CHARACTERIZING THE ROLE OF CFL, OXR AND SDR IN THE BIOSYNTHESIS OF THE STREPTOMYCES SCABIES COR-LIKE METABOLITES

by

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ABSTRACT

Streptomyces is the largest genus of actinobacteria and consists of Gram-positive filamentous organisms that mainly inhabit soil environments. Some members of this genus have the ability to cause economically important crop diseases such as potato common scab (CS), which is characterized by the formation of superficial, raised or pitted corky-like lesions on the surface of potato tubers. Among the virulence factors produced by the best characterized CS-causing pathogen, *S. scabies* are the phytotoxic secondary metabolites called the COR-like metabolites, which resemble the coronatine (COR) phytotoxin produced by the plant pathogenic bacterium *Pseudomonas syringae*. The objective of this study was to characterize the role of three *S. scabies* genes (*cfl*, *oxr* and *sdr*) in the biosynthesis of the COR-like metabolites by constructing gene deletion mutants and examining the effect of each mutation on metabolite biosynthesis and bioactivity. The results of this study indicate that all three genes are necessary for normal production of the COR-like metabolites in *S. scabies*, and possible roles for each gene in the biosynthetic pathway are discussed.

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List of Symbols, Nomenclature and Abbreviations

α: Alpha
Δ: Delta or deletion
λ: Lambda bacteriophage
μ: Micro
Ω: Ohm
Φ: Phi

ACN: Acetonitrile
amp^r: Ampicillin resistant
apra^r: Apramycin resistant

bp: Base pair

C: Carbon
cam^r: Chloramphenicol resistant
CFA: Coronafacic acid
CMA: Coronamic acid
COR: Coronatine
CS: Common scab

DNA: Deoxyribonucleic acid
dNTP: Deoxyribonucleoside triphosphate
DMSO: Dimethylsulfoxide

EDTA: Ethylene diamine tetra-acetic acid
EtBr: Ethidium bromide
EtOAc: Ethyl acetate

FMN: Flavin mononucleotide

g: g-force
g: Grams
HPLC: High-performance liquid chromatography
hr: Hour
hyg^r: Hygromycin B resistant

Ile: Isoleucine
IPTG: Isopropyl β-D-thiogalactopyranoside
ISP-4 medium: International *Streptomyces* Project medium 4

kan^s: Kanamycin sensitive
kan^r: Kanamycin resistant

kb: kilobase

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

m: Milli
M: Molar
Mb: Megabase
MeOH: Methanol
min: Minute(s)
mol: Mole(s)
msec: Milliseconds

n: Nano
NA: Nutrient agar
NAD: Nicotinamide adenine dinucleotide
NADP: Nicotinamide adenine dinucleotide phosphate
NaOAc: Sodium acetate

OBA: Oat bran agar
OD: Optical density

p: Pico
PCR: Polymerase chain reaction
PKS: Polyketide synthase
pv: pathovar

rpm: Revolutions per minute

SD: Standard deviation
SDR: Short chain dehydrogenase/reductase
sec: Second(s)
SFMA: Soy flour mannitol agar
SFMB: Soy flour mannitol broth
SOB: Super optimal broth
SOC: Super optimal broth with catabolite repression
spp.: species

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

UV: Ultraviolet

V: Volt(s)
Val: Valine
v/v: volume per volume

w/v: Weight per volume
w/w: Weight per weight

X-gal: 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Overview and general features of *Streptomyces* bacteria

Streptomyces is the largest genus of actinobacteria and includes over 500 species (Garrity et al. 2007). *Streptomyces* spp. are Gram-positive, filamentous bacteria that mainly inhabit soil environments (Hibbing et al. 2010) but have also been isolated from aquatic environments (Stach and Bull 2005). They are obligate aerobes and can produce extracellular enzymes that degrade complex organic molecules such as cellulose, chitin and pectin, thereby allowing these organisms to grow as saprophytes and to contribute to the recycling of nutrients in the environment. The genomes of *Streptomyces* spp. are notably large compared with other bacteria, ranging from 8 to 10 Mb in size, and are typically composed of a single linear chromosome along with one or more circular and/or linear plasmids in some cases (Ventura et al. 2007). The G + C content of *Streptomyces* DNA is high and typically falls in the range of 70 – 72% (Embley et al. 1994; Lechevalier and Lechevalier 1970; Williams et al. 1983, 1989).

A notable feature of *Streptomyces* bacteria is their developmental life cycle, which is quite complex compared with other bacteria (reviewed in Flärdh and Buttner 2009). The life cycle is initiated by the germination of a single spore followed by the formation of a branched network of vegetative (substrate) hyphae (Fig. 1.1) called the substrate mycelium. In response to nutrient deprivation and possibly other signals, the organism stops vegetative growth and switches its developmental program to the formation of aerial hyphae, which grow vertically and impart a fuzzy appearance to the colony surface.

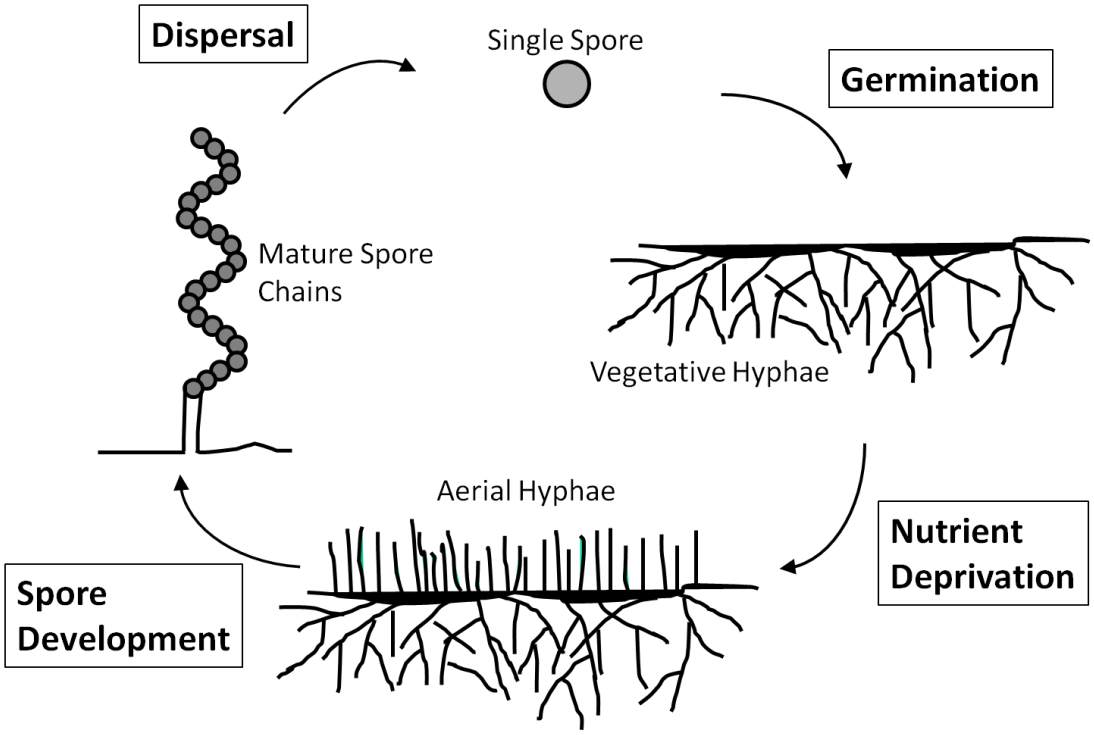


Figure 1.1: The life cycle of *Streptomyces* spp.

