabies* strains. Additionally, as previously noted by Lerat and colleagues (Lerat et al., 2010), it appeared that most of the suberin was not being consumed over the course of the experiment (7 days), and there was no effective way to measure the residual suberin in the cultures. When calculating DCW, the weight of the initial suberin added was deducted from the recorded dry weights as performed in the Lerat study, and this would not account for any differences in the breakdown of the suberin among the different cultures.

Potato is one of the natural hosts of *S. scabies,* and we expected that other molecules associated with potato tubers might trigger CFA-L-Ile production by the pathogen. However, to our surprise, supplementation of SFMB medium with blended potato tuber peel and/or potato tuber juice did not induce CFA-L-Ile production in wild-type *S. scabies,* and it inhibited CFA-L-Ile production in the overproducing strain, Δ*txtA*/pRLDB51-1 (Fig. 4.4). The reason for this is unclear; however, potato tubers are known to contain phenolic compounds such as phenolic acids and flavonoids (Deusser et al., 2012), both of which have been reported to function as antivirulence compounds that exhibit inhibitory activity against microbial virulence factors without affecting viability (Silva et al., 2016). The content of phenolic compounds varies among potato varieties (Valcarcel et al., 2016) and some potato varieties are more resistant to CS pathogens than others (Sedláková et al., 2013). It is also reported that expanding tissues are more susceptible to *S. scabies* (Khatri et al., 2011). In order to identify the potato-associated molecules affecting CFA-L-Ile production, potato varieties and developmental stages should be taken into consideration and further studies are needed.

Another aim of this study was to examine the effect of small molecules on the DNA binding activity of the CfaR CSR. CfaR contains an N-terminal PAS domain, which in other proteins has been reported to function as a sensor domain and has the potential to bind small molecule ligands (Taylor & Zhulin, 1999). We hypothesized that the final product of the biosynthetic pathway, CFA-L-Ile, may function as a ligand for CfaR and regulate its activity since it is known that intermediates and end products of specialized metabolite biosynthesis can function as ligands for some CSRs in other *Streptomyces* spp.

(Du et al., 2011; Jiang & Hutchinson, 2006; Li et al., 2013; Li et al., 2018; Mao et al., 2013; Niu et al., 2016; Tahlan et al., 2007; Xu et al., 2010). Results presented here show that pure CFA-L-Ile is able to antagonize the DNA binding activity of CfaR *in vitro* in a concentration-dependent manner (Fig. 4.5), suggesting that the activity of CfaR and the production of CFA-L-Ile is subject to negative feedback regulation in *S. scabies*. Neither the structural analog COR nor the biosynthetic intermediate CFA were able to strongly inhibit the activity of CfaR at equimolar amounts (Fig. 4.5), suggesting that the regulation of CfaR is highly specific for the end product of the biosynthetic pathway. Negative feedback regulation by biosynthetic intermediates and/or end products has been reported to control the production of antibiotic metabolites such as the sansanmycins (Li et al., 2013), jadomycin (Zhang et al., 2013), auricin (Kutas et al., 2013), simocyclinone (Horbal et al., 2012) and nosiheptide (Li et al., 2018), and it has been speculated that this may serve as a strategy to control the levels of antibiotic produced in order to ensure that the producing organism is not inhibited by its own product (Li et al., 2013). To our knowledge, ours is the first report of a phytotoxic specialized metabolite being subjected to negative feedback regulation in a streptomycete, and it is the first instance of a PAS-LuxR family regulator that can sense the end product of the biosynthetic process that it regulates. Our results together with previous reports suggest that the negative feedback regulation by end products may be a common regulatory mechanism for controlling the production of different types of bioactive specialized metabolite in *Streptomyces* spp.

In summary, our study demonstrates that the production of the CFA-L-Ile phytotoxin is likely under strict control in *S. scabies*, and additional studies will be required

to further elucidate the factors modulating production of this virulence factor. Whether host – derived signals are involved in stimulating CFA-L-Ile biosynthesis remains to be determined, but our study suggests that different signals may be involved in promoting the production of different virulence factors, perhaps as a way for the pathogen to coordinate the release of specific virulence factors at the appropriate time during the infection process.

4.6 References

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4.7 Figures and Tables

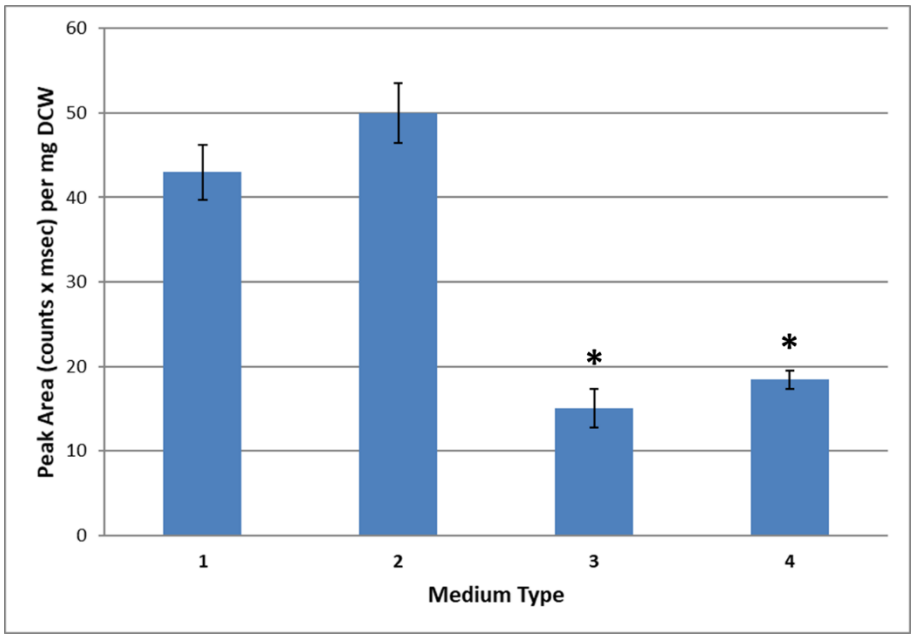
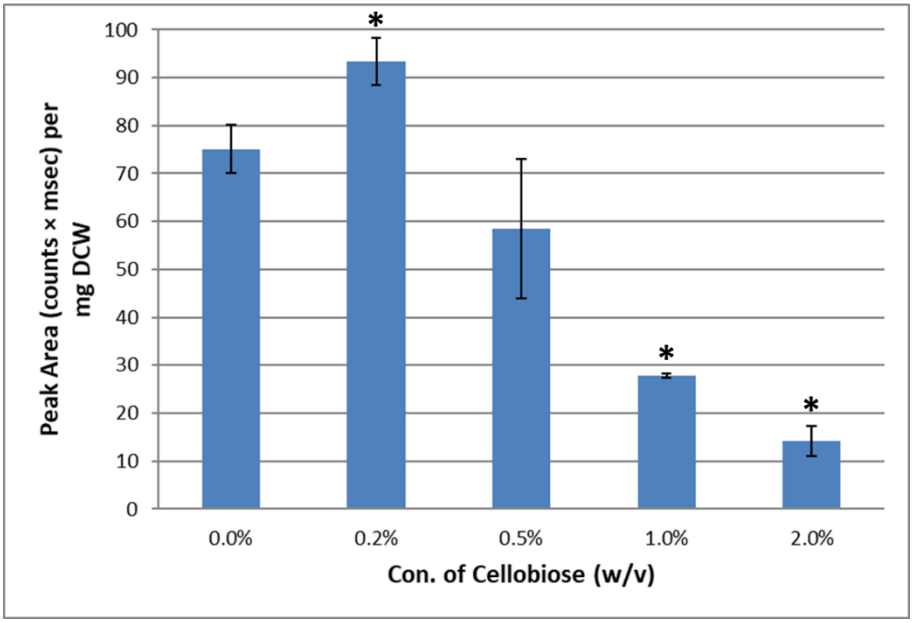


Figure 4.1. Effect of suberin and/or cellobiose on CFA-L-Ile production in *S. scabies* Δ*txtA*/pRLDB51-1. The strain was cultured in untreated SFMB medium (1) and in SFMB containing 0.1% w/v suberin (2), 0.5% w/v cellobiose (3), and 0.1% w/v suberin + 0.5% w/v cellobiose (4), after which the culture supernatants were extracted and analyzed for CFA-L-Ile using HPLC. The resulting CFA-L-Ile peak areas were normalized using the corresponding dry cell weight (DCW) measurements. The bars represent the average normalized CFA-L-Ile peak area from triplicate cultures, and the error bars represent the standard deviation from the mean. Treatments that produced a statistically significant result compared to the untreated SFMB culture are indicated by * ($p \leqslant 0.05$).

(A)



(B)

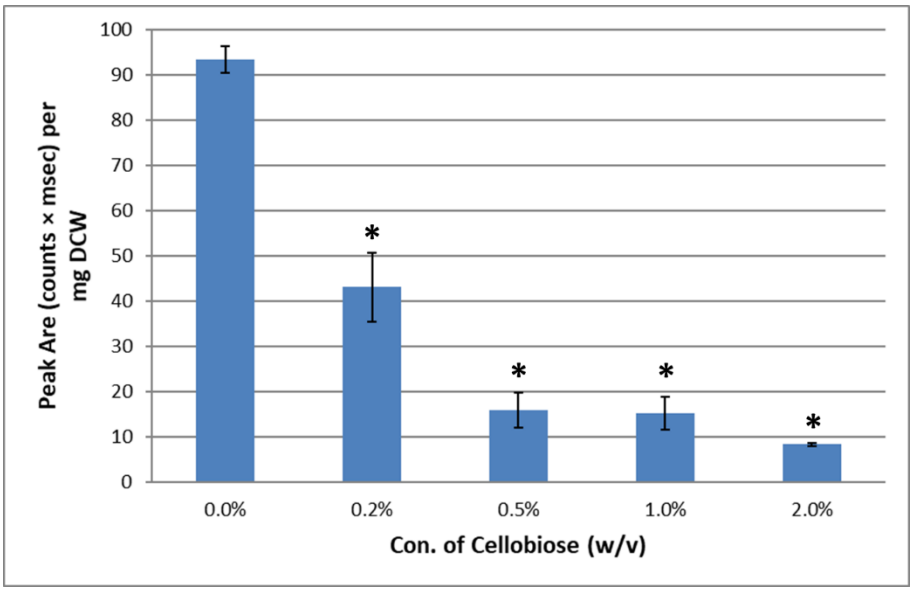
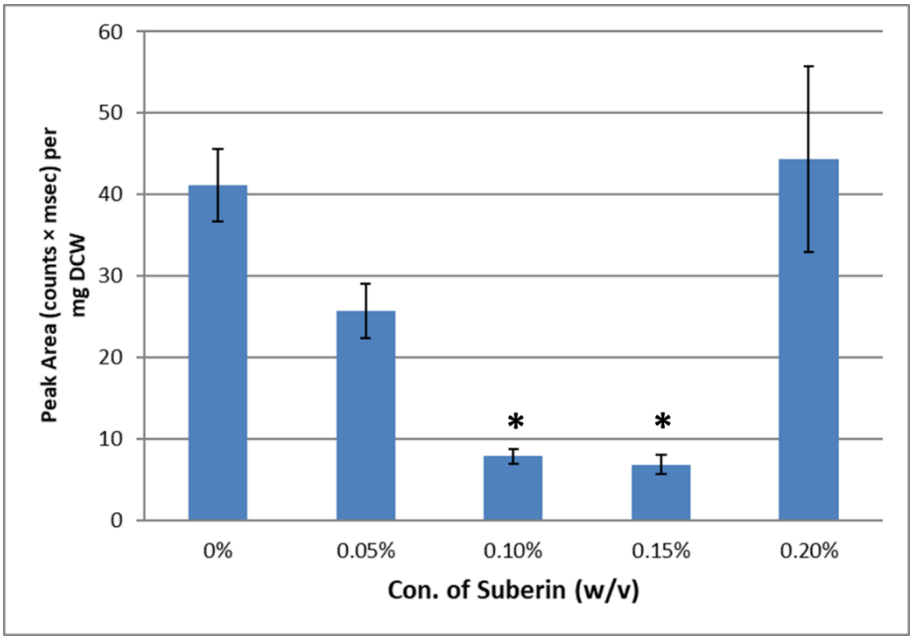


Figure 4.2. Concentration-dependent effect of cellobiose on CFA-L-Ile production in *S. scabies*. Strains 87-22/pRLDB51-1 (A) and Δ*txtA*/pRLDB51-1 (B) were cultured in SFMB containing different concentrations of cellobiose, after which the culture

supernatants were extracted and analyzed for CFA-L-Ile using HPLC. The resulting CFA-L-Ile peak areas were normalized using the corresponding dry cell weight (DCW) measurements. The bars represent the average normalized CFA-L-Ile peak area from triplicate cultures, and the error bars represent the standard deviation from the mean. Treatments that produced a statistically significant result compared to the untreated (0%) SFMB cultures are indicated by * ($p \leqslant 0.05$).

(A)


60
50
40
30
20
10
0
Peak Area (counts × msec) per mg DCW
*
*
0%
0.05%
0.10%
0.15%
0.20%
Con. of Suberin (w/v)

(B)

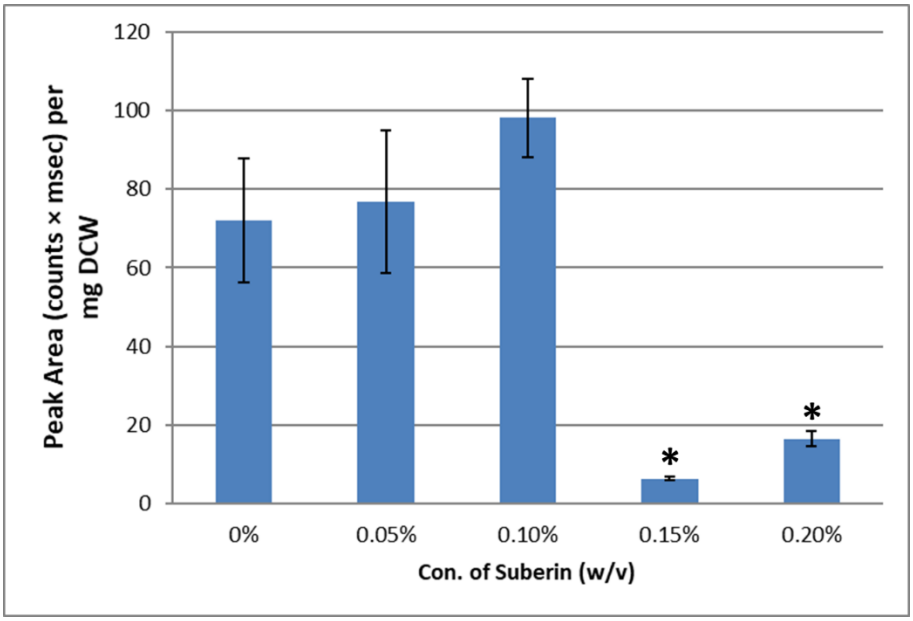

120
100
80
60
40
20
0
Peak Area (counts × msec) per mg DCW
*
*
0%
0.05%
0.10%
0.15%
0.20%
Con. of Suberin (w/v)

Figure 4.3. Concentration-dependent effect of suberin on CFA-L-Ile production in *S. scabies*. Strains 87-22/pRLDB51-1 (A) and Δ*txtA*/pRLDB51-1 (B) were cultured in SFMB medium containing different concentrations of suberin, after which the culture supernatants were extracted and analyzed for CFA-L-Ile using HPLC. The resulting CFA-L-Ile peak areas were normalized using the corresponding dry cell weight (DCW) measurements. The bars represent the average normalized CFA-L-Ile peak area from triplicate cultures, and the error bars represent the standard deviation from the mean. Treatments that produced a statistically significant result compared to the untreated (0%) SFMB cultures are indicated by ** ($p \leqslant 0.05$).

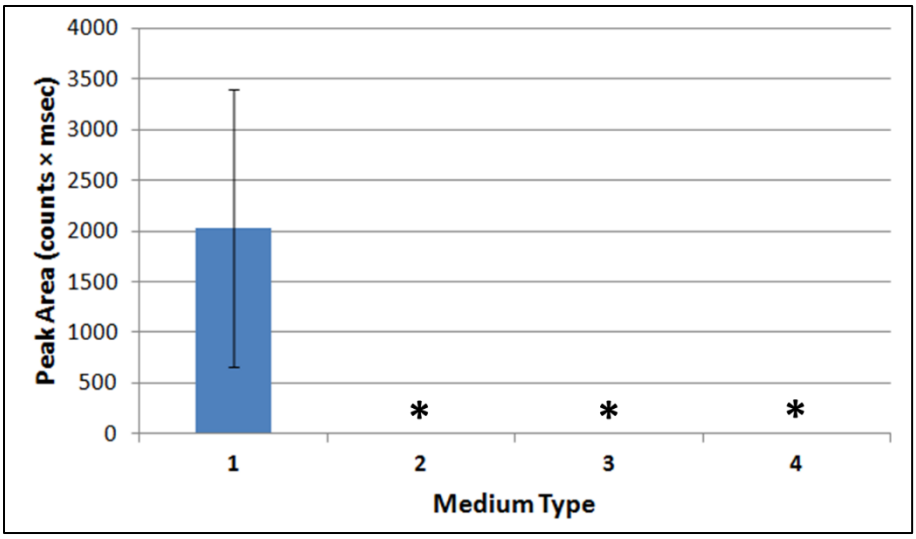
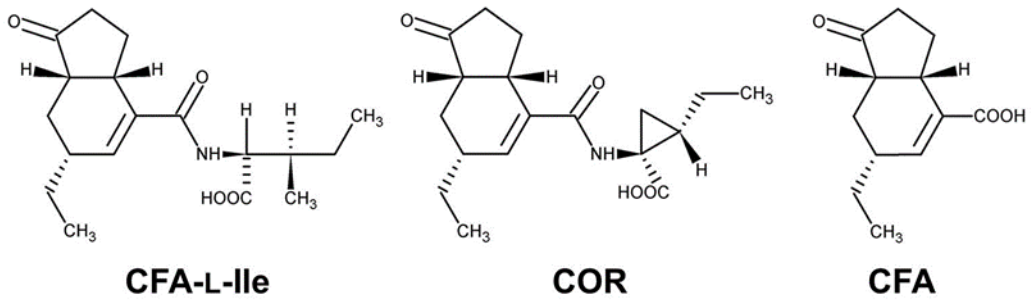
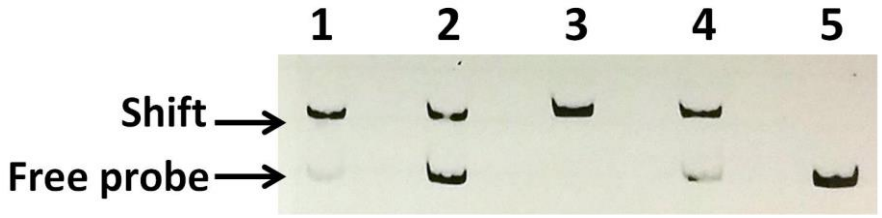


Figure 4.4. Effect of potato peel/juice on CFA-L-Ile production in *S. scabies* Δ*txtA*/pRLDB51-1. The strain was cultured in untreated SFMB medium (1) and in SFMB with added potato peel (2), potato juice (3) and potato peel + juice (4), after which the culture supernatants were extracted and analyzed for CFA-L-Ile using HPLC. The bars represent the average CFA-L-Ile peak area from triplicate cultures, and the error bars represent the standard deviation from the mean.