The aerial hyphae then undergo septation, which divides the hyphae into equal size compartments that contain a single copy of the bacterial chromosome. Such compartments eventually develop into mature spores that are coated with a gray pigment, which turns the aerial hyphae from white to gray. The mature spores are not only important for dispersal of the non-motile *Streptomyces* bacteria, but they also allow for survival under conditions of desiccation and other chemical and physical challenges (McCormick and Flärdh 2012). However, the spores of *Streptomyces* bacteria are not as resistant to adverse conditions as the endospores produced by *Bacillus* and *Clostridium* spp. (Wildermuth and Hopwood 1970).

Probably the best known characteristic of *Streptomyces* spp. is their ability to produce many bioactive secondary metabolites, including nearly two thirds of the world's naturally occurring antibiotics (i.e. antibacterial, antifungal, antiparasitic agents) as well as other medically and agriculturally important compounds such as immunosuppressants, anticancer agents, pesticides, herbicides, and plant growth promoters (Bérdy 2005). Even the distinct earthy odour of soil as well as the earthy flavour of beets can be attributed to a group of volatile secondary metabolites called the geosmins, which are produced by *Streptomyces* spp. (Cane et al. 2006; Gerber and Lechevalier 1965). A more thorough discussion of *Streptomyces* secondary metabolism is provided in Section 1.2.

A less well known characteristic of *Streptomyces* bacteria is the ability of some species to function as plant pathogens. Such organisms cause diseases of the underground portions of various plants. For instance, *Streptomyces scabies* (syn. *S. scabiei*) and other species cause common scab (CS) disease of potato and other root crops, while *Streptomyces ipomoeae* causes soil rot of sweet potato, which is

characterized by necrosis of the fibrous roots and by the formation of cankers on the storage tubers (Loria et al. 2006). Other plant diseases that have been attributed to *Streptomyces* spp. include netted scab and russet scab of potato, root tumor of cucurbits, and pod wart of peanut (Loria et al. 1997, 2006). The plant pathogenic phenotype and virulence determinants of *S. scabies*, which is the focus of this thesis, will be discussed in more detail in the following sections.

1.2 Secondary metabolism in *Streptomyces*

Secondary metabolites (also known as natural products) are small molecules that are not essential for growth or reproduction of the producing organism (Woodruff 1966). In contrast to primary metabolites, which are essential for growth and are highly conserved among different organisms, secondary metabolites are mainly produced by certain organisms for specific purposes. Although the ecological role of microbial secondary metabolites is not known in many instances, it is thought that they provide an adaptive advantage to the producing organism (reviewed in O`Brien and Wright 2011).

Microbial secondary metabolites belong to a variety of chemical classes such as peptides, carbohydrates, lipids, polyketides, terpenoids, steroids and alkaloids (O′Brien and Wright 2011). In *Streptomyces* spp., the production of these molecules in liquid culture is mainly limited to the stationary phase of growth, while production on solid media coincides with the formation of aerial hyphae (Bibb 2005). Numerous environmental and physiological signals are known to affect secondary metabolism in *Streptomyces* (Bibb 2005). The genes encoding the biosynthetic enzymes for producing

secondary metabolites are most often (but not always) clustered together on *Streptomyces* chromosomes or plasmids. Such gene clusters also typically include genes encoding resistance factors that protect the producing organism from its own product (in the case of metabolites with antibacterial activity), and regulatory genes that control the expression of the metabolite biosynthesis and resistance genes (Chen et al. 2010).

There has been great debate over the ecological role(s) of some secondary metabolites produced by *Streptomyces* and other microorganisms. Iron-chelating metabolites called siderophores are secreted by various environmental microorganisms, and it is thought that they function to scavenge iron from the environment for the producing organism (Wandersman and Delepelaire 2004). In the case of antibiotics, it has long been thought that they function to kill off competing microorganisms in the soil environment, thereby providing a selective advantage to the producing organism in an environment that has a limited nutrient pool (O′Brien and Wright 2011). However, there is currently little evidence showing that antibiotics are produced in soil environments at sufficient concentrations to display toxicity against other microorganisms. More recently, it has been proposed that subinhibitory concentrations of antibiotics and other secondary metabolites can function as signaling molecules for communication between different microorganisms, and several studies appear to support this idea (Dufour and Rao 2011; Xie et al. 2007). There is also evidence that secondary metabolites can promote symbiotic relationships between bacteria and eukaryotic organisms (reviewed in Seipke et al. 2011). In some instances, such relationships are mutualistic in nature, but as discussed

in the following sections, they can also be parasitic and result in the development of disease in the eukaryotic host.

1.3 *Streptomyces* plant pathogenicity and common scab disease

Over 500 species of *Streptomyces* have been identified to date, and yet very few of these have the ability to cause plant diseases. *Streptomyces scabies* is the oldest and best characterized of the plant pathogens and has a worldwide distribution (Lambert and Loria 1989; Lerat et al. 2009b). This organism is responsible for CS disease, which is characterized by the formation of corky-like lesions on the surface of potato (*Solanum tuberosum*) tubers (Dees and Wanner 2012). These lesions are often variable in appearance and can range from shallow (superficial) brown lesions to raised (erumpent) lesions to deep, pitted lesions (Fig. 1.2). Furthermore, the lesions can cover only a small area of the tuber surface, or they can cover the entire surface. The type and severity of symptoms depends on the susceptibility of the potato cultivar, the aggressiveness and levels of the pathogen in soils, and environmental conditions (Dees and Wanner 2012). In the USA, CS has been rated among the top five diseases affecting seed potato production (Slack 1991), and in Canada, the disease was estimated to result in losses of $15.3 – 17.3 million to potato growers during the 2002 growing season (Hill and Lazarovitz 2005). In Tasmania, Australia, CS has been attributed to losses to the potato industry of up to 4% of the total crop value (Wilson 2004). The economic losses due to CS are mainly because the lesions affect the market value of the potato crop. In addition, there is evidence that infection by CS-causing pathogens can decrease the yield of the