(A)



(B)



(C)

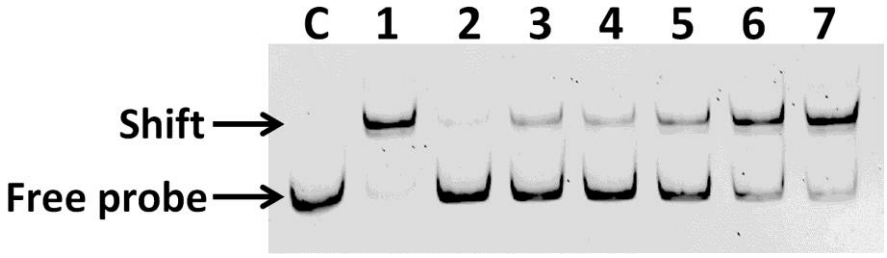


Figure 4.5. Analysis of CfaR-HIS$_6$ DNA binding activity in the presence of CFA-L-Ile and related metabolites. (A) Structure of CFA, CFA-L-Ile and COR. (B) CfaR-HIS$_6$ was incubated with the DNA probe in presence of DMSO (solvent control, lane 1), CFA-L-Ile

(lane 2), COR (lane 3), and CFA (lane 4), after which the binding reactions were subjected to electrophoresis on a 6% w/v PAGE. A reaction lacking the CfaR-HIS_6 protein (lane 5) was included as a control. The DNA bands were visualized under UV light following staining with EtBr. (C) Binding reactions were performed in the presence of different concentrations of CFA-L-Ile. The amounts CFA-L-Ile used (in nmol) are as follows: 0 (lane 1), 12.36 (lane 2); 6.18 (lane 3), 3.09 (lane 4), 1.545 (lane 5), 0.7725 (lane 6); 0.38625 (lane 7). The control (C) reaction lacked added CfaR-HIS_6 protein.

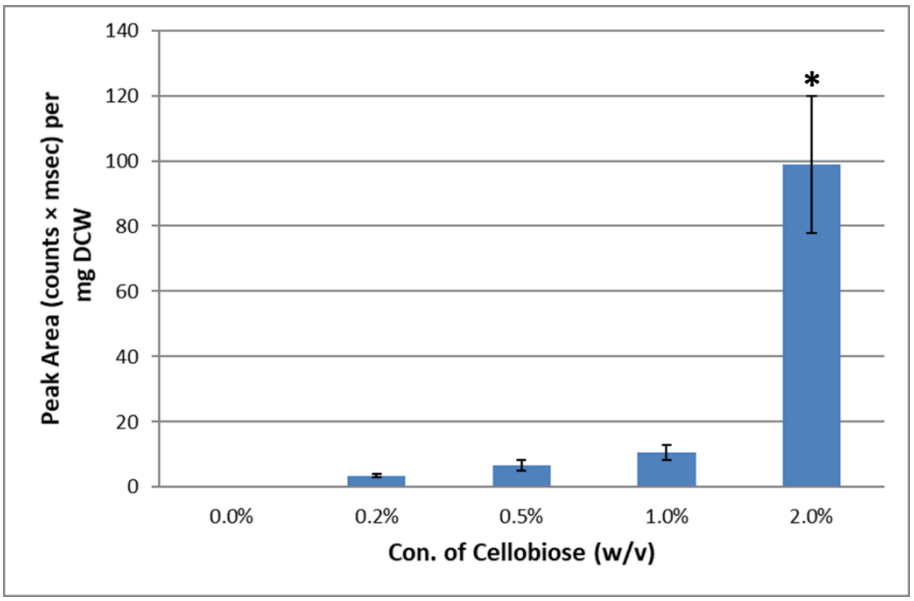
Table 4.1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Resistance†	Reference or source
***Streptomyces scabies* strains**			
87-22	Wild-type strain	n/a	(Loria et al., 1995)
Δ*txtA*	*S. scabies* 87-22 containing a deletion of the *txtA* thaxtomin biosynthetic gene	$Apra^R$	(Johnson et al., 2009)
***Escherichia coli* strains**			
DH5α	General cloning host	n/a	Gibco-BRL
NEB 5-α	DH5α derivative, high efficiency competent cells	n/a	New England Biolabs
BL21(DE3)	Protein expression strain	n/a	New England Biolabs
Plasmids			
pRLDB51-1	*scab79591* (*cfaR*) overexpression plasmid derived from pRLDB50-1a	$Apra^R$, $Thio^R$	(Bignell et al., 2010)
pET30b/CfaR	pET-30b derivative carrying a DNA fragment for expression of the full length CfaR–HIS_6 protein	Kan^R	(Cheng et al., 2015)
Cosmid			
Cosmid 158	SuperCos1 derivative containing the *S. scabies* 87-22 coronafacoyl phytotoxin biosynthetic gene cluster	Amp^R, Kan^R	(Bignell et al., 2010)

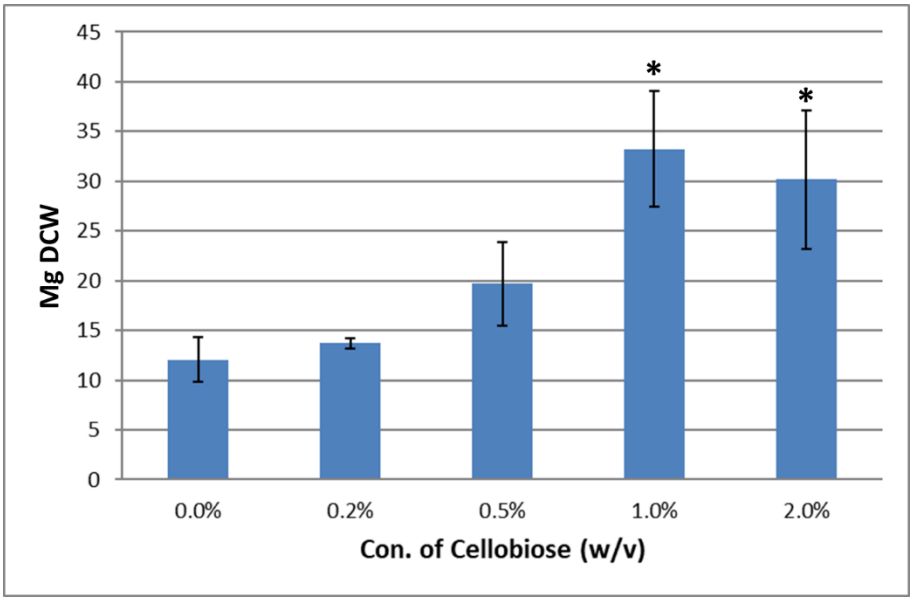
† $Apra^R$, $Thio^R$, Amp^R and Kan^R = apramycin, thiostrepton, ampicillin and kanamycin resistance, respectively. n/a = not applicable.

4.8 Supplementary Materials

(A)



(B)



(C)

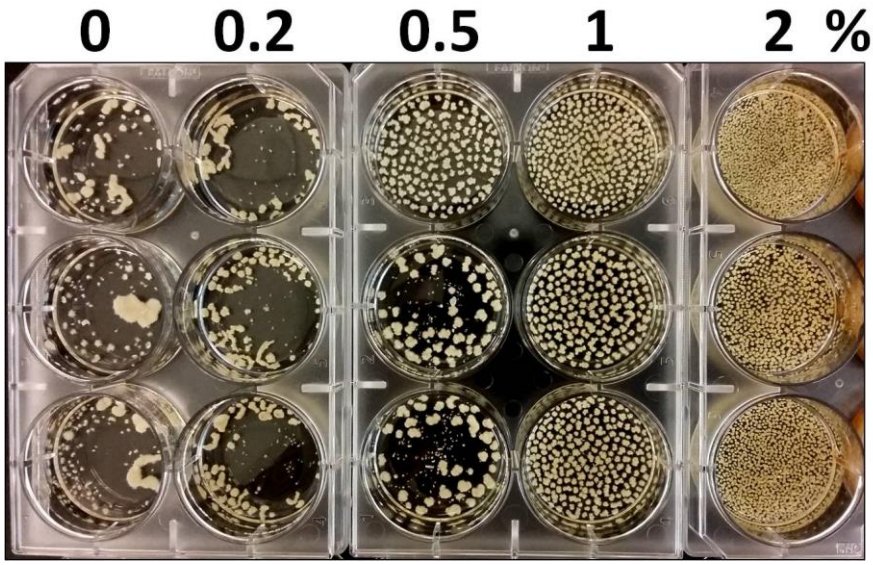


Figure S4.1: Induction of thaxtomin A production by cellobiose in *S. scabies* 87-22. The strain was cultured in MSM medium containing different concentrations of cellobiose, after which the culture supernatants were extracted and analyzed for thaxtomin A using HPLC. (A) Relative thaxtomin A production levels. The bars represent the average normalized thaxtomin A peak areas from triplicate cultures for each treatment, and the error bars represent the standard deviation from the mean. (B) Dry cell weight (DCW) measurements. The bars represent the average DCW from triplicate cultures for each treatment, and the error bars represent the standard deviation from the mean. (C) Culture growth at different concentrations of cellobiose. Treatments that produced a statistically significant result compared to the untreated (0% cellobiose) MSM cultures are indicated by * ($p \leqslant 0.05$).

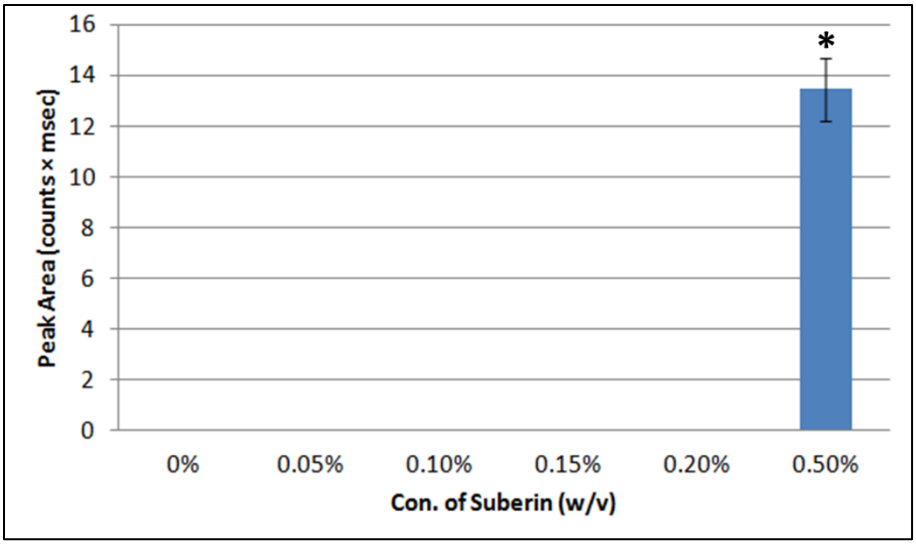


Figure S4.2. Induction of thaxtomin A production by suberin in *S. scabies* 87-22. The strain was cultured in MSM containing different concentrations of suberin, after which the culture supernatants were extracted and analyzed for thaxtomin A using HPLC. The bars represent the average thaxtomin A peak area from triplicate cultures, and the error bars represent the standard deviation from the mean.

Table S4.1. CebR site distribution in *S. scabies* 87-22 genome ϕ

CebR site	Location in genome (bp)	Inside gene		Upstream gene		Downstream gene	
		Function	Location in genome (bp)	Function	Location in genome (bp)	Function	Location in genome (bp)
1	364186 - 364195	SCAB_3301 putative oxidoreductase	362554-364266				
2	371655 - 371664	SCAB_3321 putative non-ribosomal peptide synthetase	364765-381309				
3	454473 - 454482	SCAB_3931 putative oxidoreductase	454077-455303				
4	458285 - 458294	SCAB_3971 putative MerR-family transcriptional regulator	456951-458573				
5	536852 - 536861	SCAB_4741 putative AraC-family transcriptional regulator	536034-536990				
6	564797 - 564806			SCAB_4961 putative secreted protein	563438-564742	SCAB_4971 conserved hypothetical protein	564953-565312
7	670496 - 670505			SCAB_5971 conserved hypothetical protein	669388-669951	SCAB_5981 putative secreted cellulase	670576-671718

						B precursor	
8	677470 - 677479	SCAB_6011 putative glycosyl hydrolase/ xylanase	673885-675531				
9	1004110 - 1004119			SCAB_8871 secreted endoglucanase	1001788-1004031	hypothetical protein	1004959-1007373
10	1308963 - 1308972	SCAB_11651 putative formamidopyrimidine DNA glycosylase	1308762-1309631				
11	1741764 - 1741773	SCAB_15631 putative MarR family transcriptional regulator	1741512-1742048				
12	1750086 - 1750095			SCAB_15701 1748640-1749374 putative TetR-family transcriptional regulator	1748640-1749374	SCAB_15711 putative secreted glucosidase	1750195-1751532
13	1845966 - 1845975			SCAB_16421 putative LacI-family transcriptional regulator	1844758-1845729	SCAB_16431 putative possible cellulase CELA1	1846077-1847042
14	1917856 - 1917865			SCAB_17001 putative secreted cellulase	1915844-1917556	SCAB_17011 putative secreted cellulase	1917984-1920851

15	2393851 - 2393860			SCAB_21081 putative secreted cellulose/chitin binding protein	2392612-2393685	SCAB_21091 conserved hypothetical protein	2393944-2394543
16	2401900 - 2401909	SCAB_21151 hypothetical protein	2401879-2402190				
17	2466837 - 2466846	SCAB_21571 putative transporter	2465799-2467331				
18	3144818 - 3144827	SCAB_27611 putative phage tail sheath proteins	3144285-3145862				
19	3385410 - 3385419	SCAB_29731 putative ATP-dependent protease	3383850-3386252				
20	3603291 - 3603300	SCAB_31781 txtB	3600343-3604809				
21	3610121 - 3610130			SCAB_31791 txtA	3604857-3609233	SCAB_31801 txtR	3610905-3611753
22	3912842 - 3912851			SCAB_34651 putative oxidoreductase	3911203-3912630	SCAB_34671 conserved hypothetical protein	3912912-3913304
23	3925421 - 3925430			SCAB_34791 putative sigma factor	3924409-3925164	SCAB_34801 two component system response regulator	3925472-3926149
24	4304098 - 4304107	SCAB_38281 putative long-chain-	4304062-4306020				

		fatty acid CoA ligase					
25	4342713 - 4342722			SCAB_38711 glutamate-1-semialdehyde 2,1-aminomutase	4341088-4342407	SCAB_38712 hypothetical protein	4342898-4343083
26	4376534 - 4376543	SCAB_39011 conserved hypothetical protein	4375187-4376695				
27	4582777 - 4582786			SCAB_40681 conserved hypothetical protein	4582003-4582422	SCAB_40691 conserved hypothetical protein	4582917-4588454
28	4666358 - 4666367			SCAB_41381 putative anion-transporting ATPase	4664570-4666009	SCAB_41391 putative regulatory protein	4666530-4666868
29	5102512 - 5102521	SCAB_45471 conserved hypothetical protein	5102413-5103255				
30	5264688 - 5264697	SCAB_46921 hypothetical protein	5264662-5264844				
31	5576719 - 5576728	SCAB_50031 putative phosphate transport regulator	5576308-5576997				
32	5681278 - 5681287			SCAB_51071 putative membrane protein (fragment)	5680911-5681150	SCAB_51081 putative secreted cellulase	5681330-5682880
33	6050782 - 6050791	SCAB_54441 enolase	6050006-6051286				

34	6210396 - 6210405	SCAB_55671 serine/threonine protein kinase	6210199-6211944				
35	6246345 - 6246354	SCAB_55981 putative extracellular sugar-binding protein	6246059-6247360				
36	6431114 - 6431123			SCAB_57751 putative secreted cellobiose-binding (transport system associated)	6429631-6430995	SCAB_57761 putative cellobiose transport regulator	6431608-6432666
37	6436236 - 6436245	SCAB_57831 A-factor-responsive transcriptional activator	6435842-6437065				
38	6557460 - 6557469	SCAB_58941 putative X – Pro dipeptidase/ABC transporter	6557427-6560084				
39	6896576 - 6896585			SCAB_62251 putative integral membrane transport protein	6894480-6896309	SCAB_62271 putative oxidoreductase	6896600-6897532
40	7170158 - 7170167	SCAB_64801 conserved hypothetical protein	7169561-7170280				
41	7474954 - 7474963	SCAB_67511	7474676-7475473				

		conserved hypothetical protein					
42	7906413 - 7906422	SCAB_71471 putative two-component system sensor kinase	7905868-7907013				
43	7973555 - 7973564	SCAB_72101 conserved hypothetical protein	7973136-7973969				
44	7993924 - 7993933	SCAB_72271 putative membrane protein	7993261-7994343				
45	8068704 - 8068713	SCAB_72981 putative secreted metallopeptidase	8068305-8069630				
46	8228496 - 8228505	SCAB_74401 putative binding-protein-dependent transport system ATP-binding subunit	8228134-8229168				
47	8316348 - 8316357	SCAB_75221 carbamoyl-phosphate synthase, pyrimidine-specific, small chain	8315587-8316729				
48	8585347 - 8585356			SCAB_77631	8584620-8585333	SCAB_77641	8585369-8586151

				conserved hypothetical protein		putative integral membrane protein	
49	8640581 - 8640590	SCAB_78171 conserved hypothetical protein	8640378-8640752				
50	8709271 - 8709280			SCAB_78871 putative DNA-binding protein	8708444-8708905	SCAB_78881 putative secreted glycosylhydrolase	8709354-8711024
51	8730762 - 8730771			SCAB_79011 putative secreted protein	8729576-8730712	SCAB_79031 conserved hypothetical protein	8730935-8732113
52	8973332 - 8973341	SCAB_80881 conserved hypothetical protein	8969180-8974300				
53	9057544 - 9057553	SCAB_81681 conserved hypothetical protein	9057317-9057742				
54	9101914 - 9101923	SCAB_82021 putative secreted glycosylhydrolase	9100429-9102294				
55	9103510 - 9103519			SCAB_82031 putative secreted endo-1,4-beta-xylanase	9102354-9103430	SCAB_82041 putative secreted pectate lyase	9103775-9104983
56	9353574 - 9353583	SCAB_83911 putative type I polyketide synthase	9346963-9358722				
57	9482722 - 9482731	SCAB_84861	9480583-9482997				

		putative secreted amidase					
58	9599416 - 9599425			SCAB_85831 conserved hypothetical protein	9597722-9599143	SCAB_85841 putative DNA-binding protein	9599448-9600269
59	10005451 - 10005460			SCAB_89741 putative secreted cellulose-binding protein	10004741 - 10005346	SCAB_89751 conserved hypothetical protein	10005882 - 10006544
60	10043269 - 10043278			SCAB_90061 putative cellulase	10042183 - 10043079	SCAB_90071 putative lacI-family transcriptional regulator	10043323 - 10044360
61	10045768 - 10045777			SCAB_90081 putative secreted cellulase B precursor	10044583 - 10045719	SCAB_90091 putative secreted cellulase	10045998 - 10048874
62	10048968 - 10048977			SCAB_90091 putative secreted cellulase	10045998 - 10048874	SCAB_90101 putative secreted cellulase	10049727 - 10051466
63	10049537 - 10049546			SCAB_90091 putative secreted cellulase	10045998 - 10048874	SCAB_90101 putative secreted cellulase	10049727 - 10051466
64	10096741 - 10096750	SCAB_90571 putative oxidoreductase	10096070 - 10097200				

Φ *S. scabies* 87-22 complete genome (accession number FN554889.1) was used.

For CebR sites within genes, the genes were listed under “inside gene”; for those outside of genes, the genes on both sides are listed under “upstream gene” and “downstream gene”, in accordance with the numbering of the genome.

Chapter 5: Summary and Future Directions

5.1 Summary

Together, the work presented in this thesis provides greater insights into the regulatory mechanisms controlling coronafacoyl phytotoxin biosynthesis in the common scab (CS) pathogen *Streptomyces scabies.* Prior to beginning this work, it was known that expression of the coronafacoyl phytotoxin biosynthetic genes was under control of the CfaR CSR, which functions as a positive activator (Bignell et al., 2010), and that some of the *bld* gene global regulators that are conserved in other *Streptomyces* spp. also contribute to the regulation of phytotoxin production (Bignell et al., 2010; Bignell et al., 2014). Our work reveals new information on how CfaR activates CFA-L-Ile production and how this activity may be regulated in *S. scabies,* and it uncovers for the first time the role of the *orf1* gene that is located downstream of *cfaR* in the coronafacoyl phytotoxin biosynthetic gene cluster. In addition, our work addresses the role of host-associated molecules in facilitating coronafacoyl phytotoxin production by *S. scabies.*

Previous bioinformatics analysis showed that CfaR belongs to the PAS-LuxR family of transcriptional regulators that control the production of specialized metabolites in various *Streptomyces* spp. (Bignell et al., 2010). Phylogenetic analysis of the CfaR revealed that both the PAS and LuxR domains form a distinct clade among the corresponding domains from other PAS-LuxR family proteins, indicating that CfaR is unique and the PAS and LuxR domains of CfaR are unique in this family as well. Also, CfaR is not closely related to other PAS-LuxR family members, which have been

previously reported as functionally interchangeable, suggesting that the function of CfaR may be distinct from other family members. We revealed that CfaR binds to a single site in the promoter region that drives expression of the coronafacoyl phytotoxin biosynthetic operon. The binding site is located immediately upstream of a predicted -35 hexanucleotide box within the promoter region, and it resembles the binding sites of the well characterized PAS-LuxR family member PimM. Notably, there were no other CfaR binding sites identified within the coronafacoyl phytotoxin biosynthetic gene cluster, suggesting that CfaR controls gene expression and phytotoxin production exclusively through interaction with a single binding site. Furthermore, we were unable to demonstrate binding of CfaR to its own promoter, suggesting that it may not regulate its own transcription. We established that the ability of CfaR to bind DNA and therefore activate transcription requires the PAS domain in addition to the LuxR DNA binding domain, and that the PAS domain is involved in protein dimerization. Importantly, our work is the first to demonstrate a role for the PAS domain in a member of the PAS-LuxR family of transcriptional regulators. Finally, we showed that the DNA binding activity of CfaR is inhibited *in vitro* by CFA-L-Ile but not by the CFA biosynthetic intermediate or the related coronafacoyl phytotoxin COR. Our results suggest that production of CFA-L-Ile is subjected to negative feedback control of CfaR in *S. scabies*, and we are the first to demonstrate such a regulatory strategy for a PAS-LuxR family protein.