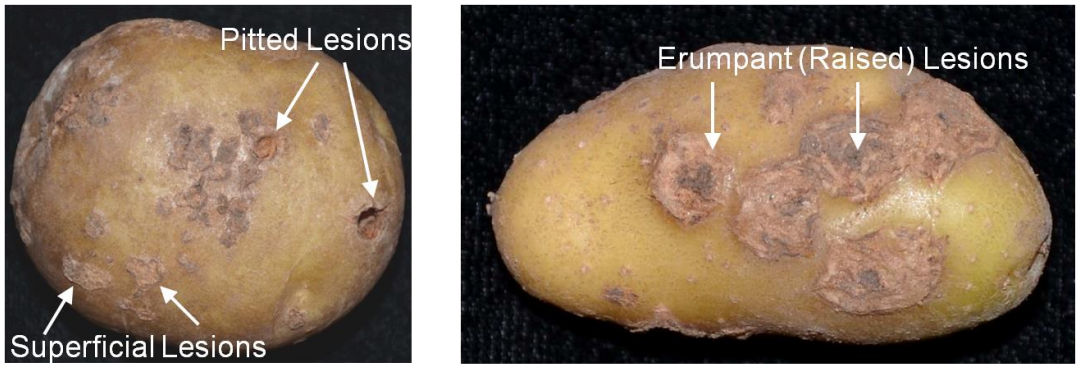


Figure 1.2: Typical CS lesions on potato tubers. The different types of lesions that can occur [superficial, erumpent (raised), and pitted] are indicated. Image courtesy of D. Bignell.

potato crop as well as increase the number of small tubers in the yield (Hiltunen et al. 2005). Control strategies that are currently used for CS disease are largely ineffective, and although there are some potato varieties that display moderate resistance to the disease, such varieties will still develop symptoms when the right conditions are present (Wanner 2009 and references therein). *S. scabies* is the most widely distributed CS pathogen, although there are at least nine other *Streptomyces* species that have been reported to cause the disease. *Streptomyces turgidiscabies* was first described as a CS pathogen from Japan and has since been isolated from Finland, China, Korea, the USA, Norway, Sweden and the UK (Faucher et al. 1992; Kreuze et al. 1999; Lehtonen et al. 2004; Loria et al. 2006; St-Onge et al. 2008; Wanner 2009). *Streptomyces europaiscabiei* is a close relative of *S. scabies* and is commonly found in Europe, Korea, the USA and Canada (Bouchek-Mechiche et al. 2000; Flores-Gonzalez et al. 2008; Song et al. 2004; Wanner 2006, 2009). *Streptomyces acidiscabies* was first isolated from low pH soils in the northeast USA and has since been found in Canada, China, Japan, Korea and the UK (Bouchek-Mechiche et al. 2000; Wanner 2006, 2009). This particular species is responsible for acid scab, which is identical to CS except that it occurs in acid soils (Loria et al. 1997). *Streptomyces stelliscabiei* has been reported as a CS pathogen in France and the USA, while *Streptomyces luridiscabiei*, *Streptomyces puniscabiei* and *Streptomyces niveiscabiei* are pathogens identified from Korea (Bouchek-Mechiche et al. 2000; Jiang et al. 2012; Park et al. 2003). Recently, two new CS pathogenic isolates (*Streptomyces* sp. IdahoX and *Streptomyces* sp. DS3024) were reported in the USA (Hao et al. 2009; Wanner 2007). Despite the fact that all of these pathogens represent distinct species, they

are all known or thought to produce the same phytotoxic secondary metabolite called thaxtomin A. This phytotoxin is discussed further in Section 1.4.1.

An interesting feature about CS-causing streptomycetes is that they are non-host specific and have the ability to infect a variety of different plants. For example, taproot crops such as beet, carrot, radish and turnip are also susceptible to CS disease and can develop the same scab lesions as seen on potato tubers (Lerat et al. 2009b; Loria et al. 2006). In addition, CS pathogens have the ability to cause root and shoot stunting, tissue swelling, chlorosis and necrosis on seedlings of both monocots and dicots in the laboratory (Loria et al. 1997, 2008). These effects are mainly due to the production of thaxtomin A and possibly other phytotoxic compounds (Loria et al. 1997). In South Africa, *S. scabies* has also been reported to cause pod wart of peanut, which is characterized by raised, necrotic lesions on the surface of the peanut shell (Loria et al. 1997). While infection by CS-causing pathogens is not host-specific, it is developmentally constrained in that only rapidly expanding plant tissue is affected (Loria et al. 2006, 2008). As discussed below, the reason for this constraint is likely due to the host target of thaxtomin A.

1.4 Virulence factors produced by CS-causing *Streptomyces* spp.

The infection of a plant host by a microbial pathogen is a complicated process. It requires specialized virulence factors that allow the pathogen to colonize the host, to induce damage to the host tissues, and to evade host defense mechanisms. Determining the mechanism of plant pathogenicity in *Streptomyces* spp. such as *S. scabies* has been the focus of intense research over the last several years. Below is a summary of our current

understanding regarding some of the virulence factors that contribute to disease development by CS-causing pathogens.

1.4.1 Thaxtomin A

Almost all of the CS-causing pathogens that have been described are known to produce a family of phytotoxic secondary metabolites called the thaxtomins. Thaxtomins are cyclic dipeptides derived from the condensation of L-phenylalanine and 4-nitro-L-typtophan residues (Johnson et al. 2009; King and Calhoun 2009). The predominant family member produced by *S. scabies, S. acidiscabies* and *S. turgidiscabies* is thaxtomin A (Fig. 1.3). This toxin can be purified from infected potato tuber tissue or from plant-based growth media (Babcock et al. 1993; Goyer et al. 1998; King and Lawrence 1996; Loria et al. 1995), and has been shown to cause necrosis of potato tuber tissue as well as scab-like symptoms on immature potato tubers (Lawrence et al. 1990; Loria et al. 2006). Several groups have also demonstrated a positive correlation between plant pathogenicity of *Streptomyces* spp. and the production of thaxtomin A (Goyer et al. 1998; King et al. 1991; Kinkel et al. 1998; Loria et al. 1995) and non thaxtomin A - producing mutants of *S. scabies* and *S. acidiscabies* do not cause the typical CS disease symptoms (Goyer et al. 1998; Healy et al. 2000). Furthermore, pure thaxtomin A has been shown to inhibit the growth of radish seedlings (Leiner et al. 1996).