Previous work had shown that *cfaR* is co-expressed with a downstream gene called *orf1*, the function of which was unknown (Bignell et al., 2010). Our work established that *orf1* is a second regulatory gene controlling CFA-L-Ile production in *S. scabies* as

overexpression of both *cfaR* and *orf1* resulted in significantly higher CFA-L-Ile production levels compared to when *cfaR* was overexpressed alone, and this was correlated with an increase in expression of the phytotoxin biosynthetic genes. Furthermore, deletion of *orf1* resulted in a significant reduction in CFA-L-Ile production, though production could still occur. Notably, deletion of *cfaR* abolished phytotoxin production, and overexpression of *cfaR* in the *orf1* deletion mutant was able to compensate for the loss of ORF1. Collectively, our work indicates that CfaR is the primary regulator of coronafacoyl phytotoxin production in *S. scabies*, while ORF1 likely functions as a "helper" protein that assists CfaR in activating phytotoxin production. ORF1 is predicted to belong to the ThiF protein family (Lehmann & Ealick, 2006; Taylor et al., 1998; Vander Horn et al., 1993), members of which function as AMPylators that catalyzes the AMPylation of a target protein or molecule (Itzen et al., 2011). Although we were unable to demonstrate AMPylation or other modifications of CfaR by ORF1 in *S. scabies*, it is possible that another protein or molecule is the target of ORF1. Given that *cfaR-orf1* homologue pairs are found in other *Streptomyces* spp. and in a *Kitasatospora* spp., it is likely that the mechanism of gene regulation by these CSRs is conserved in these other *Actinobacteria.*

Our study additionally established for the first time that CFA-L-Ile functions as a *bona fide* virulence factor during interaction of *S. scabies* with potato tuber tissue. Previous work showed that a *S. scabies* mutant unable to produce CFA-L-Ile was reduced in virulence in a tobacco seedling bioassay; however, a reduction in virulence was not observed during infection of potato tuber tissue (Bignell et al., 2010). Subsequent studies revealed that wild-type *S. scabies* 87-22 produces very little CFA-L-Ile under laboratory

conditions (Fyans et al., 2015), and this may also occur during infection of plant tissues, though in the case of tobacco seedlings the levels may be higher during infection, or the seedlings may be more susceptible to low levels of the phytotoxin. Work presented here demonstrated that *S. scabies* strains with similar thaxtomin A production levels but which varied in CFA-L-Ile production levels exhibited differences in their ability to cause necrosis and pitting of potato tuber tissue, with higher CFA-L-Ile production levels resulting in greater tissue necrosis and pitting. Our results are consistent with other studies that have shown that coronafacoyl phytotoxins are not essential for pathogenicity but instead enhance the virulence phenotype of the plant pathogens that produce these metabolites (Bignell et al., 2018). Whether CFA-L-Ile production contributes directly to the severity of CS disease symptoms caused by *S. scabies* remains to be determined; however, our results suggest that the variability in the types and severity of CS lesions observed on potatoes grown in agricultural fields might be due in part to differences in CFA-L-Ile production levels among different isolates of *S. scabies* in the environment.

Finally, the work presented in this thesis revealed new insights into the role of plant-associated molecules in mediating coronafacoyl phytotoxin production in *S. scabies.* Production of the primary pathogenicity determinant thaxtomin A is induced by cello-oligosaccharides and by potato suberin (Francis et al., 2015; Johnson et al., 2007; Jourdan et al., 2016; Lerat et al., 2010), and we hypothesized that these molecules might also control the production of other virulence factors in *S. scabies.* However, our results indicated that these molecules do not stimulate CFA-L-Ile production, and instead may inhibit production of this phytotoxin. This may indicate that *S. scabies* coordinates the production of its

virulence factors in response to different signals so that each is produced at specific stages of the infection process when they are most required. Overall, our work suggests that coronafacoyl phytotoxin production is under strict control in *S. scabies*, and more work will be needed to elucidate the signals stimulating production in this organism.

5.2 Future Directions

Several questions remain regarding the regulatory mechanisms controlling coronafacoyl phytotoxin production in *S. scabies.* ORF1 is predicted to be an AMPylator, the target of which is unknown. CfaR is a potential target of ORF1; however, we were unable to find ORF1-related modification of CfaR *in vivo*. Successful expression and purification of CfaR and ORF1 in *E. coli* would provide an opportunity to study the potential interaction between these two proteins *in vitro*. Proteomic techniques could also be employed to explore other candidate targets of ORF1. The role of the predicted ThiF and nitroreductase domains in the ORF1 protein could be further studied by constructing deletion mutants in which one or both of the domains is removed. In addition, structural analysis of ORF1 may assist in further elucidating the function of this protein.

Current culturing conditions in the lab do not induce the production of high levels of CFA-L-Ile by wild-type *S. scabies* 87-22. Infection and CS disease development by *S. scabies* is limited to developing potato tubers and is dependent on the potato cultivar (Khatri et al., 2011; Sedláková et al., 2013), possibly due to the specialized compounds produced by potatoes (Valcarcel et al., 2016). Therefore, the effects of known potato - associated compounds and of potato samples from different cultivars and developmental

stages on CFA-L-Ile production should be further explored in order to identify plant-associated inducers of phytotoxin production. As it is known that several *bld* gene global regulators control the expression of the CFA-L-Ile biosynthetic gene cluster in *S. scabies* (Bignell et al., 2014), it is possible that other pleiotropic regulators of specialized metabolite production may also play a role in modulating phytotoxin production, and thus further studies on this could be performed. Genes involved in GBL biosynthesis are present within the genome of *S. scabies*, and therefore the potential role of GBLs in inducing the production CFA-L-Ile is another area worth investigating further. Finally, it was revealed in this study that CFA-L-Ile inhibited the DNA binding activity of CfaR. Further exploration should be performed to confirm the role of CFA-L-Ile as a ligand of CfaR and to further elucidate the mechanism of protein-ligand interactions.

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REVIEW ARTICLE

Phytotoxins produced by plant pathogenic *Streptomyces* species

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Summary

Streptomyces is a large genus consisting of soil-dwelling, filamentous bacteria that are best known for their capability of producing a vast array of medically and agriculturally useful secondary metabolites. In addition, a small number of *Streptomyces* spp. are capable of colonizing and infecting the underground portions of living plants and causing economically important crop diseases such as potato common scab (CS). Research into the mechanisms of *Streptomyces* plant pathogenicity has led to the identification and characterization of several phytotoxic secondary metabolites that are known or suspected of contributing to diseases in various plants. The best characterized are the thaxtomin phytotoxins, which play a critical role in the development of CS, acid scab and soil rot of sweet potato. In addition, the best-characterized CS-causing pathogen, *Streptomyces scabies*, produces a molecule that is predicted to resemble the *Pseudomonas syringae* coronatine phytotoxin and which contributes to seedling disease symptom development. Other *Streptomyces* phytotoxic secondary metabolites that have been identified include concanamycins, FD-891 and borrelidin. Furthermore, there is evidence that additional, unknown metabolites may participate in *Streptomyces* plant pathogenicity. Such revelations have implications for the rational development of better management procedures for controlling CS and other *Streptomyces* plant diseases.

Introduction

Organisms belonging to the genus *Streptomyces* are well known for their filamentous morphology, their large genomes and their complex developmental life cycle that involves the production of desiccation-resistant spores. The vast majority of *Streptomyces* spp. are soil-dwelling saprophytes that degrade recalcitrant biological polymers and contribute to the recycling of nutrients in the environment. Furthermore, these organisms are renowned for their ability to synthesize a wide array of medically and agriculturally useful secondary metabolites such as antibiotics, immunosuppressants, anti-tumour agents, insecticides and pesticides (Berdy 2005). Such compounds may provide a selective advantage to the producing organism by allowing it to compete with other micro-organisms for limited nutrients in the soil environment, and/or they may serve as facilitators of inter- and intra-generic communication (O'Brien and Wright 2011). In addition, some secondary metabolites are thought to promote symbiotic relationships between *Streptomyces* spp. and eukaryotic organisms (Seipke *et al.* 2012). An example of this is the involvement of secondary metabolites in parasitic relationships between plant pathogenic *Streptomyces* spp. and various plant hosts, a subject that is the focus of this review.

The ability to colonize living plant tissues and to cause plant diseases is a rare trait among the streptomycetes. Species that have this ability infect the underground portions of a wide variety of economically important crops, while above-ground parts of the plant will generally remain healthy unless nutrient and water transport between the roots and the shoots is hindered by the infection (Dees and Wanner 2012). The most important

host that is affected by plant pathogenic streptomycetes is potato (*Solanum tuberosum*), and as such most of the research to date has focused on the diseases affecting this crop. However, those species causing scab disease of potato are neither tissue—nor host—specific and can infect potato as well as tap root crops such as carrot, beet, radish and parsnip under field conditions (Dees and Wanner 2012). Furthermore, such species can infect the seedlings of a variety of monocot and dicot plants under controlled conditions, leading to root and shoot stunting, cell hypertrophy and tissue necrosis (Leiner *et al.* 1996).

Potato common scab (CS) is considered the most important disease caused by *Streptomyces* spp. and is characterized by the formation of superficial, raised or pitted lesions on the surface of potato tubers (Loria *et al.* 1997). Such lesions reduce the market value of the potato crop and result in significant economic losses to growers. Several *Streptomyces* spp. are responsible for the disease (Table 1), of which *S. scabies* (syn. *S. scabiei*) was the first to be described and is the best-characterized and most widely distributed species. In addition to CS, *S. scabies* is responsible for pod wart of peanut, which is characterized by raised necrotic lesions on the peanut pericarp (Loria *et al.* 1997). Another disease, called acid scab (AS), is caused by *Streptomyces acidiscabies* and results in the same symptoms as CS except that the disease occurs in acid soils where CS is normally suppressed (Loria *et al.* 2006). Netted scab (NS) is a potato disease that has been reported mainly in Europe and is characterized by the formation of brown, superficial lesions with a netted appearance on the tuber surface. Unlike CS and AS, NS also causes severe necrosis of the fibrous roots of the potato plant and results in significant yield losses (Loria *et al.* 1997). Russet scab (RS) is similar to NS in that the lesions on the potato are superficial and are limited to the tuber periderm. However, the lesions do not have the netted pattern that is characteristic of NS, and root necrosis and yield losses have not been reported with this disease (Loria *et al.* 1997). Soil rot of sweet potato is caused by *Streptomyces ipomoeae,* which infects the fibrous roots of sweet potato (*Ipomoea batatas* (L.) Lam.), leading to tissue necrosis and death, and subsequent yield losses. Furthermore, the pathogen induces necrotic lesions on the fleshy storage roots, resulting in reduced marketability (Loria *et al.* 1997).

This review focuses on the recent progress of research into the phytotoxic secondary metabolites that contribute to *Streptomyces*—plant interactions and to the development of plant diseases. Much of the discussion will focus on the thaxtomin phytotoxins, which play a critical role in the development of CS, AS and soil rot of sweet potato; however, recent research has suggested that additional phytotoxic secondary metabolites may also contribute to the development of these and other plant diseases in natural settings, and therefore such phytotoxins will also be addressed here.

Table 1 Pathogenic *Streptomyces* spp. and associated plant disease(s) and phytotoxin(s) produced

Species	Disease(s) caused*	Phytotoxin(s) produced	Reference(s)
S. scabies (*S. scabiei*)	CS, Pod wart of peanut	Thaxtomins, concanamycin A and B, COR-like metabolite(s)	King *et al.* (1989, 1992); Natsume *et al.* (1996, 1998, 2001); Bignell *et al.* (2010b)
Streptomyces turgidiscabies	CS	Thaxtomins	Bukhalid *et al.* (1998)
Streptomyces acidiscabies	AS	Thaxtomins	Bukhalid *et al.* (1998)
Streptomyces europaeiscabiei	CS, NS	Thaxtomins	Loria *et al.* (2006)
Streptomyces reticuliscabiei	NS	Unknown	Bouchek-Mechiche *et al.* (2000)
Streptomyces stelliscabiei	CS	Thaxtomins	Loria *et al.* (2006)
Streptomyces luridiscabiei	CS	Unknown	Park *et al.* (2003)
Streptomyces niveiscabiei	CS	Unknown	Park *et al.* (2003)
Streptomyces puniciscabiei	CS	Unknown	Park *et al.* (2003)
Streptomyces spp. IdahoX	CS	Thaxtomins	Wanner (2007)
Streptomyces spp. DS3024	CS	Thaxtomins	Hao *et al.* (2009)
Streptomyces spp. GK18	CS	Borrelidin	Cao *et al.* (2012)
Streptomyces cheloniumii	RS	FD-891	Natsume *et al.* (2005)
Streptomyces spp. MAFF225003	RS	FD-891	Natsume *et al.* (2005)
Streptomyces spp. MAFF225004	RS	FD-891	Natsume *et al.* (2005)
Streptomyces spp. MAFF225005	RS	FD-891	Natsume *et al.* (2005)
Streptomyces spp. MAFF225006	RS	FD-891	Natsume *et al.* (2005)
Streptomyces ipomoeae	Soil rot of sweet potato	Thaxtomins	King *et al.* (1994); Guan *et al.* (2012)

*CS, Common scab; AS, acid scab; NS, netted scab; RS, russet scab.

Thaxtomins

The first phytotoxic secondary metabolites associated with *Streptomyces* plant pathogenicity were reported in 1989 by King and colleagues (King *et al.* 1989), who described the isolation of two members of the thaxtomin family of phytotoxins associated with CS disease. Thaxtomins are cyclic dipeptides (2,5-diketopiperazines) derived from the condensation of L-phenylalanine and L-4-nitrotryptophan moieties (reviewed in King and Calhoun 2009). Eleven members of the thaxtomin family have been identified and characterized, with each member differing only in the presence or absence of hydroxyl and *N*-methyl groups at specific sites (King and Calhoun 2009). The 4-nitro moiety, together with the L,L configuration of the tryptophan and phenylalanine groups, have been shown to be essential for the phytotoxic activity of these compounds (King *et al.* 1989, 1992). Thaxtomin A (Fig. 1a) is the primary family member produced by *S. scabies, S. acidiscabies* and *Streptomyces turgidiscabies,* although other family members have been shown to be produced in minor amounts (King and Calhoun 2009). Thaxtomin C (Fig. 1a), which is a less modified, nonhydroxylated family member, is the major product synthesized by *S. ipomoeae* (King *et al.* 1994; Guan *et al.* 2012).

Biological activity of thaxtomins

Thaxtomins have the ability to cause necrosis on excised potato tuber tissue (Loria *et al.* 2006), and they can induce scab-like lesions on aseptically cultured minitubers (Lawrence *et al.* 1990). In addition, nanomolar concentrations of thaxtomin A cause root and shoot stunting and radial swelling of monocot and dicot seedlings, effects that mimic the seedling disease symptoms caused by *S. scabies* and *S. acidiscabies* (Leiner *et al.* 1996; Loria *et al.* 1997). A positive correlation has been observed between the ability to produce thaxtomin A and the pathogenicity of scab-causing *Streptomyces* spp. (King *et al.* 1991; Loria *et al.* 1995; Goyer *et al.* 1998; Kinkel *et al.* 1998), and a constructed thaxtomin mutant of *S. acidiscabies* could not induce typical scab lesions on potato minitubers (Healy *et al.* 2000). Recently, it was shown that *S. ipomoeae* thaxtomin C mutants are unable to penetrate the intact adventitious roots of sweet potato plants (Guan *et al.* 2012). Thus, the thaxtomin phytotoxins are an essential

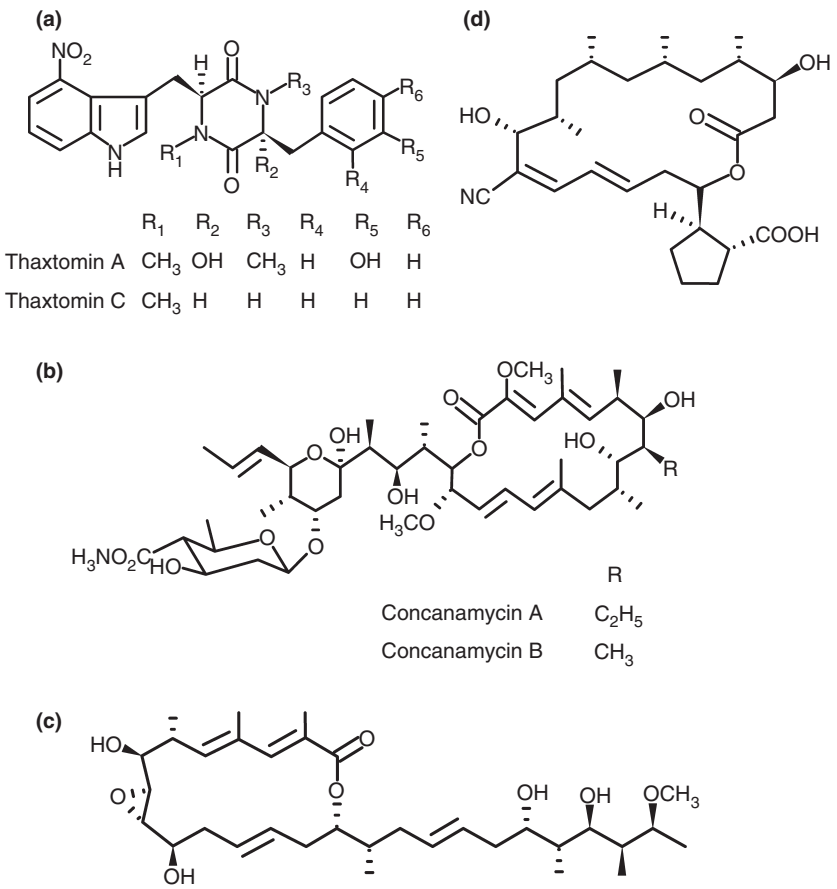

Figure 1 Molecular structure of the thaxtomin A and C (a), concanamycin A and B (b), FD-891 (c) and borrelidin (d) phytotoxins that are produced by plant pathogenic *Streptomyces* spp.

virulence factor in several plant pathogenic *Streptomyces* spp.

A number of physiological effects in plants have been reported to occur in response to thaxtomins, including alterations in plant Ca^{2+} and H^+ ion influx, induction of programmed cell death, and production of the antimicrobial plant phytoalexin scopoletin (Duval *et al.* 2005; Tegg *et al.* 2005; Errakhi *et al.* 2008; Lerat *et al.* 2009). Fry and Loria noted that nanomolar concentrations of thaxtomin A cause plant cell hypertrophy in onion seedling hypocotyls, radish seedling hypocotyls and tobacco suspension cultures (Fry and Loria 2002). It also interferes with cytokinesis in onion root tip cells, and it inhibits normal cell elongation of tobacco protoplasts (Fry and Loria 2002). This, in turn, led the authors to propose that thaxtomin A targets the plant cell wall. Further evidence for a cell wall target was provided by Scheible *et al.*, who demonstrated that thaxtomin A inhibits the incorporation of ^{14}C-glucose into the cellulosic fraction of the cell wall in *Arabidopsis thaliana* (Scheible *et al.* 2003). More recently, Bischoff *et al.* showed that thaxtomin A reduces the crystalline cellulose content of *A. thaliana* plant cell walls, and it affects the expression of cell wall synthesis genes in a similar manner as the known cellulose synthesis inhibitor isoxaben. Furthermore, spinning disc confocal microscopy revealed that thaxtomin A depletes cellulose synthase complexes from *A. thaliana* plasma membranes (Bischoff *et al.* 2009). Duval and Beaudoin used whole genome microarrays to show that thaxtomin A and isoxaben elicit a similar gene expression profile in *A. thaliana* cell suspensions (Duval and Beaudoin 2009). Taken together, the results suggest that the primary mode of action of thaxtomin A is the inhibition of cellulose biosynthesis.

Biosynthesis of the thaxtomin phytotoxins

As with other *Streptomyces* secondary metabolites, the biosynthetic genes for the thaxtomin phytotoxins (*txt*) are clustered together on the chromosome of *S. scabies, S. turgidiscabies, S. acidiscabies* and *S. ipomoeae* (Loria *et al.* 2008; Guan *et al.* 2012). The genes are arranged in at least two operons, with the first likely consisting of *txtA, txtB, txtH* and possibly *txtC,* and the second consisting of *nos*/*txtD* and *txtE,* the co-transcription of which has been confirmed (Barry *et al.* 2012). Analysis of the encoded protein products indicates a high degree of conservation among the four distantly related species, although it is apparent that the conservation is considerably higher among the scab-causing pathogens (Table 2). This, together with the localization of the *txt* gene cluster on a mobile pathogenicity island in *S. turgidiscabies* (Kers *et al.* 2005; Huguet-Tapia *et al.* 2011), suggests that horizontal gene transfer likely played a role in the acquisition of the *txt* gene cluster by *Streptomyces* spp.

The biosynthesis of the thaxtomin phytotoxins begins with the production of nitric oxide (NO) from arginine, a reaction that is catalysed by the TxtD nitric oxide synthase (Kers *et al.* 2004). NO is then used for the site-specific nitration of L-tryptophan by TxtE, which is a novel cytochrome P450 (Barry *et al.* 2012). Deletion analysis of the *txtE* gene in *S. turgidiscabies* confirmed that it is essential for thaxtomin A biosynthesis, while addition of L-4-nitrotryptophan to cultures of the Δ*txtE* strain restored thaxtomin A production (Barry *et al.* 2012). This, together with the fact that L-4-nitrotryptophan accumulates in cultures of the *S. scabies txtA* and *txtB* mutants (Johnson *et al.* 2009), indicates that the production of L-4-nitrotryptophan is the first committed step in the thaxtomin biosynthetic pathway. L-4-nitrotryptophan then serves as a substrate for the TxtB nonribosomal peptide synthetase (NRPS), while L-phenylalanine is the substrate for the TxtA NRPS (Johnson *et al.* 2009). In the case of thaxtomin A biosynthesis, the resulting cyclo-(L-4-nitrotryptophyl-L-phenylalanyl) intermediate (called thaxtomin D) is *N*-methylated on both the nitrotryptophyl and phenylalanyl moieties (Healy *et al.* 2002), and it is presumed that the methylation is

Table 2 Proteins encoded by the *S. scabies* thaxtomin biosynthetic gene cluster and their % identity/similarity to homologues in other plant pathogenic *Streptomyces* spp.

Txt Proteins from *Streptomyces scabies* 87-22	Function	% Identity/similarity to *Streptomyces turgidiscabies* Car8 Txt homologues	% Identity/similarity to *Streptomyces acidiscabies* 84.104 Txt homologues	% Identity/similarity to *Streptomyces ipomoeae* 91-03 Txt homologues
TxtA	Synthesis of thaxtomin backbone	90/93	100/100	50/60
TxtB	Synthesis of thaxtomin backbone	90/93	99/99	60/70
TxtC	Hydroxylation of thaxtomin backbone	90/93	100/100	Absent
TxtD	Nitration of L-tryptophan precursor	91/93	100/100	75/83
TxtE	Nitration of L-tryptophan precursor	88/94	100/100	85/91
TxtH	Unknown	81/85	100/100	63/69
TxtR	Regulation of thaxtomin biosynthesis	78/83	100/100	42/58

catalysed by the *S*-adenosylmethionine-dependent *N*-methyltransferase domain found in both TxtA and TxtB. It has previously been reported that *N*-methyl-L-4-nitrotryptophan can accumulate in the culture supernatants of wild-type *S. scabies* (King and Lawrence 1995) and of a *S. scabies* Δ*txtA* mutant (Johnson *et al.* 2009), which suggests that the *N*-methylation occurs prior to cyclic dipeptide formation. Interestingly, the *S. ipomoeae* TxtAB homologues are also predicted to each contain an *N*-methyltransferase domain, and yet thaxtomin C is only *N*-methylated on the nitrotryptophanyl moiety (Fig. 1a). The final step in thaxtomin A biosynthesis is the addition of hydroxyl groups to the phenylalanyl moiety of thaxtomin D by the TxtC P450 monooxygenase. Deletion analysis of *txtC* in *S. acidiscabies* led to the accumulation of thaxtomin D in the culture supernatant, confirming the role of TxtC in postcyclization hydroxylation (Healy *et al.* 2002). Notably, *txtC* is absent from the *S. ipomoeae txt* gene cluster, and no homologue appears to exist anywhere else in the *S. ipomoeae* genome (Bignell *et al.* 2010a; Guan *et al.* 2012), an observation that is consistent with the fact that this organism does not produce thaxtomin A (King *et al.* 1994).

An additional gene (*txtH*) that was recently identified in the thaxtomin biosynthetic gene cluster of *S. scabies* (Bignell *et al.* 2010a) is predicted to encode a member of the MbtH-like protein superfamily. MbtH-like proteins are small proteins (normally 62–80 amino acids) that are often associated with NRPS gene clusters (reviewed in Baltz 2011). Deletion studies have shown that some MbtH-like proteins are necessary for production of the corresponding metabolite, whereas in other instances, deletion of the MbtH-like protein-encoding gene does not have any effect. The latter is often due to the presence of other MbtH-like protein-encoding genes elsewhere in the genome that can cross complement the deleted gene with varying efficiencies. Recent biochemical studies have shown that some MbtH-like proteins can be co-purified with their cognate NRPS and that they function to facilitate the adenylation reaction catalysed by the NRPS adenylation domain (Baltz 2011 and references therein). The *S. scabies txtH* gene is conserved in the *txt* gene clusters of *S. turgidiscabies, S. acidiscabies* and *S. ipomoeae,* suggesting that it may be important for the biosynthesis of thaxtomins (Table 2). However, it is noteworthy that the genome sequences for all four pathogens contain multiple predicted MbtH-like protein-encoding genes, and therefore the possibility exists for cross-complementation to occur in each organism.

Regulation of thaxtomin biosynthesis

The production of thaxtomin A by scab-causing streptomycetes is affected by several physiological and environmental signals. For example, production does not take place in common microbiological growth media such as LB and tryptic soy broth (Loria *et al.* 1995), whereas it readily occurs in living host tissue or in plant-based media such as potato broth, oatmeal broth or oat bran broth (Babcock *et al.* 1993; Loria *et al.* 1995; King and Lawrence 1996; Goyer *et al.* 1998). Glucose appears to repress the biosynthesis of thaxtomin in liquid growth media (Babcock *et al.* 1993; Loria *et al.* 1995), a phenomenon that has been reported for other *Streptomyces* secondary metabolites (Ruiz *et al.* 2010). Aromatic amino acids such as tryptophan, tyrosine and phenylalanine have also been shown to inhibit phytotoxin biosynthesis, whereas aliphatic amino acids have no effect (Babcock *et al.* 1993; Lauzier *et al.* 2002).

Recent work has identified specific plant-based compounds that are capable of stimulating thaxtomin A biosynthesis. Wach *et al.* (2007) demonstrated that the addition of xylans, glucans and cellobiose to oat bran broth medium stimulates higher levels of thaxtomin A production in *S. acidiscabies* compared to the unamended control, while Johnson *et al.* (2007) showed that the cellobiose and cellotriose could stimulate *txt* gene expression and phytotoxin production in a defined minimal medium. Moreover, suberin, which is a complex plant polymer found on the surface of potato tubers, has been shown to stimulate phytotoxin production in a minimal medium (Beausejour *et al.* 1999), and more recently it was demonstrated that the addition of both suberin and cellobiose to a minimal medium stimulates much higher *txt* gene expression and thaxtomin A production then when cellobiose or suberin are added separately (Lerat *et al.* 2010).

Embedded within the *txt* gene clusters of *S. scabies, S. turgidiscabies* and *S. acidiscabies* is a gene (*txtR*) that encodes an AraC-family transcriptional regulator (Table 2; Joshi *et al.* 2007). Given that regulatory genes are often associated with secondary metabolite biosynthetic gene clusters and that they function to control the production of the corresponding metabolite (van Wezel and McDowall 2011), it was hypothesized that TxtR likely serves as a regulator of thaxtomin biosynthesis in these organisms. This was confirmed by constructing a *S. scabies* Δ*txtR* mutant and showing that it produced only trace levels of thaxtomin A, was reduced in expression of the thaxtomin biosynthetic genes, and was avirulent on tobacco and radish seedlings (Joshi *et al.* 2007; Loria *et al.* 2008). Interestingly, the expression of the *txtR* gene in *S. scabies* and *S. turgidiscabies* was shown to be dependent on cellobiose (Johnson *et al.* 2007; Joshi *et al.* 2007), and cellobiose was demonstrated to serve as a ligand for the *S. scabies* TxtR protein in a pull-down assay (Joshi *et al.* 2007). Given that thaxtomin A targets

cellulose biosynthesis and that cellobiose is the smallest subunit of cellulose, it has been proposed that cellobiose and possibly other cello-oligosaccharides may serve as a signal for the presence of active plant cell growth and tissue expansion where cellulose synthesis takes place, and that stimulation of thaxtomin A production by cellobiose may allow penetration of the expanding tissue by the pathogen (Loria *et al.* 2008). Whether suberin or breakdown products of suberin also serve as signals that are sensed by TxtR remains to be determined; however, as it was recently shown that suberin induces the onset of morphological differentiation and secondary metabolism in both pathogenic and nonpathogenic streptomycetes (Lerat *et al.* 2012), it is likely that the effect of suberin is not specific to the thaxtomin phytotoxins.

Recently, a *txtR* homologue was reported in the *txt* gene cluster of *S. ipomoeae* (Guan *et al.* 2012). The resulting protein product shows only weak similarity to the TxtR protein from *S. scabies* (Table 2), which might reflect differences in the regulation of thaxtomin production in the scab-causing pathogens and in *S. ipomoeae*. Specifically, thaxtomin C in *S. ipomoeae* is not produced in the same plant-based media that induce thaxtomin A production (King *et al.* 1994; Guan *et al.* 2012), which suggests that cello-oligosaccharides do not function as inducers of thaxtomin C production. The exact ligand(s) that interacts with the *S. ipomoeae* TxtR remains to be determined, but it is intriguing to speculate that the ligand(s) is a plant-derived molecule that is specific to the *Convolvulaceae* family, and that this might account for the observed narrow host range of *S. ipomoeae* as compared to the scab-causing pathogens (Guan *et al.* 2012).

Concanamycins

In addition to thaxtomins, *S. scabies* has been reported to produce two members of the concanamycin family of secondary metabolites (Table 1). Concanamycins are polyketide macrolides that were first isolated from the culture medium of *S. diastatochromogenes* (Kinashi *et al.* 1984). They are characterized by an 18-membered tetraenic macrolide ring with a methyl enol ether and a β-hydroxyhemiacetyl side chain (Fig. 1b), and they function as vacuolar-type ATPase inhibitors and exhibit antifungal and anti-neoplastic activity but not antibacterial activity (Kinashi *et al.* 1984; Seki-Asano *et al.* 1994). Natsume and colleagues were the first to report the isolation of *S. scabies* strains from Japan that produced concanamycin A and B, and rice seedling bioassays demonstrated that the pure compounds exhibit root growth inhibitory activity (Natsume *et al.* 1996, 1998). The genome sequence of *S. scabies* 87–22 contains a biosynthetic gene cluster that is highly similar to the concanamycin biosynthetic gene cluster from *Streptomyces neyagawaensis* (Haydock *et al.* 2005), suggesting that this strain of *S. scabies* also produces concanamycins. The contribution of concanamycins to CS disease needs further clarification given that other characterized CS pathogens do not appear to produce these compounds (Natsume *et al.* 1998, 2001, 2005).

COR-like metabolites

Genome sequencing of *S. scabies* strain 87–22 revealed the presence of a biosynthetic gene cluster that is highly similar to the coronafacic acid (CFA) biosynthetic gene cluster from the Gram-negative plant pathogens *Pseudomonas syringae* and *Pectobacterium atrosepticum* (Bignell *et al.* 2010b). CFA (Fig. 2a) is the polyketide component of coronatine (COR) (Fig. 2b), which is a nonhost specific phytotoxin produced by different pathovars of *Ps. syringae* (Gross and Loper 2009). The COR molecule consists of CFA linked via an amide bond to an

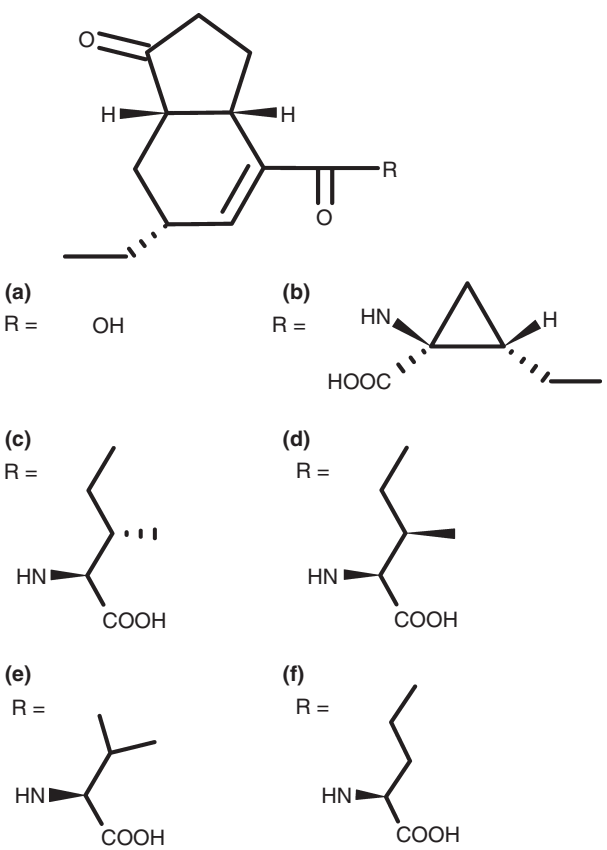

Figure 2 Molecular structure of coronafacic acid (CFA) (a), coronatine (COR) (b), CFA-isoleucine (c), CFA-*allo*-isoleucine (d), CFA-valine (e) and CFA-norvaline (f) produced by *Pseudomonas syringae*.

ethylcyclopropyl amino acid called coronamic acid (CMA), which is derived from L-*allo*-isoleucine (Gross and Loper 2009). Although COR is the primary metabolite produced by *Ps. syringae* and is the most toxic, other coronafacoyl compounds in which CFA is linked to various amino acids have been reported, including CFA-isoleucine, CFA-*allo*-isoleucine, CFA-valine and CFA-norvaline (Fig. 2c–f; Bender *et al.* 1999).

The CFA-like biosynthetic gene cluster identified in *S. scabies* 87–22 consists of at least 15 genes, nine of which are homologous to genes from the CFA biosynthetic gene clusters of *Ps. syringae* pv. *tomato* and *P. atrosepticum* (Bignell *et al.* 2010b). These include the *cfa1-5* genes that encode enzymes believed to synthesize the 2-carboxy-2-cyclopentenone intermediate (CPC), as well as the *cfa6* and *cfa7* genes, which encode the large multidomain polyketide synthases (PKSs) that generate the CFA backbone from CPC (Rangaswamy *et al.* 1998). In addition, the *cfl* gene, which in *Ps. syringae* encodes an enzyme that is believed to catalyse the adenylation of CFA and the ligation of the CFA adenylate to CMA (Bender *et al.* 1999), is also conserved in *S. scabies.* Although *S. scabies* is unable to produce COR due to the absence of the CMA biosynthetic genes in the genome (Bignell *et al.* 2010b), it is likely that this organism produces one or more COR-like metabolites that are similar to the minor coronafacoyl compounds that are generated by *Ps. syringae* (Fig. 2c–f).

It is interesting to note that there are six genes within the *S. scabies* CFA-like biosynthetic gene cluster that are absent from the *Ps. syringae* and *P. atrosepticum* CFA biosynthetic gene clusters, and at least three of these genes are predicted to encode enzymes that could potentially modify the CFA polyketide backbone (Bignell *et al.* 2010b). Furthermore, the *S. scabies* Cfa7 enzyme contains an enoyl reductase domain that is absent from the Cfa7 homologues in *Ps. syringae* and *P. atrosepticum* (Bignell *et al.* 2010b), and if active, this domain would presumably reduce the carbon double bond that is present in CFA (Fig. 2b). Purification and structural analysis of the COR-like metabolite is currently ongoing within our laboratory, and this will provide insight into whether the molecule is novel in structure as compared to COR and the COR analogues produced by *Ps. syringae.*

Bioactivity of the *S. scabies* COR-like metabolite

Gene deletion studies in *S. scabies* have demonstrated that the COR-like metabolite contributes to the development of root disease symptoms in tobacco seedlings (Bignell *et al.* 2010a,b), and this correlates with the observed role of COR as an important contributor of disease symptom development during *Ps. syringae* infections (Xin and He 2013). Whether the COR-like metabolite also influences the severity of CS disease symptoms has not been determined, but is something that does warrant further investigation. However, it is likely that the metabolite is not required for CS disease development as other CS pathogens do not appear to produce it (Bignell *et al.* 2010b). It is noteworthy that the metabolite can cause hypertrophy of potato tuber tissue in a similar manner as COR (Fig. 3), suggesting that it may share the same target(s) in the plant host. It has been determined that COR functions as a molecular mimic of jasmonoyl—isoleucine (JA-Ile), which is the active form of the jasmonic acid (JA) plant hormone (Katsir *et al.* 2008a,b). JA-Ile controls the expression of genes involved in plant growth, development and defence against herbivores and necrotrophic pathogens (Browse and Howe 2008). When JA-responsive genes are activated, this leads to suppression of salicylic acid (SA)—mediated defence pathways, which are important for defence against biotrophic pathogens such as *Ps. syringae* (Koornneef and Pieterse 2008). Thus by functioning as a molecular mimic of JA-Ile, COR suppresses the plant defence response that is most important for combating infection by *Ps. syringae.* It is possible that the *S. scabies* COR-like metabolite also functions in a similar manner to allow the pathogen to overcome the host immune response, an idea that is currently under investigation in our laboratory.

Regulation of COR-like metabolite production in *S. scabies*

Embedded within the CFA-like biosynthetic gene cluster in *S. scabies* is a gene (*scab79591;* referred to herein as *cfaR*) that was previously shown to modulate the expression of the biosynthetic genes within the cluster (Bignell *et al.* 2010b). The encoded protein belongs to a novel family of transcriptional regulators that are only found in actinobacteria and are characterized by a C-terminal LuxR- family DNA-binding domain and an N-terminal

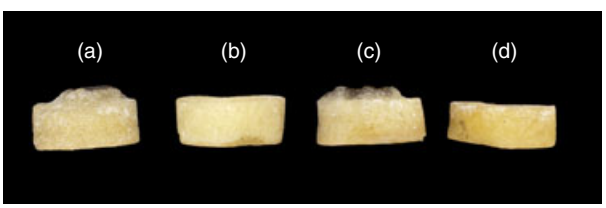

Figure 3 Induction of potato tissue hypertrophy by the *S. scabies* coronatine (COR)-like metabolite. Potato tuber disks were treated with COR (250 ng) (a) or with culture extract from a *S. scabies* COR-like metabolite-producing strain (c). Control treatments included 100% methanol (b) and culture extract from a *S. scabies* COR-like metabolite nonproducing strain (d).