There is a large amount of evidence to suggest that the principal mode of action of thaxtomin A is the inhibition of cellulose biosynthesis, although it is still unclear how this occurs. Fry and Loria (2002) showed that thaxtomin A causes isotropic expansion of cells in radish and onion seedlings and in tobacco suspension cultures, and that it

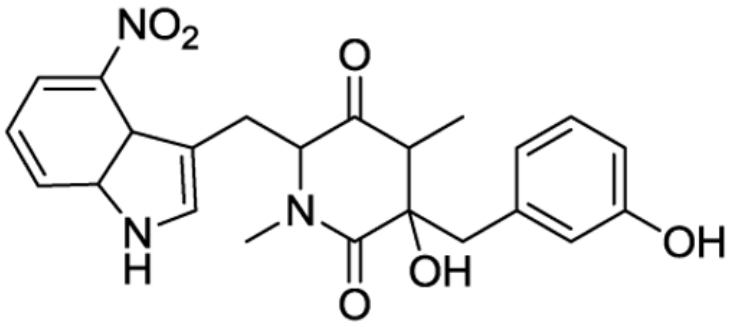


Figure 1.3: Structure of the phytotoxic secondary metabolite thaxtomin A produced by scab-causing *Streptomyces* spp.

interferes with normal cell division in onion root tip cells. Scheible et al. (2003) provided more substantial evidence by showing that pure thaxtomin A greatly reduces the incorporation of ^{14}C-glucose into the cellulosic fraction of the plant cell wall, and more recently it was demonstrated that the amount of crystalline cellulose in the cell wall is reduced in response to thaxtomin A (Bischoff et al. 2009). Thaxtomin A has also been shown to affect the expression of cell wall synthesis genes, and it depletes the number of cellulose synthase complexes in the Arabidopsis plasma membrane (Bischoff et al. 2009). Furthermore, Duval and Beaudoin (2009) showed that thaxtomin A affects the expression of genes in Arabidopsis in a similar manner to the known cellulose synthesis inhibitor isoxaben. Given that cellulose biosynthesis mainly occurs near the meristematic tissues of plants where the cells are actively dividing and expanding (Loria et al. 2008), the targeting of cellulose biosynthesis by thaxtomin A explains why infection by CS-causing streptomycetes is developmentally constrained.

A number of additional physiological effects have been observed in plants in response to thaxtomin A. Tegg et al. (2005) reported that thaxtomin A induces Ca^{2+} influx and H^{+} efflux across the cell walls of Arabidopsis and tomato plants prior to the inhibition of root elongation. At around the same time, Duval et al. (2005) demonstrated that thaxtomin A induces programmed cell death in Arabidopsis cell suspension cultures, and more recently it was shown that the influx of Ca^{2+} ions induced by thaxtomin A in Arabidopsis is a prerequisite to cell death and to other toxin-induced responses (Errakhi et al. 2008). Furthermore, thaxtomin A has also been shown to induce the production of the antimicrobial plant phytoalexin scopoletin (Lerat et al. 2009a).

The current model for infection by CS-causing streptomycetes has thaxtomin A functioning as an initiator of plant tissue penetration. Bacteria growing on the surface of plant tissues may be stimulated to produce thaxtomin A in response to plant-derived signals. Studies have shown that cello-oligosaccharides and suberin, which is a lipid plant polymer found on the surface of potato tubers, induce thaxtomin A production in *Streptomyces* spp. (Johnson et al. 2007; Lerat et al. 2009c). Thaxtomin A then might allow the bacteria to penetrate the expanding plant tissues and to grow both intercellularly and intracellularly, a phenomenon that has been described for *S. scabies* and other CS-causing streptomycetes (Loria et al. 2008).

1.4.2 Nec1

Although thaxtomin A is the primary virulence factor produced by CS pathogens, it is now known that other virulence factors contribute to plant pathogenicity in these organisms. The first identified *Streptomyces* virulence-associated gene, *nec1,* was discovered in a genetic screen meant for the identification of the thaxtomin biosynthetic genes. When a *S. scabies* cosmid carrying the *nec1* gene was introduced into the non-pathogenic streptomycete *Streptomyces lividans,* the resulting strain was able to necrotize and colonize potato tuber slices and cause scab-like symptoms on immature potato minitubers (Bukhalid and Loria 1997). Gene deletion studies later confirmed that *nec1* is important for the virulence phenotype of *S. turgidiscabies* (Joshi et al. 2007). The *nec1* gene is conserved among many CS-causing *Streptomyces* spp. (Bukhalid et al. 1998; Wanner 2009) and has a G+C content of 54%, which is much lower than the average G+C content of a typical *Streptomyces* genome and suggests that it was acquired from

another genus by horizontal gene transfer (Bukhalid and Loria 1997). Interestingly, no homologs of the *nec1* protein product exist in the publically available databases. Recent work by Joshi et al. (2007) demonstrated that Nec1 is a necrogenic protein that is secreted via N-terminal processing. Furthermore, in contrast to wild-type *S. turgidiscabies,* a Δ*nec1* mutant was unable to aggressively colonize and infect the root meristem of radish seedlings (Joshi et al. 2007). Although the role of Nec1 in *Streptomyces* plant pathogenicity is unknown, it has been suggested that it may function to suppress the plant defense response (Joshi et al. 2007).

1.4.3 TomA

The *tomA* gene was first discovered in *S. turgidiscabies* in the vicinity of the *nec1* gene (Kers et al. 2005) and encodes a saponinase enzyme that resembles tomatinase enzymes produced by microbial tomato pathogens (Seipke and Loria 2008). Saponinases are glycosyl hydrolases that detoxify saponins (phytoanticipins), which are preformed antimicrobial compounds produced by plants (Seipke and Loria 2008; VanEtten et al. 1994). Tomatinases are specifically involved in the degradation of α-tomatine, which is a saponin produced by tomato plants (Morrissey and Osbourn 1999). The *tomA* gene is conserved in *S. scabies*, *S. turgidiscabies* and *S. acidiscabies* (Kers et al. 2005), and functional analysis of the *S. scabies* TomA protein demonstrated that it possesses a similar enzymatic activity as other characterized tomatinases (Seipke and Loria 2008). Although a *S. scabies* Δ*tomA* mutant was identical to the wild-type strain in terms of its virulence phenotype, the conservation of *tomA* in pathogenic streptomycetes suggests a