PAS fold domain. The best-characterized member of this family is PimM, which controls the production of the polyene macrolide pimaricin in *Streptomyces natalensis*. PimM is required for expression of the pimaricin biosynthetic genes and for pimaricin production (Anton *et al.* 2007), and it has been shown to directly bind eight promoter regions within the biosynthetic gene cluster (Santos-Aberturas *et al.* 2011b). In addition, a Δ*pimM* mutant can be complemented by other closely related members of the PAS-LuxR family such as *amphRIV, nysRIV* and *pteF,* which are associated with the amphotericin, nystatin and filipin polyene macrolide biosynthetic clusters, respectively, and heterologous expression of *pimM* can enhance the biosynthesis of amphotericin and filipin in the respective producing organisms (Santos-Aberturas *et al.* 2011a). Together, this suggests that there is functional conservation among these members of the PAS-LuxR protein family. Genetic studies have shown that CfaR functions as a positive activator of gene expression in the CFA-like gene cluster (Bignell *et al.* 2010b), and electrophoretic mobility shift assays have confirmed that the protein directly binds to DNA within the cluster (Z. Cheng, unpublished data). It is currently not clear how the DNA binding activity of CfaR is regulated, although this is presumed to somehow involve the associated PAS domain. Interestingly, phylogenetic analysis suggests that CfaR may represent a novel member of the PAS-LuxR family as the protein does not appear to cluster with other family members in the database (Fig. 4).

FD-891

Streptomyces cheloniumii (Table 1) is a new species of *Streptomyces* that was isolated in Japan and causes RS but not CS on potato tubers (Oniki *et al.* 1986). In 2005, Natsume and colleagues reported the isolation of a new phytotoxin produced by *S. cheloniumii* and four other *Streptomyces* strains isolated from Japan (Natsume *et al.* 2005). Bioassays indicated that the phytotoxic compound induces necrosis of potato tuber tissue and causes stunting of rice and alfalfa seedlings, indicating that like thaxtomin A, it is a nonspecific phytotoxin. Purification and structural analysis of the phytotoxic compound identified it as the 16-membered macrolide FD-891 (Fig. 1c; Seki-Asano *et al.* 1994; Eguchi *et al.* 2004). FD-891 was previously reported to have cytocidal activity against animal cells (Seki-Asano *et al.* 1994), and the report by Natsume *et al.* is the first to describe its phytotoxicity (Natsume *et al.* 2005). Although FD-891 has a similar structure to the concanamycins (Fig. 1), the mode of action of the two types of metabolites appears to be different (Kataoka *et al.* 2000). It is currently not clear whether other RS-causing pathogens from other parts of the world also produce FD-891, and the contribution of FD-891 to RS disease symptom development also remains to be determined.

Borrelidin

Recently, a new pathogenic strain of *Streptomyces* was isolated from a scab lesion on a potato grown in Iran (Cao *et al.* 2012). The strain (GK18) was shown to induce deep pitted lesions on potato tubers rather than the raised lesions that are typically caused by *S. scabies* and other thaxtomin-producing species, and it also caused severe stunting of potato plants grown in pots. Interestingly, the authors could not detect thaxtomin A production by this strain, nor could they detect the *txtA* gene using Southern analysis. Instead, the strain was shown to produce the 18-membered polyketide macrolide borrelidin (Fig. 1d), which was first identified as an antibacterial antibiotic produced by *Streptomyces rochei* (Berger *et al.* 1949). Southern analysis confirmed that strain GK18 contains genes involved in the biosynthesis of borrelidin, and bioassays using potato tuber slices and radish seedlings demonstrated that the borrelidin purified from GK18 culture extracts exhibits phytotoxic activity. Interestingly, borrelidin was reported to cause deep, black holes on the potato tuber slices, an effect that is reminiscent of the disease symptoms caused by *Streptomyces* spp. GK18 on mini tubers. Thaxtomin A, on the other hand, produced more shallow, brown lesions on the potato tuber slices. Thus, it appears as though different *Streptomyces* phytotoxins can contribute to the production of distinct types of scab symptoms on potato tubers, and that production of different phytotoxins by different pathogenic streptomycetes might explain in some instances why there are several types of disease symptoms associated with CS disease in natural settings.

Borrelidin has been shown to exhibit anti-bacterial, antiviral, anti-malarial and anti-angiogenic activity (Dickinson *et al.* 1965; Wakabayashi *et al.* 1997; Otoguro *et al.* 2003); however, the report by Cao and colleagues is the first to demonstrate that this metabolite also exhibits phytotoxic activity (Cao *et al.* 2012). Furthermore, the report supports previous findings (Park *et al.* 2003; Wanner 2004) that some CS-causing streptomycetes do not produce thaxtomin A. It is noteworthy that Cao and colleagues were able to isolate 17 additional CS-causing streptomycetes, none of which produced thaxtomin A or borrelidin (Cao *et al.* 2012). Furthermore, research in our own laboratory has led to the isolation of two *Streptomyces* strains from Newfoundland, Canada that are pathogenic on radish seedlings (Fig. 5) and on potato tuber disks (data not shown), and yet they do not appear to produce thaxtomins, borrelidin or concanamycins (J. Fyans, unpublished). It therefore appears as though additional, unknown phytotoxic

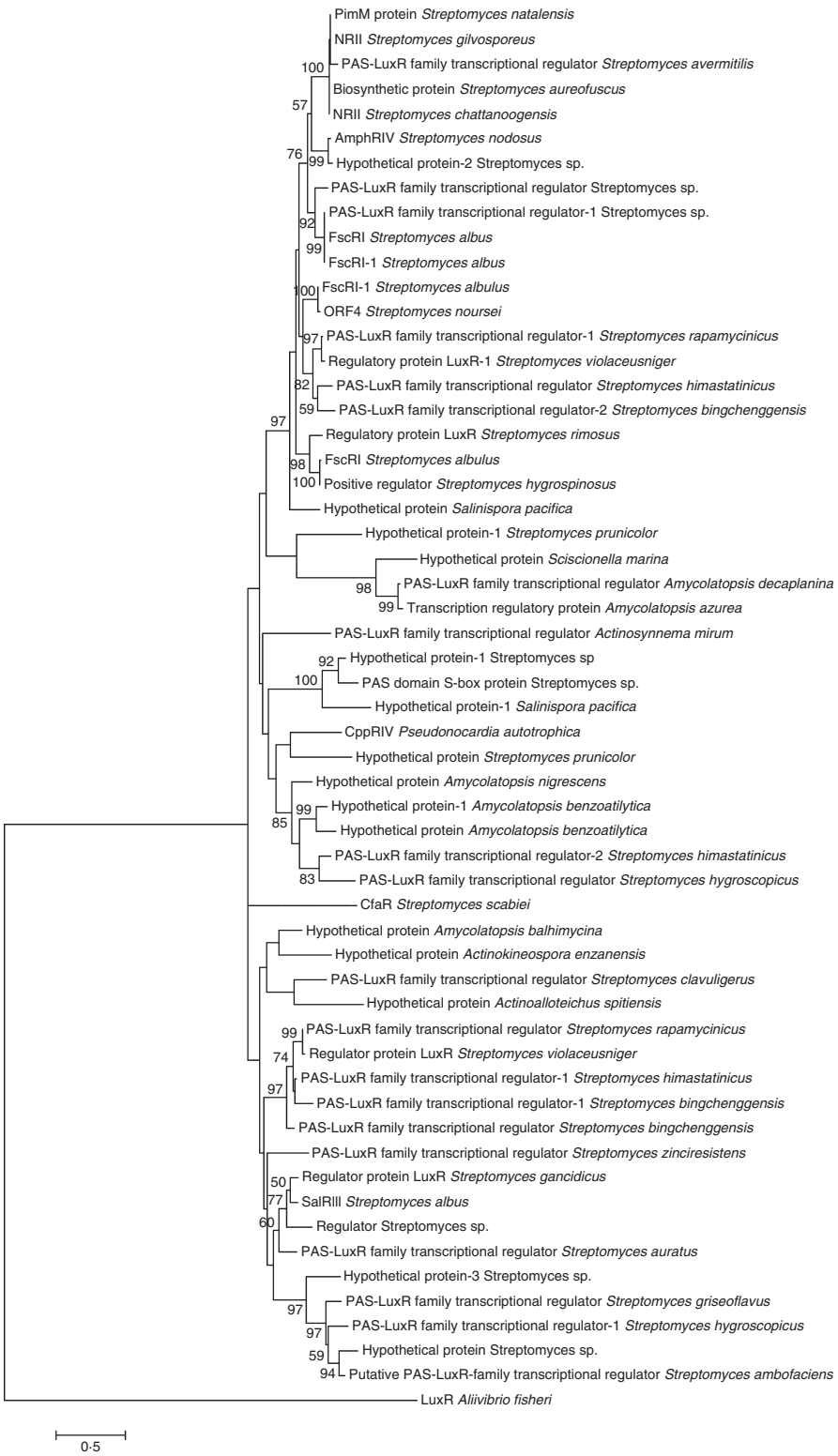

Figure 4 Phylogenetic analysis of PAS-LuxR family proteins from *Streptomyces* and other actinomycetes. The tree was constructed using the MEGA 5.2 software (Tamura *et al.* 2011) with the maximum likelihood algorithm. Bootstrap values ≥50% for 1000 repetitions are indicated. The scale bar indicates the number of amino acid substitutions per site. Accession numbers for the protein sequences used in this analysis are listed in Table S1. The *Aliivibrio fischeri* LuxR protein was included as an outgroup.

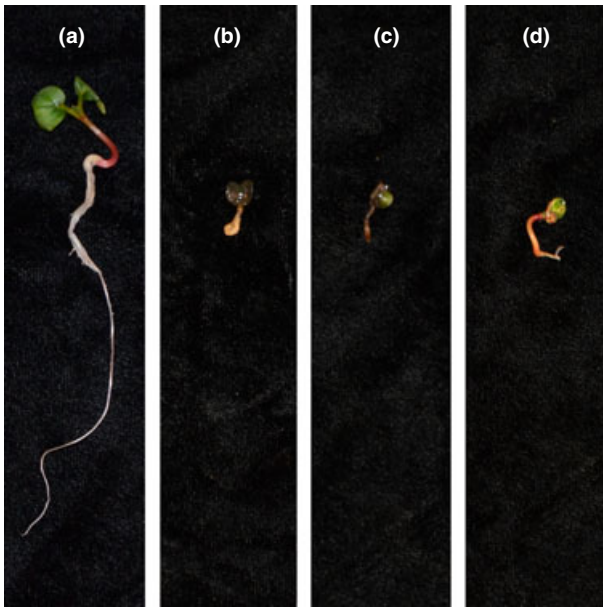

Figure 5 Virulence phenotype of nonthaxtomin-producing plant pathogenic *Streptomyces* strains isolated from Newfoundland, Canada. Radish seedlings were inoculated with *Streptomyces* spp. 11-1-2 (c) and 11-2-4 (d), whereas control seedlings were treated with water (a) or with *S. scabies* 87-22 (b).

secondary metabolites are possibly contributing to *Streptomyces* plant pathogenicity in the environment, and the identification and characterization of such metabolites will undoubtedly contribute to a better understanding of the mechanisms of disease development by these organisms.

Concluding remarks

Research over the last several years has provided important insights into plant pathogenic *Streptomyces* spp. and the phytotoxins that they produce to colonize and infect living plant tissues. Such information has assisted in the development of better procedures for detecting the pathogens in agricultural settings, and has provided new ideas for developing better control methods for reducing the economic impact of CS and other diseases. For example, the thaxtomin phytotoxins are now known to function as key virulence factors that are produced by several different CS and AS pathogens, and recent work using thaxtomin A as a selective agent has provided promising results for the development of potato lines that display elevated resistance to CS (Wilson *et al.* 2010; Hiltunen *et al.* 2011). This is significant given that CS is ubiquitous and notoriously difficult to effectively manage, and there are currently no potato cultivars that are completely resistant to the responsible pathogens (Dees and Wanner 2012). But, as discussed in this review, it is now apparent that multiple phytotoxic secondary metabolites are likely playing a role in the pathogenic phenotype of *Streptomyces* spp. in the environment, and thaxtomins are not always involved in the development of CS disease. This has important implications for control strategies that specifically target thaxtomin as such strategies will likely not be universally effective against all CS pathogens. Therefore, it is vital that we continue to decipher the role of secondary metabolism in the development of economically important crop diseases by *Streptomyces* spp. as this information is critical for the rational development of control strategies that will be effective in the long term. In addition, the functional analysis of *Streptomyces* secondary metabolites will help to further elucidate the complex mechanisms involved in host–pathogen interactions, which are ever evolving and dynamic processes.

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Conflict of interest

No conflict of interest is declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Accession numbers of the PAS-LuxR protein sequences used for construction of the phylogenetic tree.

The Coronafacoyl Phytotoxins: Structure, Biosynthesis, Regulation and Biological Activities

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Abstract

Phytotoxins are secondary metabolites that contribute to the development and/or severity of diseases caused by various plant pathogenic microorganisms. The coronafacoyl phytotoxins are an important family of plant toxins that are known or suspected to be produced by several phylogenetically distinct plant pathogenic bacteria, including the gammaproteobacterium *Pseudomonas syringae* and the actinobacterium *Streptomyces scabies.* At least seven different family members have been identified, of which coronatine (COR) was the first to be described and is the best-characterized. Though nonessential for disease development, coronafacoyl phytotoxins appear to enhance the severity of disease symptoms induced by pathogenic microbes during host infection. In addition, the identification of coronafacoyl phytotoxin biosynthetic genes in organisms not known to be plant pathogens suggests that these metabolites may have additional roles other than as virulence factors. This review focuses on our current understanding of the structures, biosynthesis, regulation, biological activities and evolution of coronafacoyl phytotoxins as well as the different methods that are used to detect the metabolites and the organisms that produce them.

Key Words

Phytotoxins; Secondary metabolites; Coronatine; *N*-Coronafacoyl-L-isoleucine; *Pseudomonas*; *Streptomyces*

Introduction

Phytotoxins are secondary metabolites that are produced by many phytopathogenic bacteria and fungi. They exhibit adverse effects on plants at very low concentrations and can be critical to the development of disease by pathogenic organisms (Bender et al. 1999; Strange 2007). Phytotoxins can be host specific and affect only those plant species that can be infected by the producing organism, or they can be non-host specific and exert toxic effects against a broad range of plant species that are not infected by the pathogen. Although some phytotoxins are essential for pathogenicity, many are not required for disease development but instead contribute to the virulence phenotype of the producing organism. In this case, disease may still occur in the absence of the phytotoxin, however the severity of disease symptoms is greatly enhanced when the phytotoxin is present (Bender et al. 1999; Strange 2007).

Coronatine (COR) is a non-host specific phytotoxin that was first described in 1977 by Ichihara and colleagues (Ichihara et al. 1977). It is produced by different pathogenic variants (pathovars or pv) of the Gram-negative plant pathogen *Pseudomonas syringae,* which causes economically important diseases in a variety of plant species (Xin and He 2013). COR belongs to a family of phytotoxins called the coronafacoyl phytotoxins, members of which are now known or suspected to be produced by several phylogenetically distinct plant pathogenic bacteria, including the potato common scab pathogen *Streptomyces scabies* (syn. *S. scabiei*) and the potato blackleg pathogen *Pectobacterium atrosepticum* (Bell et al. 2004; Fyans et al. 2015). Though nonessential for pathogenicity, there is convincing evidence that COR and COR-like molecules are important virulence factors that contribute to the severity of disease symptoms induced

by the producing organisms (Bell et al. 2004; Bignell et al. 2010; Panda et al. 2016; Slawiak and Lojkowska 2009; Xin and He 2013). A number of recent studies have provided significant insights into the bioactivity and mode of action of COR and other coronafacoyl phytotoxins, while other studies have focused on understanding the biosynthesis, regulation and evolution of production of these metabolites in different microorganisms. An updated overview of coronafacoyl phytotoxins is long overdue and is the focus of this review.

Chemical structures and producing organisms

All coronafacoyl phytotoxins characterized to date consist of the bicyclic hydrindane ring - based polyketide coronafacic acid (CFA, Fig. 1A) linked via an amide bond to an amino acid or amino acid derivative. In the case of COR (Fig. 1B), the molecule attached to CFA is coronamic acid (CMA), an ethylcyclopropyl amino acid derived from L-isoleucine via its diastereoisomer L-*allo*-isoleucine (Parry et al. 1994). Production of COR has been demonstrated in three *P. syringae* pathovars (*tomato*, *maculicola*, *actinidiae*) and in *P. coronafaciens* pv *atropurpurea*, *P. cannabina* pv *alisalensis* (formerly *P. syringae* pv *alisalensis*), *P. amygdali* pv *morsprunorum* (formerly *P. syringae* pv *morsprunorum*) and *P. savastanoi* pv *glycinea* (formerly *P. syringae* pv *glycinea*) (Table 1). Other pathovars of *P. syringae, P. coronafaciens, P. savastanoi, P. cannabina* and *P. amygdali* as well as a closely related *Pseudomonas* spp. (*P. temae*) most likely produce COR due to the presence of the biosynthetic genes for CFA and CMA in the respective genome sequences (Table 1). COR production has also been reported in the New Zealand flax pathogen *Xanthomonas campestris* pv

phormiicola and most recently in a novel plant pathogenic strain of *Pectobacterium cacticidum* (Table 1). *S. scabies* does not produce COR but instead produces *N*-coronafacoyl-L-isoleucine (CFA-Ile; Fig. 1C) as the primary coronafacoyl phytotoxin as well as other minor compounds (Fyans et al. 2015). Coronafacoyl compounds containing the amino acids L-valine (CFA-Val, Fig. 1D), L-isoleucine, L-*allo*-isoleucine (CFA-*a*Ile, Fig. 1E), L-serine (CFA-Ser, Fig. 1F), L-threonine (CFA-Thr, Fig. 1G) and a methyl-substituted CMA derivative (norcoronatine, Fig. 1H) have also been identified in culture extracts of COR-producing *X. campestris* pv *phormiicola* and/or *Pseudomonas* spp. (Table 1). Studies conducted in *P. savastanoi* pv *glycinea* and *P. syringae* pv *tomato* indicate that COR is the most abundant compound produced, while CFA-Val is usually the second most abundant and the rest are produced as minor components (Mitchell 1991).

Genome sequencing data have revealed that many more bacteria may produce coronafacoyl phytotoxins than previously realized. In 2004, it was reported that the genome sequence of the potato blackleg pathogen *P. atrosepticum* SCRI1043 harbours homologues of the *P. syringae cfa1-8* and *cfl* genes (Bell et al. 2004), and since then the genes have been found in the genome sequences of other plant pathogenic *Pectobacterium* spp. and in strains of *Dickeya* spp., *Brenneria* spp. and *Lonsdalea quercina*, all of which are close relatives of *Pectobacterium* (Table 1). Interestingly, a recent analysis of the NCBI database revealed that coronafacoyl phytotoxin biosynthetic genes are not confined to plant pathogenic bacteria species but can also be found in species that are not known to be pathogenic, including *Pseudomonas psychrotolerans,*

Azospirillum sp. B510, *Streptomyces griseoruber*, *Streptomyces graminilatus*, *Streptomyces* sp. NRRL WC-3618, *Kitasatospora azatica* and *Zymobacter palmae* (Table 1). This suggests that the production of coronafacoyl phytotoxins may serve other purposes rather than functioning exclusively as virulence factors (Bown et al. 2017). Currently, it is not known which specific coronafacoyl phytotoxin(s) is produced by most organisms, though it is likely that the majority do not produce COR due to the absence of the CMA biosynthetic genes.