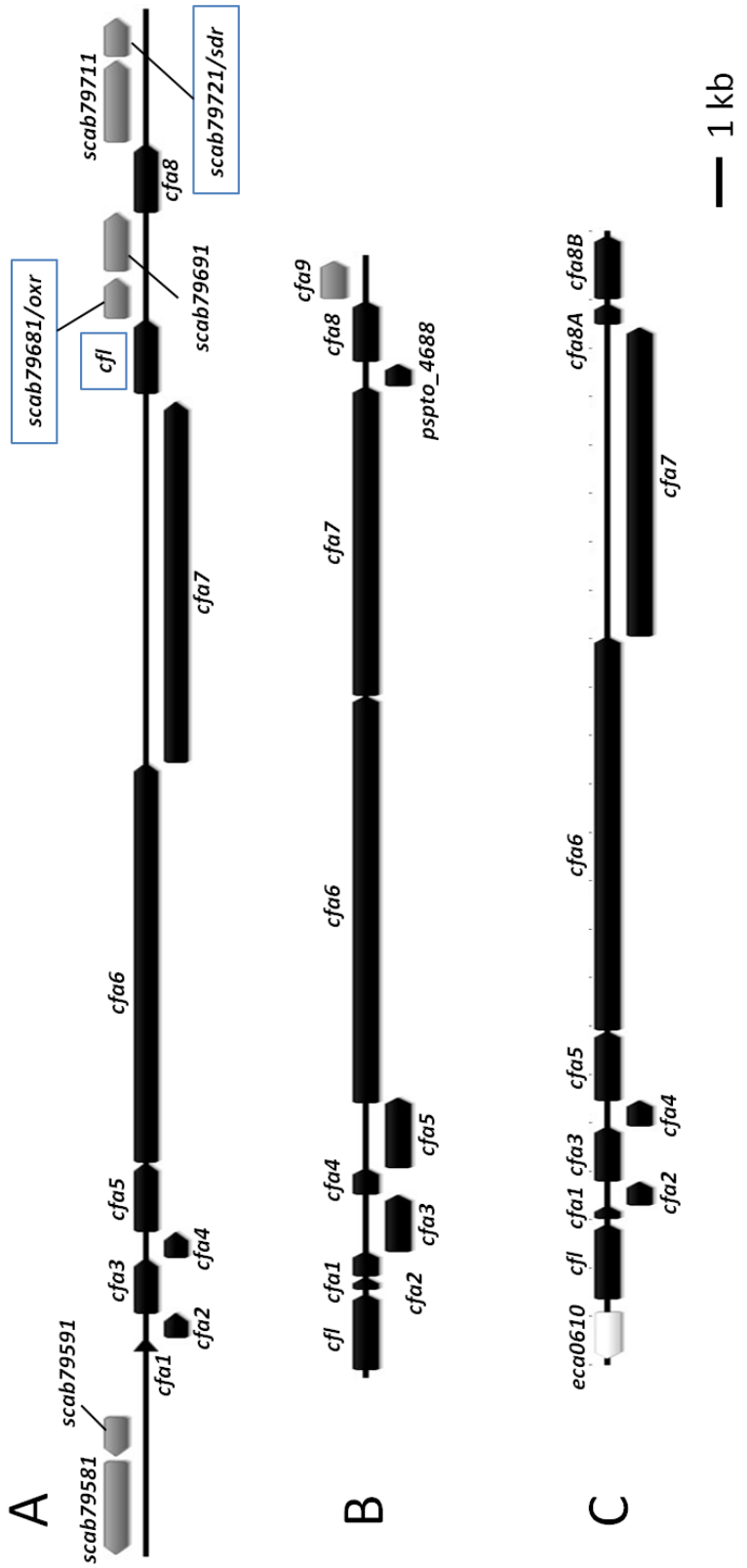
role for this gene in plant-microbe interactions. One possibility is that it helps to suppress induced defense responses in the plant host, a function that has been demonstrated for a tomatinase enzyme from the fungal pathogen *Septoria lycopersici* (Bouarb et al. 2002).

In *S. turgidiscabies*, the *tomA* gene is localized together with *nec1* and the thaxtomin A biosynthetic genes on a large (660 kb) pathogenicity island (PAI) that can be transferred to other *Streptomyces* spp. under laboratory conditions (Kers et al. 2005). It is thought that the mobilization of this PAI among *Streptomyces* spp. may be a contributing factor to the emergence of new pathogenic species in the environment (Loria et al. 2006).

1.5 The *S. scabies* COR-like metabolites

The recently available genome sequence for *S. scabies* 87-22 (http://strepdb.streptomyces.org.uk) has made it possible to use comparative genomic analyses for identifying genes that are conserved in other plant pathogenic microorganisms, and therefore may contribute to the virulence phenotype of *S. scabies* (Bignell et al. 2010a). Such an approach led to the initial discovery of the *S. scabies* CFA-like gene cluster, which as discussed below is involved in the biosynthesis of COR-like molecules by this organism.

The CFA-like biosynthetic gene cluster is ~31 kb in size and consists of at least 15 genes (Fig. 1.4). It is absent from the genome sequences of other *Streptomyces* bacteria; however, it is highly similar in structure and organization to the CFA biosynthetic gene cluster found in several pathovars of the Gram-negative plant pathogenic bacterium *Pseudomonas syringae*, and to the predicted CFA biosynthetic gene cluster that was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cfa1
cfa2
cfa3
cfa4
cfa5
cfa6
cfa7
cfa8A
cfa8B
1 kb

Figure 1.4: The CFA-like gene cluster from *Streptomyces scabies* 87-22 (A) and the CFA gene cluster from *Pseudomonas syringae* pv *tomato* DC3000 (B) and *Pectobacterium atrosepticum* SCRI1043 (C). Black arrows indicate genes that are conserved in all three clusters, the white arrow indicates a putative regulatory gene in the *P. atrosepticum* cluster, and the gray arrows indicate genes that are only found in the respective gene clusters. The genes in the *S. scabies* cluster that are the focus of this study are indicated by the blue boxes.

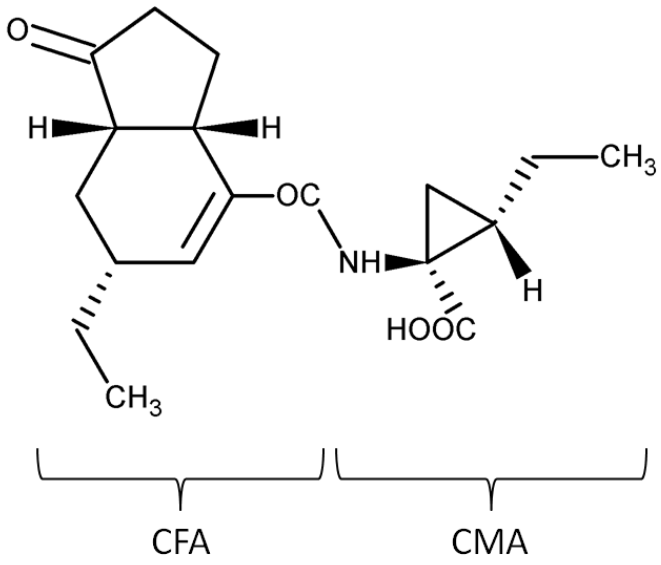

Figure 1.5: Structure of the *Pseudomonas syringae* COR phytotoxin consisting of CFA linked to CMA.

identified in the genome sequence of the potato soft rot pathogen *Pectobacterium atrosepticum* (Fig. 1.4; Bignell et al. 2010b). In *P. syringae,* the CFA biosynthetic gene cluster is responsible for producing CFA, which is the polyketide component of the COR phytotoxin (Fig. 1.5) (Bender et al. 1999a,b). COR is required for the full virulence phenotype of *P. syringae* (Bender et al. 1996, 1999a,b; Uppalapati et al. 2008), and the CFA gene cluster in *P. atrosepticum* has also been shown to contribute to the virulence phenotype of that organism (Bell et al. 2004). Recently, it was shown that the *S. scabies* CFA-like gene cluster is expressed *in planta,* and that a mutant containing a deletion of the *cfa6* gene in the cluster caused reduced stunting and tissue necrosis of tobacco (*Nicotiana tabacum*) roots as compared to the wild-type strain (Bignell et al. 2010a,b). This suggests that the CFA-like gene cluster also contributes to host-pathogen interactions, though it is not essential for plant pathogenicity in *S. scabies*.