Biosynthesis

Initial investigations into the biosynthesis of the CMA moiety in *Pseudomonas* spp. led to the discovery of three genes (*cmaATU*; Fig. 2 and Table 2) that are required for the production of CMA (Ullrich & Bender 1994). The *cmaA* gene product appeared to be a didomain protein containing an adenylation (A) domain and a thiolation (T) domain, while the *cmaT* gene product resembled thioesterases and the *cmaU* gene product did not show similarity to any known proteins and its function is currently unknown (Couch et al. 2004; Ullrich and Bender 1994). Subsequent investigations led to the discovery and characterization of additional CMA biosynthetic genes, namely the *cmaBCDE* genes (Fig. 2 and Table 2) (Vaillancourt et al. 2005). Biosynthesis of the CMA moiety begins with CmaA catalyzing the adenylation of L-*a*Ile and attachment of L-*a*Ile to the T domain of CmaA (Fig. 3) (Couch et al. 2004). Recent work identified a gene, *cmaL* (PSPTO4723), which encodes a predicted DUF1330 protein and is likely involved in the biosynthesis of L-*a*Ile from L-Ile (Fig. 3 and Table 2) (Worley et al. 2013). Although *cmaL* is separated from the rest of the *cma* genes by 8.8 kb in *P. syringae* pv *tomato*

DC3000, there is evidence that the gene is co-regulated with the other *cma* genes (Worley et al. 2013). The aminoacyl form of L-*a*Ile is then transported to the phosphopantetheinyl arm of the CmaD protein, which is a stand-alone T domain without a corresponding A domain, by the aminoacyltransferase CmaE (Strieter et al. 2007; Vaillancourt et al. 2005). While attached to CmaD, the L-*a*Ile is chlorinated at the γ position to form γ-chloro-L-*a*Ile by the CmaB protein, which is a member of the non-haem Fe^{2+} α-ketoglutarate - dependant enzyme superfamily (Vaillancourt et al. 2005). The CmaC enzyme then catalyzes the cyclization of the intermediate through the removal of the γ-chloro group by the nucleophilic attack of the α-carbon from L-*a*Ile to produce a cyclopropane ring (Kelly et al. 2007; Vaillancourt et al. 2005). It is believed that the covalent linkage attaching the now cyclised L-*a*Ile is then hydrolyzed by the CmaT thioesterase to release the fully formed CMA substrate (Patel et al. 1998).

The *Pseudomonas* CFA biosynthetic gene cluster is composed of 10 structural genes designated *cfa1-9* and *cfl*, which are organized as a single 19 kb transcriptional unit (Fig. 2 and Table 2) (Bender et al. 1999). *cfa1*, *cfa2* and *cfa3* encode proteins showing significant similarity to acyl carrier proteins (ACP), fatty acid dehydratases (DH) and a β-ketoacyl synthetases (KS) of type II polyketide synthases (PKS), respectively (Penfold et al. 1996). The *cfa4* protein product does not show significant similarity to sequences in the database and its function is unknown, though it has been suggested that it may function as a cyclase (Rangaswamy et al. 1998a). *cfa5* encodes a predicted acyl-CoA ligase, and *cfa6* and *cfa7* encode large, multifunctional enzymes resembling modular type I PKSs (Penfold et al. 1996; Rangaswamy et al. 1998a). The *cfa8* protein product shows

similarity to crotonyl-CoA reductase/carboxylase (CCR) enzymes that catalyze the reductive carboxylation of (*E*)-crotonyl-CoA to (2*S*)-ethylmalonyl-CoA, which in turn is used as an extender unit for polyketide biosynthesis (Wilson and Moore 2012). Mutagenesis experiments indicated that *cfa8* is essential for CFA and COR production in *P. savastanoi* pv *glycinea* (Rangaswamy et al. 1998b). The final gene in the CFA biosynthetic gene cluster is *cfa9,* which encodes a protein showing similarity to thioesterases and is dispensable for CFA and COR production (Rangaswamy et al. 1998b).

The exact pathway of CFA biosynthesis in *Pseudomonas* spp. is not well understood, though a hypothetical pathway has been proposed (Rangaswamy et al. 1998a). Precursor feeding studies using ^{13}C – labeled substrates indicated that CFA is synthesized from one unit of pyruvate, one unit of butyrate and three units of acetate (Parry et al. 1994). The theorized starting precursor is α-ketoglutarate, which is generated by carboxylation of pyruvate to form oxaloacetate and then conversion of oxaloacetate to α-ketoglutarate via the TCA cycle (Parry et al. 1996). Rangaswamy and colleagues proposed that α-ketoglutarate is decarboxylated to produce succinic semialdehyde, which is then converted into a CoA ester, possibly by Cfa5. Cfa1, Cfa3, and Cfa4 are then predicted to create enzyme – bound 2-carboxy-3-hydroxycyclopentenone, which is dehydrated by Cfa2 to produce enzyme – bound 2-carboxy-2-cyclopentenone (CPC; Fig. 4) (Rangaswamy et al. 1998a). CPC may then be passed to the loading module of Cfa6, which catalyzes the extension of CPC by a butyrate unit followed by complete reduction of the β-keto ester to form enzyme – bound 2-[1-oxo-2-cyclopenten-2-ylmethyl]butanoic

acid (CPE; Fig. 4). The predicted CCR encoded by *cfa8* is thought to provide the (2*S*)-ethylmalonyl-CoA used for extension of CPC by Cfa6 (Rangaswamy et al. 1998b). Next, CPE is transferred to Cfa7, which catalyzes the extension of CPE by a malonate unit. This is followed by an intramolecular 6 - *endo* - trig cyclization of the tethered intermediate to produce the bicyclic hydrindane ring - containing intermediate, which then undergoes ketoreduction and dehydration by the Cfa7 ketoreductase (KR) and DH domains, respectively, to form the complete CFA moiety (Fig. 4) (Strieter et al. 2009). Cfa7 also harbours a thioesterase (TE) domain that would allow for the release of free CFA, which may be enhanced by the activity of the free Cfa9 thioesterase (Rangaswamy et al. 1998b). The final step in the production of COR is the ligation of the CFA and CMA moieties, which is predicted to be catalyzed by the coronafacate ligase (Cfl) enzyme encoded by the *cfl* gene (Bender et al. 1999).

Sequencing of the *S. scabies* 87-22 genome revealed the presence of a gene cluster that is highly similar to the *Pseudomonas* CFA biosynthetic gene cluster (Table 2 and Fig. 2) (Bignell et al. 2010). Homologues of the *cfa1-8* and *cfl* genes were identified in a similar arrangement as in *Pseudomonas* spp.; however, no homologues of the *cma* genes were found anywhere in the genome, suggesting that *S. scabies* cannot make COR. Interestingly, six additional genes that are absent from the *Pseudomonas* gene cluster were found associated with the *S. scabies cfa* and *cfl* genes. Four of these genes, *SCAB79681* (also known as *oxr*), *SCAB79691* (also known as *CYP107AK1)*, *SCAB79711* and *SCAB79721* (also known as *sdr*) were predicted to encode biosynthetic enzymes and were shown to be co-transcribed with the *cfa* and *cfl* genes, while the other two genes

(*SCAB79581/orf1*, *SCAB79591/cfaR*) are divergently co-transcribed from the other genes and have been shown to be involved in regulation (Fig. 2) (Bignell et al. 2010; Cheng et al. 2015). The *SCAB79711* gene product shows similarity to 3-hydroxybutyrl-CoA dehydrogenases that are typically involved in the reduction of acetoacetyl-CoA to 3-hydroxybutyryl-CoA, an intermediate in the biosynthesis of crotonyl-CoA (Chan et al. 2009). It is therefore likely that SCAB79711 works together with the CCR encoded by *cfa8* to produce the (2*S*)-ethylmalonyl-CoA extender unit used for polyketide biosynthesis (Fig. 5) (Bignell et al. 2010). It was initially thought that *oxr*, *CYP107AK1* and *sdr*, which encode a predicted F_{420}-dependent oxidoreductase, a cytochrome P450 and a short chain dehydrogenase/reductase, respectively (Table 2), may function as tailoring enzymes and contribute to the production of one or more novel coronafacoyl phytotoxins in *S. scabies* (Bignell et al. 2010). Although subsequent structural characterization of the primary *S. scabies* phytotoxin indicated that it is the known coronafacoyl phytotoxin CFA-Ile (Fyans et al. 2015), gene deletion analysis revealed that all three genes are required for normal production of CFA-Ile, with *CYP107AK1* being essential (Bown et al. 2016; Bown et al. 2017). Both the Δ*CYP107AK1* and Δ*sdr* mutants accumulated biosynthetic intermediates that were purified and structurally characterized, and based on this it was proposed that *CYP107AK1* and *sdr* are responsible for introducing the keto group that is present on the bicyclic hydrindane ring of CFA (Fig. 5) (Bown et al. 2016; Bown et al. 2017). In contrast, no biosynthetic intermediates were isolated from the Δ*oxr* mutant, and so it role in CFA-Ile biosynthesis remains unclear (Bown et al. 2016).

Given the absence of similar genes in the CFA biosynthetic gene cluster and elsewhere in the genome sequences of *Pseudomonas* spp., the involvement of *oxr*, *CYP107AK1* and *sdr* in CFA-Ile biosynthesis suggests that *S. scabies* and *Pseudomonas* spp. utilize distinct biosynthetic pathways for producing the same family of phytotoxins. The proposed hypothetical pathway for CFA biosynthesis in *P. syringae* suggests that the keto group originates from the α-ketoglutarate precursor (Fig. 4). In contrast, studies in *S. scabies* suggest that it is the CYP107AK1 enzyme that introduces the oxygen via a hydroxyl group, which then converted to the keto group by the Sdr enzyme (Bown et al. 2016; Bown et al. 2017). The scheme shown in Fig. 5 proposes that these steps take place after polyketide biosynthesis by Cfa6 and Cfa7, though it cannot yet be ruled out that CYP107AK1 and Sdr generate the keto group within an earlier precursor molecule. Although the exact function of the Oxr enzyme in CFA-Ile biosynthesis is currently unknown, it has been suggested that it might introduce the carbon – carbon double bond that is found within the cyclohexene ring of CFA (Fig. 5) (Bown et al. 2016). In *P. syringae*, this double bond results from the reduction and dehydration of the hydrindane ring - containing intermediate by Cfa7 (Fig. 4) (Rangaswamy et al. 1998a; Strieter et al. 2009), whereas the *S. scabies* Cfa7 enzyme is proposed to contain an extra enoyl reductase (ER) domain that is believed to be functional and would reduce the double bond that is formed by the DH domain (Fig. 5) (Bignell et al. 2010; Bown et al. 2017). If this is the case, then the double bond must be reintroduced at a later step, possibly by Oxr, though further studies are needed to confirm this. The final ligation of CFA to L-Ile by the Cfl homologue was confirmed by deletion of the *S. scabies cfl* gene, which

resulted in accumulation of CFA in the mutant culture supernatants (Fyans et al. 2015). Interestingly, the *S. scabies* Cfl is able to utilize CFA biosynthetic intermediates as substrates for ligation to Ile, suggesting that the enzyme lacks a rigid specificity for the polyketide substrate (Bown et al. 2016; Bown et al. 2017).

Regulation of production

Production of COR in *Pseudomonas* spp. is regulated by multiple nutritional and environmental factors, including pH, carbon sources, osmolarity and nutrient levels (Li et al. 1998; Palmer and Bender 1993). In *P. savastanoi* pv *glycinea* PG4180, temperature has a major effect on COR production as production rates are much higher (more than 60 fold) at 18°C than at 28°C, the latter being the optimum growth temperature for the organism (Budde et al. 1998; Palmer and Bender 1993; Weingart et al. 2004). The observed thermoregulation of COR production is mainly due to the temperature-dependent transcription of the *cma* and *cfl/cfa* genes (Budde et al. 1998; Liyanage et al. 1995; Rangaswamy et al. 1997), which in the case of the *cma* genes also occurs *in planta* (Weingart et al. 2004). In addition, Budde and colleagues showed that the stability of the CmaB protein is greater at 18°C than at 28°C, indicating that the thermoregulation is also influenced by post-translational factors (Budde et al. 1998). In contrast, the biosynthesis of COR and expression of the *cma* genes is not significantly affected by temperature in *P. syringae* pv *tomato* DC3000 (Weingart et al. 2004), although an earlier study has suggested that it was (Rohde et al. 1998). COR gene expression in *P. syringae* pv *tomato* DC3000 is strongly activated when the cells are cultured *in planta* or in basal medium containing plant extracts (Boch et al. 2002; Li et al. 1998; Ma et al. 1991; Weingart et al.

2004), whereas COR production is not influenced by plant extracts or plant-derived secondary metabolites in *P. savastanoi* pv *glycinea* PG4180 (Palmer and Bender 1993). This suggests that the signals for inducing COR biosynthesis are different in these two organisms.

The DNA region responsible for the thermoregulation of COR production in *P. savastanoi* pv *glycinea* PG4180 is localized in between the CFA and CMA biosynthetic gene clusters and consists of three genes, *corP*, *corS* and *corR*, which encode components of a modified two-component regulatory system (Fig. 2 and Table 2) (Sreedharan et al. 2006; Ullrich et al. 1995). CorR and CorP both show similarity to response regulators that function as mediators of the cellular response in two-component regulatory systems, while CorS shows similarity to histidine protein kinases that serve as environmental sensors (Ullrich et al. 1995). CorR has the N-terminal receiver domain and the C-terminal DNA binding and effector domain with a helix-turn-helix (HTH) motif that is typical of response regulators (Pao et al. 1994). It has been shown to function as a positive activator of COR gene expression and to bind to the promoter regions controlling expression of the *cfl/cfa* and *cma* genes in a temperature-dependent manner (Fig. 6A) (Penaloza-Vazquez and Bender 1998; Wang et al. 1999). CorP is similar to CorR but it lacks the C-terminal HTH DNA binding domain and does not bind to the promoter regions driving COR production (Wang et al. 1999). Instead, CorP is required for the activation of CorR by CorS at low temperature, though its exact function remains unclear (Fig. 6A) (Wang et al. 1999). CorS is thought to be a membrane-embedded histidine protein kinase that responds to changes in temperature by modulating its conformation within the cell

membrane (Braun et al. 2007; Smirnova and Ullrich 2004). Rangaswamy and Bender (2000) demonstrated that CorS can autophosphorylate *in vitro* and can transphosphorylate CorR but not CorP (Fig. 6A), which is consistent with the presence of the conserved receiver aspartate residue in the former protein but not in the latter (Rangaswamy and Bender 2000). Although *corP* and *corR* are expressed constitutively at 18 and 28°C, *corS* expression is highest at 18°C and minimal at 28°C, suggesting that CorS may autoregulate its own expression (Ullrich et al. 1995).

A database search revealed that part or all of the CorRPS two-component regulatory system is conserved in several *Pseudomonas* species that also harbour the *cfl/cfa* and *cma* genes. In *P. syringae* pv *tomato* DC3000, the CorR homolog also functions as a positive activator of CFA and CMA structural gene expression and COR production (Sreedharan et al. 2006). In turn, *corR* expression is dependent on *hrpL*, which encodes an extracytoplasmic function sigma factor that also regulates the expression of the virulence-associated type III secretion system (Sreedharan et al. 2006). Mutations in *corR* were found to reduce the expression of *hrpL*, and a putative CorR binding site was identified within the *hrpL* promoter, suggesting that CorR may enhance the early expression of *hrpL* (Sreedharan et al. 2006). Whether CorS and CorP are involved in activating CorR via phosphorylation, and what signal(s) is sensed by CorS in *P. syringae* pv *tomato* DC3000 is currently unclear; however, the absence of thermoregulation of COR production in this organism suggests that the signal sensed by CorS is likely different than in *P. savastanoi* pv *glycinea* PG4180.

The regulation of coronafacoyl phytotoxin production has also been studied in *S. scabies.* Production of CFA-Ile was found to mainly occur in media containing plant-based components, with the highest levels observed in soy flour – based media (Fyans et al. 2015). Production levels were also found to be greater at 25°C than at the optimum growth temperature of 28°C (Fyans et al. 2015). The *S. scabies* CFA-Ile biosynthetic genes are transcribed as a single large mRNA transcript from the *cfa1* promoter, and a gene that is divergently transcribed was shown to function as a positive activator of the biosynthetic genes (Bignell et al. 2010). Overexpression of the gene, designated *SCAB79591*/*cfaR,* leads to enhanced expression of the biosynthetic genes and enhanced CFA-Ile production (Bignell et al. 2010; Fyans et al. 2015). CfaR belongs to a family of actinobacterial transcriptional regulators characterized by an N-terminal PAS (PER-ARNT-SIM) sensory domain and a C-terminal LuxR-type DNA binding domain (Taylor and Zhulin 1999; Fuqua et al. 1994). Electrophoretic mobility shift assays demonstrated that CfaR binds specifically to a single site located immediately upstream of the -35 hexanucleotide box within the *cfa1* promoter region (Fig. 6B) (Cheng et al. 2015). The binding site identified (5′-CTAGGGATTCTCCTAG-3′) is a 16 bp palindromic sequence that is highly similar to the binding site consensus sequence of the PAS-LuxR family regulator PimM, which controls the production of the polyene antifungal compound pimaricin in *Streptomyces natalensis* (Anton et al. 2007; Santos-Aberturas et al. 2011). CfaR DNA binding activity requires both the LuxR and PAS domains, with the latter playing a role in protein homodimer formation (Cheng et al. 2015). Intriguingly, the activation of CFA-Ile biosynthesis by CfaR is significantly enhanced by *SCAB79581/orf1*

(Fig. 6B), which is located downstream of and is co-transcribed with *cfaR* (Fig. 2) (Bignell et al. 2010; Cheng et al. 2015). *orf1* encodes a protein with a predicted ThiF family domain and a nitroreductase domain, and while overexpression of *orf1* alone has no effect on CFA-Ile production, overexpression of both *cfaR* and *orf1* leads to significantly greater CFA-Ile production levels than when *cfaR* alone is overexpressed (Cheng et al. 2015). Although the exact function of *orf1* is unclear, it was recently noted that homologues of both *cfaR* and *orf1* are conserved in other Actinobacteria that harbour coronafacoyl phytotoxin biosynthetic genes, suggesting that both genes play a role in regulating metabolite production in multiple species (Bown et al. 2017).

In addition to *cfaR* and *orf1,* other genes appear to regulate the production of CFA-Ile in *S. scabies* (Fig. 6B). Deletion of the *bldA* gene, which encodes the only tRNA that efficiently translates the rare UUA codon in *Streptomyces* mRNA (Chater 2006), led to a reduction in expression of the CFA-Ile biosynthetic genes (Bignell et al. 2010). An analysis of the *cfaR* coding sequence revealed the presence of a single TTA codon, suggesting that CfaR is not efficiently translated in the *bldA* mutant (Bignell et al. 2010). *bldA* is a member of the *bld* (bald) gene family of global regulators that control both morphological differentiation and secondary metabolism in *Streptomyces* spp. (Barka et al. 2016). Other *bld* genes such as *bldD*, *bldG* and *bldH* were recently shown to control the expression of *cfaR* and/or *cfa1* in *S. scabies* (Fig. 6B) (Bignell et al. 2014).

Biological activities and mode of action

Studies on the biological activity of coronafacoyl phytotoxins have mainly focused on COR, which is the most toxic family member (Bender et al. 1999). One of the most obvious effects of COR is the induction of diffuse chlorosis in various plants, including soybean [*Glycine max*; (Gnanamanickam et al. 1982)], tomato [*Lycopersicon esculentum*; (Uppalapati et al. 2005)] and *Nicotiana benthamiana* (Worley et al. 2013). In *Arabidopsis thaliana* and tomato, COR promotes anthocyanin accumulation and inhibits root elongation (Bent et al. 1992; Uppalapati et al. 2005; Ichihara and Toshima 1999). It also induces hypertrophy of potato tuber tissue (Gnanamanickam et al. 1982; Sakai et al. 1979; Volksch et al. 1989) and it stimulates ethylene production in bean (*Phaseolus vulgaris* L.) and tobacco (*Nicotiana tabacum*) leaves (Ferguson and Mitchell 1985; Kenyon and Turner 1992). Other effects attributed to COR include cell wall thickening, changes in chloroplast structure and accumulation of proteinase inhibitors (Uppalapati et al. 2005; Palmer and Bender 1995). Related phytotoxins such as CFA-Ile and CFA-Val are also biologically active and can induce chlorosis, inhibit root elongation and stimulate potato tuber tissue hypertrophy, though they are not as active as COR in inducing these effects (Fyans et al. 2015; Mitchell 1984; Mitchell and Young 1985; Shiraishi et al. 1979; Uppalapati et al. 2005). In contrast, CFA alone exhibits very little to no biological activity (Shiraishi et al. 1979; Uppalapati et al. 2005) indicating that the attached amino acid is necessary for the observed effects of coronafacoyl phytotoxins.