The CFA-like gene cluster of *S. scabies* 87-22 contains homologues of the *cfl* and *cfa1-8* genes that are present in the CFA gene clusters from *P. syringae* and *P. atrosepticum* (Fig. 1.4). In *P. syringae, cfa1-3* encode type II PKS proteins (Bender et al. 1999a,b; Penfold et al. 1996), which together with the *cfa4* and *cfa5* protein products are thought to participate in the biosynthesis of 2-carboxy-2-cyclopentenone (CPC) from ∝-ketoglutarate and malonyl-CoA (Fig. 1.6). CPC is then believed to serve as a starter unit for polyketide biosynthesis by the modular type I PKS encoded by *cfa6* and *cfa7* (Fig. 1.6). The production of CFA from CPC requires ethylmalonyl-CoA and malonyl-CoA as extender units, and the *cfa8*-encoded crotonyl-CoA reductase (CCR) is believed to synthesize ethylmalonyl-CoA from crotonyl-CoA as a means of ensuring a sufficient

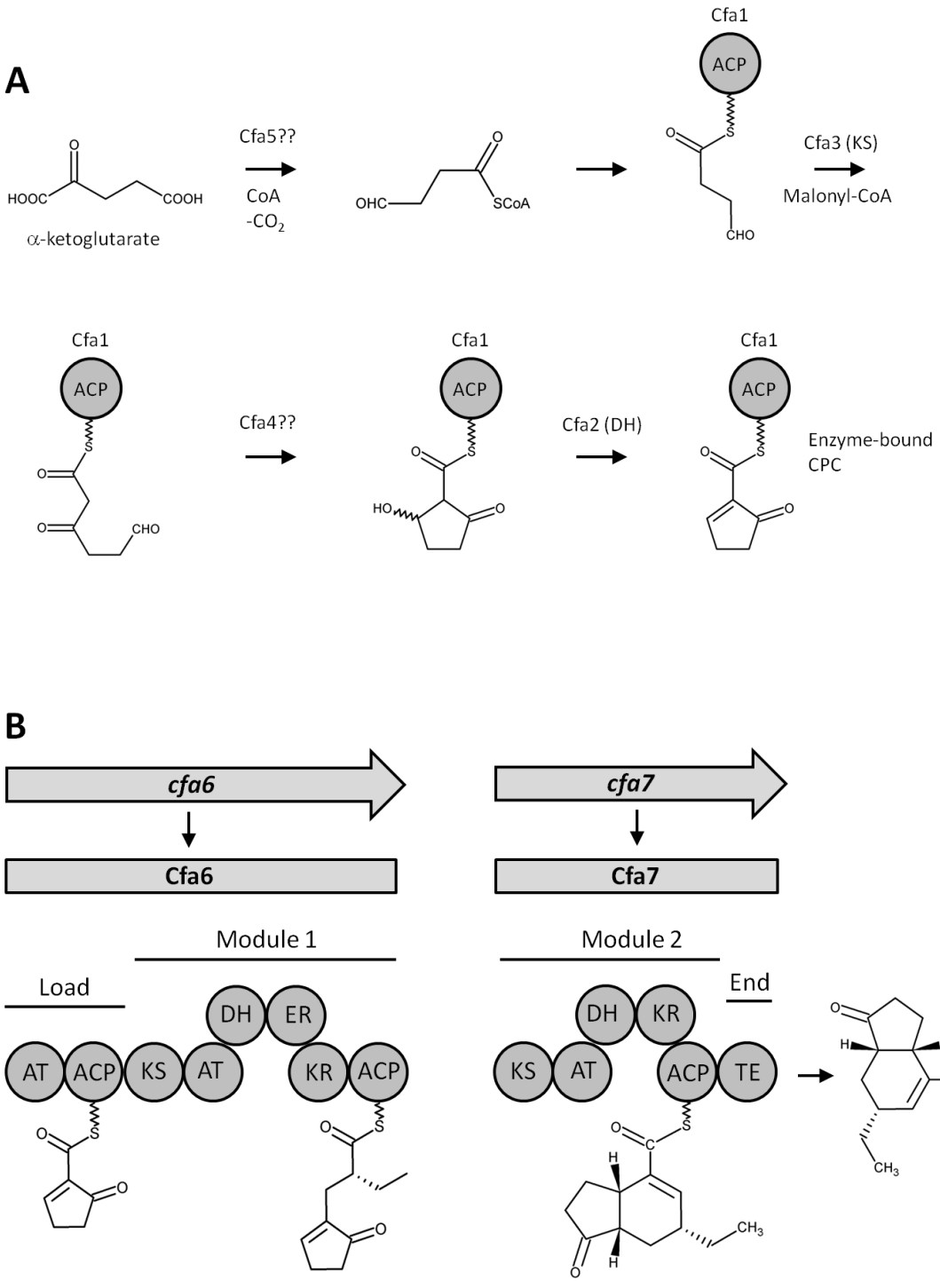
A
Cfa1
ACP
HOOC
COOH
α-ketoglutarate
Cfa5??
CoA
-CO2
OHC
SCoA
Cfa3 (KS)
Malonyl-CoA
CHO
Cfa1
ACP
Cfa4??
Cfa1
ACP
HO
Cfa2 (DH)
Cfa1
ACP
Enzyme-bound
CPC
CHO
B
cfa6
Cfa6
cfa7
Cfa7
Module 1
Module 2
Load
End
DH
ER
AT
ACP
KS
AT
KR
ACP
DH
KR
KS
AT
ACP
TE
COOH
CFA
CH3
CH3

Figure 1.6: The hypothetical biosynthetic pathway for CFA in *Pseudomonas syringae.* (A) The type II PKS enzymes Cfa1, Cfa2 and Cfa3, together with Cfa4 and Cfa5, are thought to be involved in the biosynthesis of 2-carboxy-2-cyclopentenone (CPC). CPC may then be directly transferred to the Cfa6 type I PKS. (B) Organization of the *cfa6* and *cfa7* type I PKS genes and their corresponding protein domains. The PKS consists of a load module, which specifies the starter unit (CPC), and two chain extension modules (module 1 and 2), which specify the extension units ethylmalony-CoA and malonyl-CoA, respectively. The completed CFA molecule is then released from the Cfa7 PKS by the thioesterase domain. CoA, coenzyme A; KS, β-ketoacyl synthase; ACP, acyl carrier protein; DH, dehydratase; AT, acyltransferase; ER, enoylreductase; KR, β-ketoacyl reductase; TE, thioesterase. Adapted from Rangaswamy et al. (1998).