Several studies have demonstrated that COR is an important virulence factor in *Pseudomonas* spp. COR – deficient Tn5 mutants were shown to still be pathogenic; however, they produced little or no chlorosis and smaller necrotic lesions, and their

population sizes were significantly lower *in planta* as compared to COR – producing strains (Bender 1999). It was noted early on by several research groups that COR is structurally similar to the plant hormone jasmonic acid (JA), and more specifically the L-isoleucine conjugate of JA (JA-Ile), which is the most bioactive form (Fonseca et al. 2009; Staswick 2008). JA is a critical phytohormone that is responsible for regulating various biological processes in plants, including defence against necrotrophic pathogens and herbivores (Wasternack and Hause 2013). COR and JA induce similar responses in plants and regulate similar genes, and an Arabidopsis *coi1* (*coronatine insensitive 1*) mutant was shown to be insensitive to both COR and JA (Xin and He 2013). Together, this suggested that COR and JA share a similar mode of action. COI1 is the F-box component of SCF^{COI1}, a member of the Skip/Cullin/F-box (SCF) family of E3 ubiquitin ligases that target proteins for degradation by the 26S proteasome (Staswick 2008). JA-Ile promotes the binding of COI1 to several members of the JAZ (jasmonate ZIM – domain) family of repressor proteins, thereby leading to degradation of the JAZ repressors and activation of JA responsive genes (Chini et al. 2007; Katsir et al. 2008; Melotto et al. 2008a; Thines et al. 2007). Intriguingly, COR also promotes the formation of COI1-JAZ complexes *in vitro*, but it is ~ 1000 times more active than JA-Ile at promoting this interaction (Katsir et al. 2008). JA-Ile can compete with COR for binding to the COI1-JAZ complexes, and a crystal structure of the COI1-JAZ complex showed that JA-Ile and COR bind to the same ligand binding pocket, indicating that COR functions as a molecular mimic of JA-Ile (Katsir et al. 2008; Sheard et al. 2010). Activation of JA signalling by COR leads to suppression of the salicylic acid (SA) – mediated signalling

pathway, which is important for regulating plant defence against biotropic and hemibiotropic pathogens like *P. syringae* (Xin and He 2013). More recently, COR was also shown to suppress callose deposition in an SA – independent manner, and it enables bacterial entry into the plant host by overcoming stomatal defenses (Geng et al. 2012; Melotto et al. 2008b). Thus, COR contributes significantly to the virulence of *Pseudomonas* spp. by facilitating host invasion, by promoting bacterial multiplication within the plant through suppression of both SA – dependent and SA – independent defense responses, and by contributing directly to disease symptom development.

Currently, it is not clear whether other members of the coronafacoyl phytotoxin family also contribute to host invasion and/or suppression of plant defense responses during pathogen infection. The structural similarity between JA-Ile and other family members such as CFA-Ile, together with similarities in biological activity between COR and its relatives suggests that they may also function as JA-Ile mimics and induce JA – responsive genes in a COI1 – dependent manner, though likely not with the same efficiency as COR. There is evidence that COR may have additional targets within plant cells other than COI1 (Geng et al. 2012), and the same may apply to the related compounds. Studies of *cfa* mutants of both *P. atrosepticum* and *S. scabies* have indicated that the resulting coronafacoyl phytotoxins contribute to the virulence phenotype of each organism (Bell et al. 2004; Bignell et al. 2010; Panda et al. 2016), and strains of *P. carotovorum* subsp. *carotovorum* and *Dickeya* spp. harbouring the *cfa/cfl* genes cause more severe disease symptoms than strains that lack these genes (Slawiak and Lojkowska 2009). It therefore appears that the production of COR and COR-like molecules provides

an adaptive advantage to a broad range of plant pathogenic bacteria during host colorization and infection, either by stimulating the JA signalling pathway or by interacting with other targets within plant cells. Furthermore, as non-pathogenic bacteria also appear to have the capability to produce coronafacoyl phytotoxins, there are likely additional roles for these metabolites that remain to be discovered.

Evolution of coronafacoyl phytotoxin production

The identification of coronafacoyl phytotoxin biosynthetic genes in phylogenetically distinct bacteria suggests that the production of these compounds is widespread in nature and that horizontal gene transfer has played an important role in the dissemination of the biosynthetic genes. A recent study from our lab attempted to investigate the evolution of coronafacoyl phytotoxin production by examining the genetic architecture and phylogenetic relationships of the CFA biosynthetic gene clusters from different organisms (Bown et al. 2017). A comparison of the gene clusters from members of the *Gammaproteobacteria*, *Alphaproteobacteria* and *Actinobacteria* revealed that the *cfa1-7* and *cfl* genes are conserved among all of the gene clusters, and all but the *cfl* gene were found in the identical arrangement in each gene cluster. Differences were observed with regards to the presence or absence of other genes, including the CCR – encoding *cfa8* gene and the thioesterase – encoding *cfa9* (Bown et al. 2017). The actinobacterial gene clusters were all found to contain homologues of the *cfaR* and *orf1* regulatory genes as well as the *oxr* and *CYP107AK1* biosynthetic genes from *S. scabies*, and at least one gene cluster additionally contained homologues of *SCAB79711* and *sdr*. Quite possibly, these genes represent subclusters that were recently joined to an ancestral core gene

cluster given that they are only found within the actinobacterial CFA biosynthetic gene clusters (Bown et al. 2017). Phylogenetic analysis of the core (*cfa1-7, cfl*) biosynthetic genes indicated that the *S. scabies* gene cluster is most closely related to the other actinobacterial gene clusters, and in turn these gene clusters share a common ancestor with the identified cluster from *Azospirillum* sp. B510, an alphaproteobacterium. As further evidence of a close relationship between these clusters, the Cfa7 PKS encoded within these clusters all contain the ER domain that was first identified in the *S. scabies* Cfa7 (Fig. 5), whereas none of the Cfa7 proteins identified from the *Gammaproteobacteria* contain this domain (Bown et al. 2017). The phylogenetic analysis also indicated that the *Pseudomonas* core biosynthetic genes form a distinct clade that shares a common ancestor with the actinobacterial and *Azospirillum* sp. B510 gene clusters (Bown et al. 2017). Analysis of the GC content of the *Pseudomonas* core *cfa/cfl* genes indicated that most of the genes have a significantly higher GC content than the average GC content of the corresponding genomes (Online Resource 1), suggesting that the genes in the pseudomonads may have originated from an actinobacterium or another high GC organism. Although the *cfa/cfl* genes could be identified in phylogenetically distinct bacteria, the *cma* genes were only found in a small subset of known or predicted CFA producers (Bown et al. 2017). This may indicate that the ability to produce CMA and COR is a more recently acquired trait, a notion that has been suggested previously (Mitchell 1991).

How were the coronafacoyl phytotoxin biosynthetic genes acquired by different bacteria? It was noted early on that the *cfa/cfl* genes in different *Pseudomonas* spp. are

localized together with the *cma* genes on large (80 - 100 kb) indigenous plasmids of the pT23A family, which are readily transferred between different strains by conjugation (Bender et al. 1999; Sundin 2007). In *P. syringae* pv *tomato* DC3000, the *cfa/cfl* genes are chromosomally localized and are separated from the *cma* genes by 26 kb, and both regions are rich in mobile genetic elements that could allow for gene transfer (Gross and Loper 2009). Similarly, an analysis of the *S. scabies* 87-22 genome sequence has indicated the presence of mobile genetic elements in the vicinity of *cfa/cfl* genes (Z. Cheng and D. Bignell, unpublished). The *cfa/cfl* genes in *P. atrosepticum* and in blackleg – causing *P. carotovorum* strains are located on a putative horizontally acquired island, HAI2, which is an integrative and conjugative element (Bell et al. 2004; Panda et al. 2016). Studies have shown that HAI2 can excise from the chromosome at low frequency, including *in planta*, providing a means for lateral transfer of the genes that it harbours (Panda et al. 2016; Vanga et al. 2012; Vanga et al. 2015).

Detection of coronafacoyl phytotoxins and toxin – producing organisms

A qualitative bioassay for detecting COR and other coronafacoyl phytotoxins in culture filtrates has been described that makes use of the chlorosis – inducing activity of these compounds in various plants (Gnanamanickam et al. 1982). In addition, the hypertrophy – inducing activity of coronafacoyl phytotoxins on potato tuber tissue has been used in numerous studies for detecting these compounds and characterizing the activity of biosynthetic intermediates (Fig. 7) (Bown et al. 2016; Bown et al. 2017; Fyans et al. 2015; Gnanamanickam et al. 1982; Valenzuela-Soto et al. 2015). Völksch et al. (1989) demonstrated that the observed hypertrophy – inducing activity could be

developed into a semi-quantitative assay for detecting COR in culture filtrates (Volksch et al. 1989). As little as 0.8 nmol of COR can be detected using this bioassay (Fig. 7), though it has been noted that there can be some variability in the hypertrophy response depending on the potato cultivar that is used and the age of the tissue (Bender et al. 1999). Also, the activity of other coronafacoyl phytotoxins in comparison to COR is significantly less in both the chlorosis – inducing bioassay and the hypertrophy – inducing bioassay (Fig. 7) (Fyans et al. 2015; Uppalapati et al. 2005), which reflects the fact that COR is the most toxic family member.

Analytical methods involving small – scale extraction of culture supernatants and HPLC – based detection of coronafacoyl phytotoxin production have been described (Fyans et al. 2015; Panchal et al. 2017). Extractions are typically performed using 0.5 – 1 ml of acidified culture supernatants and either ethyl acetate or chloroform, and the compounds are separated from other components of the extract using a C8 or C18 reverse phase column with a detection wavelength of 208 – 230 nm. Such methods allow for absolute quantitative analysis of phytotoxin production when a standard curve is generated using known amounts of the target compound (Panchal et al. 2017), or relative quantitative analysis when comparing the phytotoxin peak area in a mutant strain with that from a wild-type strain (Bown et al. 2016; Bown et al. 2017; Fyans et al. 2015). Jones and colleagues developed an indirect competitive ELISA assay using monoclonal antibodies specific for COR. The assay was able to quantify COR with a detection limit of 1 ng/ml and could also detect CFA-Val with similar efficiency and CFA-Ile and CFA-*a*Ile with less efficiency (Jones et al. 1997). More recently, Schmeltz and colleagues

described a metabolic profiling approach using vapor phase extraction and GC-MS for directly quantifying COR in *P. syringae* –infected plant tissues. The method requires very little plant material and can be used to simultaneously quantify numerous interacting phytohormones and phytotoxins in plants (Schmelz et al. 2003).

Molecular approaches such as PCR and Southern analysis have been used to detect *Pectobacterium* spp., *Dickeya* spp. and *Streptomyces* spp. that are capable of producing coronafacoyl phytotoxins (Bignell et al. 2010; Slawiak and Lojkowska 2009). Such approaches involved the use of PCR primers or DNA probes specific for the *cfa6, cfa7* genes and *cfl* genes (Fig. 2). With the advent of inexpensive next generation sequencing technologies, the search for coronafacoyl phytotoxin producers has now become much easier as entire genome sequences can now be screened for the presence of the CFA and CMA biosynthetic gene clusters. This has led to the identification of bacteria that were not previously known to produce coronafacoyl phytotoxins, including several non-pathogenic species (Table 1) (Bown et al. 2017).

Concluding remarks

Although much has been learned about the coronafacoyl phytotoxin family since the discovery of COR, there are still many interesting questions that remain to be explored. For example, while most predicted coronafacoyl phytotoxin producers cannot make COR due to the absence of the *cma* genes, it is still unclear which specific family members are made by these organisms. Is there a preference for the production of CFA-Ile and/or CFA-Val over other family members in the absence of CMA, as observed in *S.*

scabies? Also, is there an ecological explanation for why COR production appears to be limited to a relatively small number of coronafacoyl phytotoxin producers? There are additionally unanswered questions regarding the coronafacoyl phytotoxin biosynthetic pathway, which largely remains hypothetical in *Pseudomonas* spp. and in other organisms. The role of several genes within the *cfa/cfl* gene cluster have yet to be verified using genetic and/or biochemical approaches. In *S. scabies*, we are particularly interested in whether the ER domain of Cfa7 is active given its absence in the *Pseudomonas* Cfa7 homologs, as well as the precise function of Oxr in the biosynthesis of CFA-Ile. Also, the role of Orf1 in the regulation of CFA-Ile biosynthesis is the subject of ongoing research in our lab. It remains to be determined whether all coronafacoyl phytotoxins function as suppressors of plant defense responses by behaving as JA mimics, or whether there are other roles for these compounds in plant cells. Finally, it will be interesting to establish whether non-pathogenic bacteria can produce coronafacoyl phytotoxins and what role(s) the metabolites play for these organisms. It is noteworthy that at least three of the non-pathogenic organisms that harbour the phytotoxin biosynthetic genes (*Pseudomonas psychrotolerans, Azospirillum* sp. B510, *Zymobacter palmae*) are known to be associated with plants, and one (*Azospirillum* sp. B510) has been reported to promote rice plant growth and resistance to fungal and bacterial pathogens (Kaneko et al. 2010; Midha et al. 2016; Okamoto et al. 1993). It is intriguing to speculate that coronafacoyl phytotoxin production by these organisms may contribute to beneficial interactions with plants rather than toxic interactions, an idea worth investigating further.

Compliance with ethical standards

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Figures and Tables

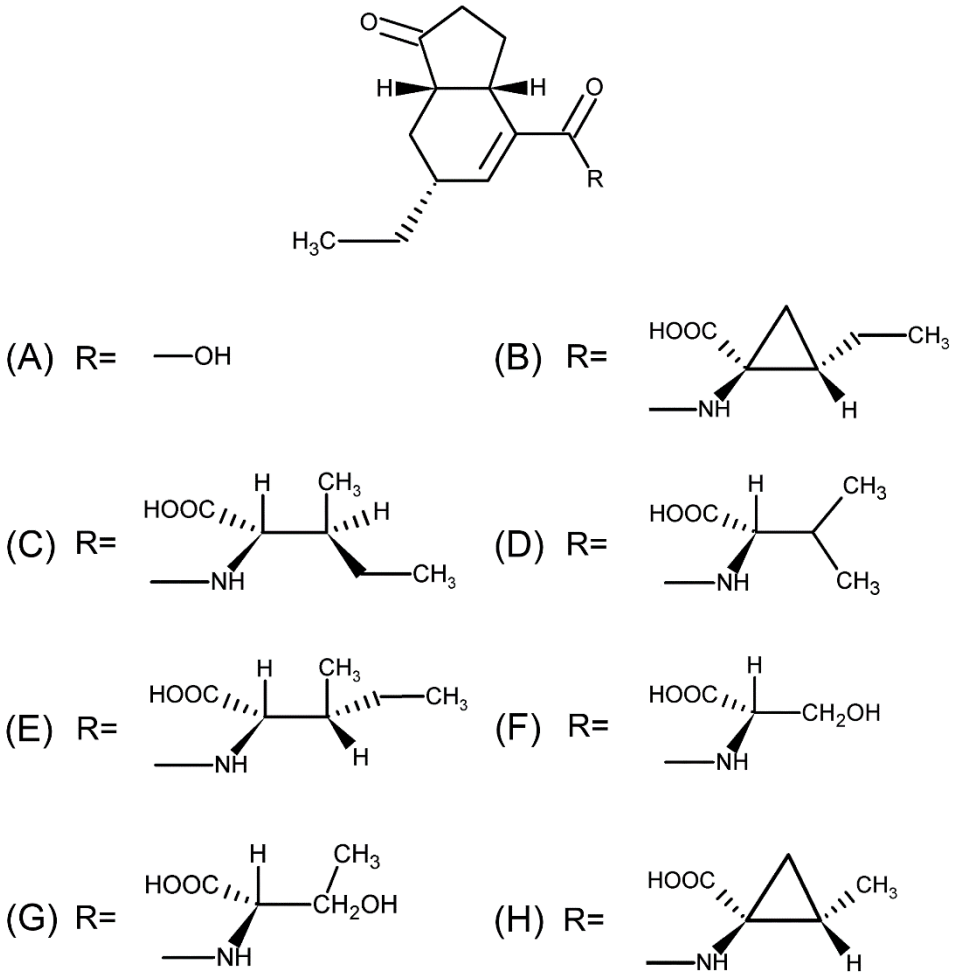

Fig. 1 Chemical structure of (A) coronafacic acid (CFA), (B) coronatine (COR), (C) *N*-coronafacoyl-L-isoleucine (CFA-Ile), (D) *N*-coronafacoyl-L-valine (CFA-Val), (E) *N*-coronafacoyl-L-*allo*-isoleucine (CFA-*a*Ile), (F) *N*-coronafacoyl-L-serine (CFA-Ser), (G) (D) *N*-coronafacoyl-L-threonine (CFA-Thr) and (G) norcoronatine (norCOR).

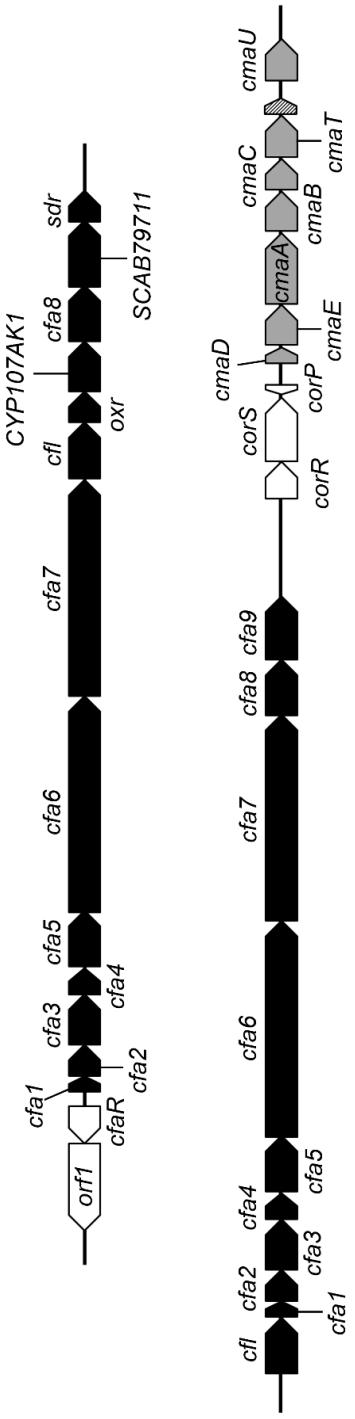

Fig. 2 Organization of the gene clusters involved in coronafacoyl phytotoxin biosynthesis in *S. scabies* (top) and *P. savastanoi* pv *glycinea* (bottom). The *cfa/cfl* operon is shown in

black, the *cma* operon is in gray, and regulatory genes are in white. The gene within the *cma* operon that is indicated with the hatched lines has not been shown to be involved in CMA biosynthesis.

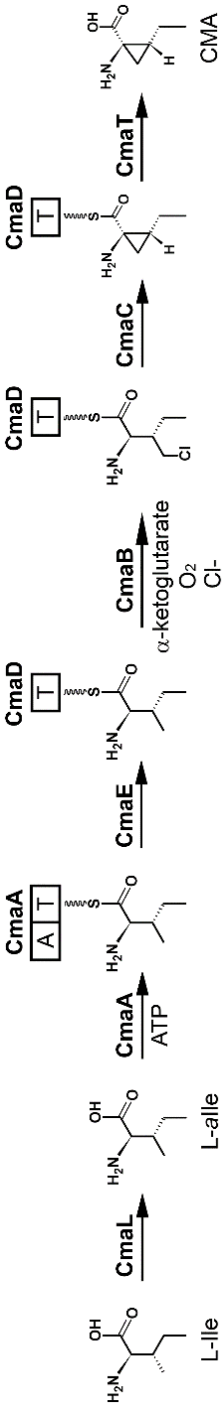

Fig. 3 Biosynthetic pathway for production of CMA in *Pseudomonas* spp.

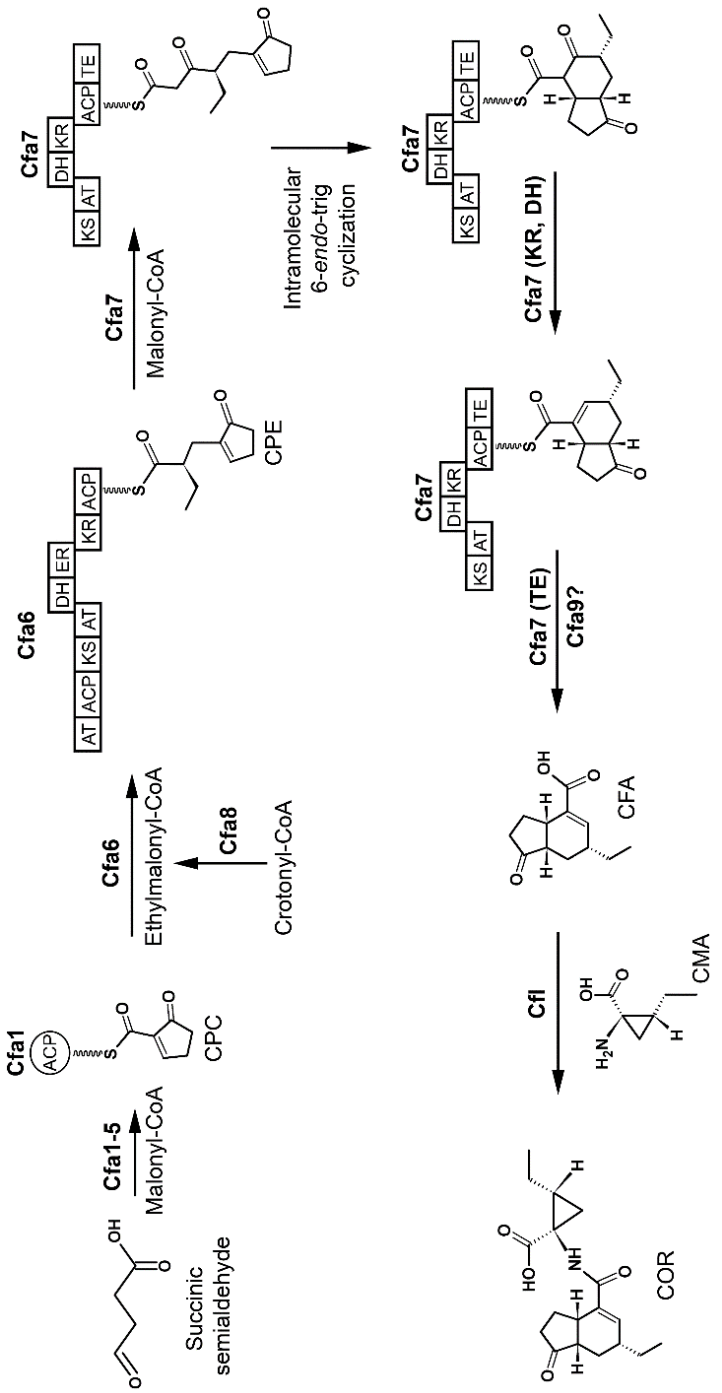

Fig. 4 Hypothetic biosynthetic pathway for CFA production and ligation of CFA with CMA to produce COR in *Pseudomonas* spp.

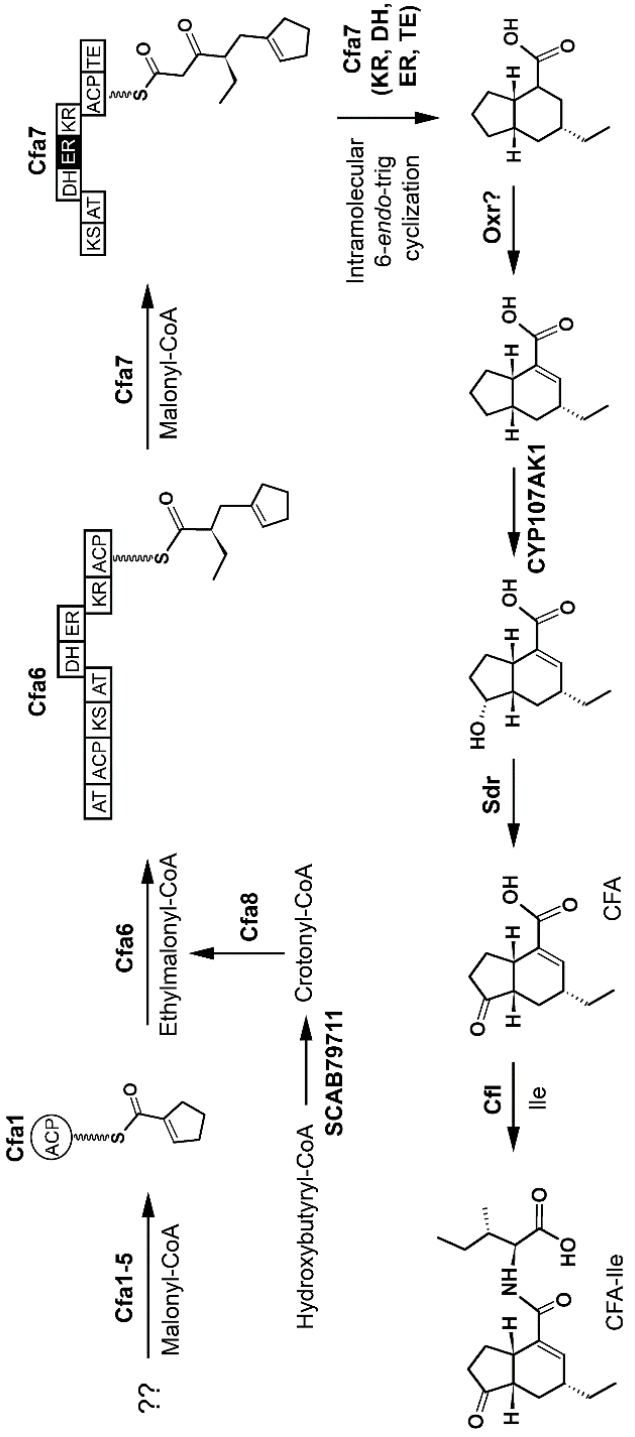

Fig. 5 Hypothetical biosynthetic pathway for the production of CFA-Ile in *S. scabies.* The starting precursor may or may not be the same as in *Pseudomonas* spp. and thus is

indicated by the question marks. The enoyl reductase (ER) domain that is present in the *S. scabies* Cfa7 and is absent from the Cfa7 homolog in *Pseudomonas* spp. is indicated in black.

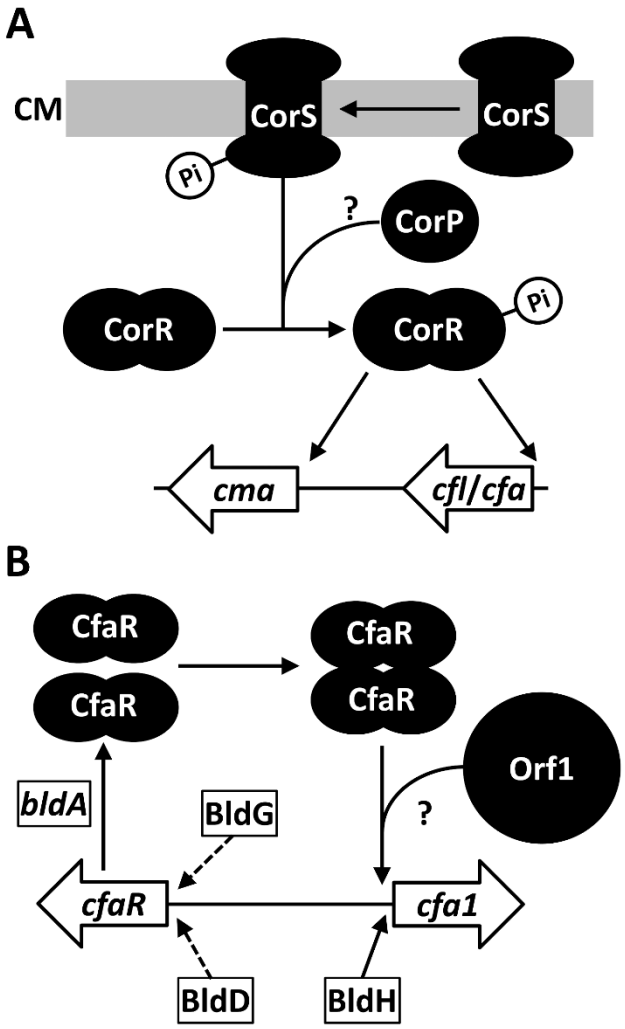

Fig. 6 Regulation of coronafacoyl phytotoxin production. (A) The production of COR in *P. savastanoi* pv *glycinea* is regulated by the histidine protein kinase CorS and the response regulators CorR and CorP. CorS is thought to be localized in the cell membrane (CM) and to autophosphorylate in response to changes in temperature. It then transphosphorylates CorR, which in turn activates expression of the *cma* and *cfl/cfa* operons. CorP is required for the activation of CorR by CorS through an unknown mechanism. (B) The regulation of CFA-Ile production in *S. scabies* involves the PAS-

LuxR family regulator CfaR, which binds to the *cfa1* promoter as a dimer and activates expression of the *cfa* operon. The Orf1 protein enhances the activation of CFA-Ile production by CfaR through an unknown mechanism. Several *bld* gene global regulators also modulate the expression of *cfaR* and/or *cfa1*. *bldA* is required for translation of the TTA codon within the *cfaR* coding sequence, while BldD and BldG are predicted to indirectly control the expression of *cfaR*, and BldH is predicted to directly control expression of the *cfa1* promoter (Bignell et al. 2014). Solid arrows in both (A) and (B) are used to indicate direct regulation, while dashed arrows indicate indirect regulation.

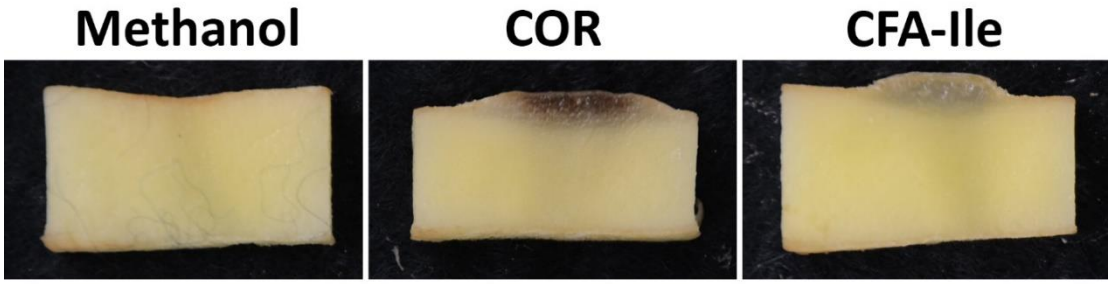

Fig. 7 Hypertrophy – inducing activity of coronafacoyl phytotoxins on potato tuber tissue. Tuber disks were treated with pure COR (0.8 nmol), CFA-Ile (16 nmol) or methanol (solvent control).

Table 1. Organisms known or suspected to produce coronafacoyl phytotoxins.

Organism	Subspecies or pathovar	Plant pathogen (host)	Coronafacoyl phytotoxin(s) produced	Reference(s)
***Azospirillum* sp. B510**	NA[a]	No	Unknown, may produce COR due to presence of *cfa*, *cfl* and *cma* genes	(Bown et al. 2017; Kaneko et al. 2010)
***Brenneria* sp. EniD312**	NA	Yes (multiple)	Unknown, harbours *cfa* genes and *cfl*, no *cma* genes present	(Bown et al. 2017; Brady et al. 2012)
Dickeya dadantii	*dieffenbachiae*	Yes (*Dieffenbachia* spp)	Unknown, harbours *cfa* genes and *cfl*, no *cma* genes present	(Brady et al. 2012; Samson et al. 2005); this study[b]
Kitasatospora azatica	NA	No	Unknown, harbours *cfa* genes and *cfl*, no *cma* genes present	(Bown et al. 2017)
Lonsdalea quercina	*britannica, iberica, quercina*	Yes (multiple)	Unknown, harbours *cfa* genes and *cfl*, no *cma* genes present	(Brady et al. 2012); this study[b]
Pectobacterium atrosepticum	NA	Yes (*Solanum tuberosum*)	Unknown, harbours *cfa* genes and *cfl*, no *cma* genes present	(Bell et al. 2004; Panda et al. 2016; Slawiak and Lojkowska 2009)
Pectobacterium betavasculorum	NA	Yes (*Beta vulgaris* subsp. *vulgaris*)	Unknown, harbours *cfa* genes and *cfl*, no *cma* genes present	(Bown et al. 2017; Gardan et al. 2003)
Pectobacterium cacticidum	NA	Yes	COR	(Valenzuela-Soto et al. 2015)
Pectobacterium carotovorum	*basiliensis, carotovorum, actinidiae*	Yes (multiple)	Unknown, harbours *cfa* genes and *cfl*, no *cma* genes present	(Panda et al. 2016); this study[b]

Pseudomonas amygdali	*tabaci, lachrymans , aesculi, ulmi, morsprunorum*	Yes (multiple)	COR	(Baltrus et al. 2011; Bender et al. 1999; Gardan et al. 1999; Jeong et al. 2015); this study[b]
Pseudomonas cannabina	*cannabina, alisalensis*	Yes (multiple)	Unknown, may produce COR due to presence of *cfa*, *cfl* and *cma* genes	(Bull et al. 2010; Cintas et al. 2002; Sarris et al. 2013)
Pseudomonas coronafaciens	*atropurpurea, porri, zizaniae, oryzae*	Yes (multiple)	COR, CFA-Val	(Baltrus et al. 2011; Mitchell 1984; Nishiyama et al. 1976); this study[b]
Pseudomonas psychrotolerans	NA	No	Unknown, may produce COR due to presence of *cfa*, *cfl* and *cma* genes	(Bown et al. 2017; Hauser et al. 2004)
Pseudomonas savastanoi	*glycinea*	Yes (multiple)	COR, CFA-Val, CFA-Ile, CFA-*allo*-Ile, CFA-Ser, CFA-Thr, nor COR	(Bender et al. 1999; Qi et al. 2011); this study[b]
Pseudomonas syringae	*tomato, maculicola, actinidiae, persicae, berberidis, spinaceae*	Yes (multiple)	COR, CFA-Val, CFA-Ser, CFA-Thr	(Bender et al. 1999; Han et al. 2003); this study[b]
Pseudomonas tremae	NA	Yes (*Trema orientalis*)	Unknown, likely produces COR due to presence of *cfa*, *cfl* and *cma* genes	(Gardan et al. 1999); this study[b]
***Streptomyces* sp. NRRL WC-3618**	NA	No	Unknown, harbours *cfa* genes and *cfl*, no *cma* genes present	(Bown et al. 2017)
Streptomyces	NA	No	Unknown,	This study[b]

graminilatus			harbours *cfa* genes and *cfl*, no *cma* genes present	
Streptomyces griseoruber	NA	No	Unknown, harbours *cfa* genes and *cfl*, no *cma* genes present	(Bown et al. 2017)
Streptomyces scabies	NA	Yes (multiple)	CFA-Ile, other minor compounds	(Fyans et al. 2015)
Xanthomonas campestris	*phormiicola*	Yes (New Zealand flax)	COR, CFA-Ile, CFA-Val	(Mitchell 1991; Tamura et al. 1992)
Zymobacter palmae	NA	No	Unknown, harbours *cfa* genes and *cfl*, no *cma* genes present	This study[b]

[a] NA, not applicable

[b] Based on identification of homologues of the *S. scabies* 87-22 and *P. syringae* pv *tomato* DC3000 phytotoxin biosynthetic enzymes using NCBI BlastP

Table 2. Genes and predicted gene function for the CMA and CFA biosynthetic gene clusters in *Pseudomonas* spp. and in *Streptomyces scabies*

Gene	Predicted protein product[a]	Predicted function	Organism
Biosynthetic			
cfa1	Acyl carrier protein (ACP)	Starter unit biosynthesis	conserved
cfa2	Type II fatty acid dehydratase (DH)	Starter unit biosynthesis	conserved
cfa3	Type II β-ketoacyl synthase (KS)	Starter unit biosynthesis	conserved
cfa4	Unknown	Starter unit biosynthesis	conserved
cfa5	Acyl-CoA ligase	Starter unit biosynthesis	conserved
cfa6	Type I PKS	CFA polyketide backbone biosynthesis	conserved
cfa7	Type I PKS	CFA polyketide backbone biosynthesis	conserved
cfa8	Crotonyl-CoA reductase/ carboxylase (CCR)	Ethylmalonyl-CoA biosynthesis	conserved
cfl	Acyl-CoA ligase	Ligation of CFA to amino acid	conserved
SCAB79681/oxr	F_{420}-dependent oxidoreductase	CFA biosynthesis	*S. scabies*
SCAB79691/CYP107AK1	P450 monooxygenase	CFA biosynthesis	*S. scabies*
SCAB79711	Hydroxybutyryl-CoA dehydrogenase	Ethylmalonyl-CoA biosynthesis	*S. scabies*
SCAB79721/sdr	Short chain dehydrogenase/reductase	CFA biosynthesis	*S. scabies*
cfa9	Thioesterase	Hydrolysis of thioester bond	*Pseudomonas* spp.
cmaD	Free standing thiolation	Allows	*Pseudomonas*

	(T) protein	formation of cyclopropyl ring	spp.
cmaE	Aminoacyltransferase	Intermediate shuttle	*Pseudomonas* spp.
cmaA	NRPS like adenylation-thiolation (A-T) didomain	Adenylation of L-*allo*-isoleucine	*Pseudomonas* spp.
cmaB	Non-heme Fe^{2+} α-ketoglutarate – dependent halogenase	Chlorination of L-*allo*-isoleucine	*Pseudomonas* spp.
cmaC	Vicinal oxygen chelate homologue	Cyclopropane ring construction	*Pseudomonas* spp.
cmaT	Thioesterase	Hydrolysis of thioester bond	*Pseudomonas* spp.
cmaU	Unknown	Unknown	*Pseudomonas* spp.
cmaL	Domain of unknown function; DUF1330	L-*allo*-isoleucine biosynthesis	*Pseudomonas* spp.
Regulatory			
SCAB79581/orf1	ThiF superfamily protein	Unknown	*S. scabies*
SCAB79591/cfaR	PAS-LuxR DNA binding protein	Transcriptional activator	*S. scabies*
corR	Two-component response regulator	Transcriptional activator	*Pseudomonas* spp.
corS	Two-component histidine protein kinase	Signal transduction	*Pseudomonas* spp.
corP	Two-component response regulator	Unknown	*Pseudomonas* spp.

[a] CoA = coenzyme A

Table S1. %GC content of the core coronafacoyl phytotoxin biosynthetic genes from different organisms.

Organism	**GC Content (%)[a]**								
	cfa1	***cfa2***	***cfa3***	***cfa4***	***cfa5***	***cfa6***	***cfa7***	***cfl***	**Genome**
Azospirillum sp. B510	54.2	61.8	69.9	66.2	66.5	69.6	69.7	62.2	**67.6**
Kitasatospora azatica KCTC 9699	56.5	63.3	73.0	70.3	71.5	73.7	74.4	71.2	**71.6**
Streptomyces scabies 87-22	60.1	62.4	73.4	70.6	71.1	74.8	74.8	71.7	**71.5**
Streptomyces sp. NRRL WC-3618	57.6	62.4	73.4	70.3	71.1	74.1	75.4	71.4	**72**
Streptomyces griseoruber DSM 40281	62.0	63.0	70.5	70.3	72.6	74.5	74.9	70.8	**71.55**
Pseudomonas coronafaciens pv *porri* LMG 28495	58.7	62.0	67.1	65.1	64.8	67.8	68.0	62.4	**57.8**
Pseudomonas syringae pv *tomato* DC3000	58.7	62.0	67.0	65.2	64.9	67.6	68.0	62.4	**58.3**
Pseudomonas savastanoi pv *glycinea* B076	58.7	62.2	67.4	64.6	65.3	67.8	68.6	62.9	**57.8**
Pseudomonas psychrotolerans NS274	57.1	63.3	68.4	66.9	68.4	69.8	70.1	65.2	**64.7**
Pseudomonas amygdali pv *morsprunorum* HRI-W5269	59.1	62.2	67.3	64.6	65.3	67.8	68.5	62.9	**58.1**
Pectobacterium carotovorum subsp. *carotovorum* UGC32	35.1	43.4	49.3	50.0	51.9	55.5	54.7	35.6	**51.1**
Pectobacterium atrosepticum SCRI1043	36.6	42.4	48.8	49.3	51.7	55.6	55.0	35.8	**51**
Pectobacterium betavasculorum NCPPB 2795	34.8	43.6	49.6	49.1	51	56.1	55.8	36.1	**54**
Brenneria sp. EniD312	36.6	43.8	50.3	49.4	50.9	54.7	53.7	36.9	**55.9**

[a] % GC values greater than the average % GC content of the corresponding genome are indicated in red, % GC values less than the average % GC content of the corresponding genome are indicated in green, and % GC values that are within ± 2% of the average % GC content of the corresponding genome are indicated in gray.