supply of the extender unit for CFA biosynthesis (Rangaswamy et al. 1998). Once completed, the CFA molecule is coupled to CMA via an amide bond to form the final COR phytotoxin (Fig. 1.7). CMA is an ethylcyclopropyl amino acid derived from isoleucine (Fig. 1.7), and the genes involved in its biosynthesis (*cmaABCDELT)* form a cluster that is separate from the CFA gene cluster (Brooks et al. 2004; Mitchell et al. 1994; Rangaswamy et al. 1998). Interestingly, the genome sequences of *P. atrosepticum* and *S. scabies* do not contain homologues of the *cma* genes, indicating that neither of these organisms is able to produce COR (Bell et al. 2004; Bignell et al. 2010b). However, the *cfl* gene, which encodes the coronafacate ligase that is thought to link CMA and CFA together during COR biosynthesis (Fig. 1.7; Bender et al. 1999a,b) is conserved in *P. atrosepticum* and *S. scabies* (Fig. 1.4). It is believed that the Cfl enzyme has a relaxed substrate specificity as *P. syringae* is able to produce a variety of COR-like molecules in which CFA is linked to different amino acids such as isoleucine, valine, norvaline, serine and threonine (Fig. 1.7; Bender et al. 1999a,b; Ichihara and Toshima 1999), and a similar situation is thought to occur in *P. atrosepticum* and *S. scabies*. In addition, it has been reported that the *P. syringae cfl* may also play a role in CFA biosynthesis as a nonpolar *cfl* mutant was unable to accumulate CFA in culture supernatants (Rangaswamy et al. 1997).

In addition to the *cfl* and *cfa1-8* genes, the *S. scabies* CFA-like biosynthetic gene cluster contains six genes that are not present in the *P. syringae* and *P. atrosepticum* CFA clusters (Fig. 1.4). One gene, *scab79711*, encodes a putative 3-hydroxybutyryl-CoA dehydrogenase that potentially functions to reduce acetoacetyl-CoA to 3-hydroxybutyryl-CoA, which is an intermediate in crotonyl-CoA biosynthesis (Chan et al. 2009). As

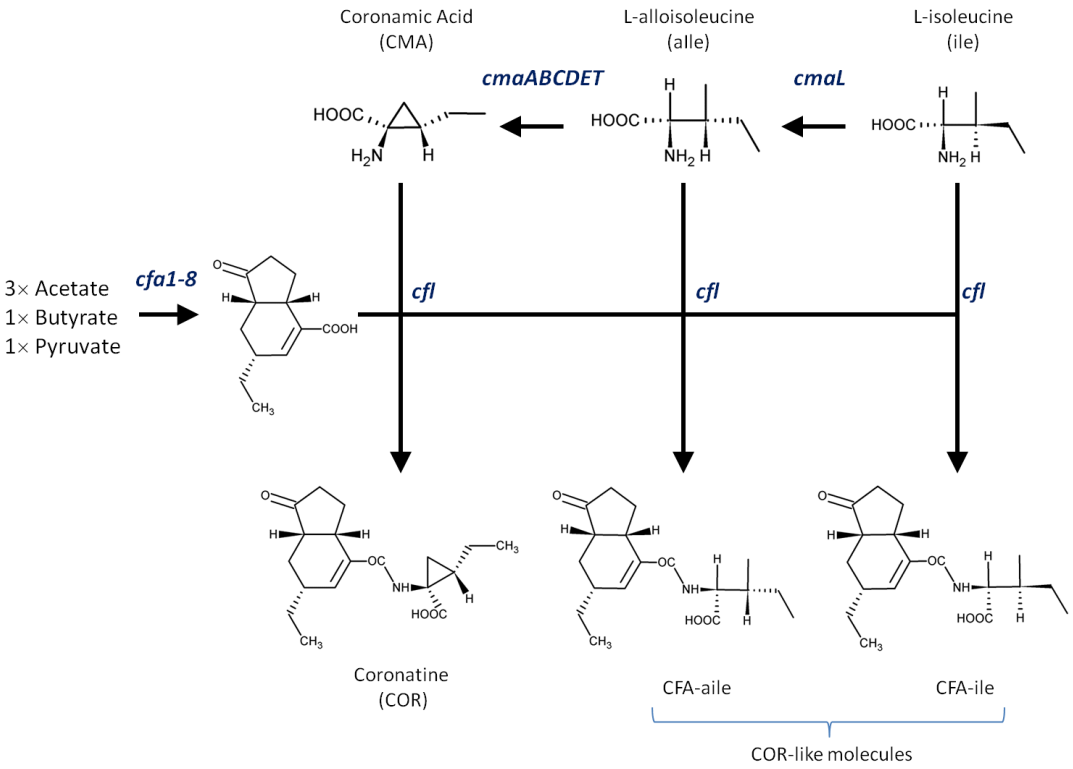

Figure 1.7: Schematic diagram of the biosynthetic pathways leading to production of COR and COR-like molecules in *Pseudomonas syringae.* Adapted from Bender et al. (1999b).

mentioned above, crotonyl-CoA is a substrate for the *cfa8*- encoded CCR enzyme, and it has been proposed that the inclusion of both *cfa8* and *scab79711* in the CFA-like gene cluster ensures that there is a sufficient supply of the ethylmalonyl-CoA extender unit for polyketide biosynthesis (Bignell et al. 2010b). The gene *scab79691* encodes a putative P450 monooxygenase that might function to modify the CFA backbone following polyketide biosynthesis. Another gene, *scab79591,* encodes a protein that belongs to a novel family of *Streptomyces* transcriptional regulators associated with antibiotic biosynthetic gene clusters. The protein contains an N-terminal PAS domain and a C-terminal LuxR-family DNA binding domain, and it has been demonstrated that it functions as a transcriptional activator of the CFA-like biosynthetic genes (Bignell et al. 2010b). *scab79591* is co-transcribed with a downstream gene (*scab79581*) that encodes a ThiF-family protein of unknown function (Bignell et al. 2010b). Finally, *scab79681* (*oxr*) and *scab79721* (*sdr*) encode a putative oxidoreductase and a short chain dehydrogenase/reductase, respectively, and it has been proposed that they may play a role in modification of the CFA backbone (Bignell et al. 2010b). As discussed in Section 1.7, the role(s) of these two genes in the biosynthesis of the *S. scabies* COR-like metabolites is the focus of the research presented in this thesis.

1.6 Possible roles of the *S. scabies* COR-like metabolites in plant pathogenicity

Based on sequence analysis of the CFA-like gene cluster, it was previously proposed that *S. scabies* may synthesize novel COR-like metabolites that may or may not function in a similar manner as the COR phytotoxin (Bignell et al. 2010b). COR is produced by at least five pathovars of *P. syringae,* including *atropurpurea, glycinea,*

maculicola, *morsprunorum*, and *tomato*, and it plays several roles during the host infection process (reviewed in Xin and He 2013). For example, many plants treated with pure COR will develop diffuse chlorosis (the exception is *Arabidopsis thaliana*, which develops a purple colour at the site of inoculation due to anthocyanin accumulation; Bent et al. 1992), and COR-deficient mutants of *P. syringae* pv *tomato* cause reduced chlorosis and necrosis of plant tissue as compared to the wild-type strain (Uppalapati et al. 2008; Worley et al. 2013; Zeng et al. 2011). Furthermore, COR is known to induce hypertrophy of potato tuber tissue, and it can inhibit root elongation in different plants (Bender et al. 1999a; Kenyon and Turner 1992; Sakai et al. 1979). Together, these results suggest that COR contributes to disease symptom development during infection by *P. syringae.* COR also has a role in promoting the growth and maintenance of *P. syringae* inside the plant apoplast, it assists *P. syringae* in overcoming stomatal defenses, and it induces disease susceptibility in systemic plant tissues (Bender et al.1987, 1999b; Brooks et al. 2004; Cui et al. 2005; Elizabeth and Bender 2007; Melotto et al. 2006; Uppalapati et al. 2007). At the molecular level, COR has been shown to function as an antagonist of the JA-Ile receptor in plants (Geng et al. 2012; Ichihara and Toshima 1999), thereby allowing it to activate the expression of JA-responsive genes (Lee et al. 2013; Uppalapati et al 2005). This, in turn, leads to suppression of SA-mediated defense signaling, and SA-mediated signaling is vital for host resistance against *P. syringae* (Brooks et al. 2005; Geng et al. 2012; Lee et al. 2013). There is also evidence that COR suppresses plant defenses in an SA-independent manner (Geng et al. 2012), which suggests that COR is a multifunctional suppressor of host defense responses.

Preliminary results from the Bignell laboratory indicate that the metabolites

produced by the *S. scabies* CFA-like gene cluster have similar chemical and bioactive properties as COR. The compounds can be extracted from *S. scabies* culture supernatants using the same conditions described previously for COR (Palmer and Bender 1993), and the resulting extracts have the ability to induce hypertrophy of potato tuber tissue in a similar manner as pure COR (Bignell et al. 2014). When the extracts are subjected to HPLC analysis, at least five different UV-absorbing peaks can be detected that correspond to metabolites associated with the CFA-like gene cluster, of which two have been identified as the COR-like molecules CFA-Val and CFA-Ile (J. Fyans, personnel communication). CFA-Val has been reported to cause similar effects on plants as COR, although its activity is reduced in comparison to COR (Uppalapati et al. 2005). As such, it is hypothesized that the *S. scabies* COR-like metabolites function in the suppression of plant defense responses, and they may have other roles during *S. scabies* infection process as seen with COR. For example, they may enhance the severity of disease symptoms induced by *S. scabies*, and they may also promote the growth of the organism inside the plant host.

1.7 Thesis objectives

The *S. scabies* COR-like metabolites are known to contribute to host-pathogen interactions, and as such it is important to understand how such molecules are produced in this organism. The CFA-like gene cluster responsible for the biosynthesis of the metabolites contains a number of genes that do not exist in the corresponding CFA gene cluster in *P. syringae* (Fig. 1.4). Two of these genes, *oxr* and *sdr*, are predicted to encode an oxidoreductase and a short chain dehydrogenase, respectively. Previously, it was

demonstrated that both genes are co-transcribed with the other biosynthetic genes in the cluster (Bignell et al. 2010b), suggesting that they are somehow involved in the biosynthesis of the metabolites. However, the lack of homologues in the *P. syringae* CFA biosynthetic gene cluster has made it difficult to predict their role in the metabolite biosynthetic pathway. As such, an objective of this research was to further investigate the involvement of *oxr* and *sdr* in the biosynthesis of the COR-like metabolites in *S. scabies*.

The *cfl* gene in *P. syringae* has been predicted to participate in the linking of CFA to CMA and other amino acids to form COR and COR-like molecules, respectively (Fig. 1.7). As *S. scabies* produces at least two of the COR-like metabolites made by *P. syringae* (CFA-Val and CFA-Ile), and the CFA-like gene cluster contains a *cfl* homologue (Fig. 1.4), it is likely that the *S. scabies cfl* gene plays a similar role in COR-like metabolite biosynthesis as in *P. syringae*. Furthermore, there is evidence from mutational studies in *P. syringae* that *cfl* may also be involved in the biosynthesis of CFA itself (Rangaswamy et al. 1997), though this has not been conclusively demonstrated. As such, another objective of this study was to characterize the role(s) of the *S. scabies cfl* gene in the biosynthesis of the COR-like molecules.

The involvement of *cfl, oxr* and *sdr* in metabolite biosynthesis was addressed by constructing deletion mutants for each gene in *S. scabies* and then assessing each mutant for the ability to produce the COR-like metabolites using HPLC. In addition, the mutants were assessed using a potato tuber disk bioassay in order to determine the effect of each gene deletion on COR-like metabolite bioactivity. The results of this study indicate that all three genes are necessary for normal production of the COR-like metabolites in *S. scabies*, and possible roles for each gene in the biosynthetic pathway are discussed.

CHAPTER 2: MATERIALS AND METHODS

2.1 Bacterial strains, cosmids and plasmids

2.1.1 *Escherichia coli* and *Streptomyces scabies* strains

Escherichia coli and *Streptomyces scabies* strains used in this study are listed in Table 2.1.

2.1.2 Cosmid and plasmid vectors

All cosmids and plasmids that were used or constructed in this study are listed in Table 2.2.

2.2 General DNA techniques

2.2.1 Cloning and digestion of DNA

Plasmid DNA was routinely digested with FastDigest restriction enzymes (Fermentas) according to the manufacturer's instructions. When necessary, the DNA was simultaneously treated with FastAP alkaline phosphatase (Thermo Scientific) in order to prevent the recircularization of the DNA in ligation reactions. Ligation reactions were typically performed using gel-purified vector and insert DNA (See Sections 2.2.3 and 2.2.4) in a 10 μl reaction volume using T4 DNA ligase (New England Biolabs) according to the manufacturer's instructions. A molar ratio of 3 (insert):1 (vector) was used in all cases.

Table 2.1: Bacterial strains used in this study

Strains	**Description**	**Antibiotic Resistance**	**Reference or Source**
***E. coli* Strains**			
DH5α	General cloning host	N/A	GibroBRL
NEB 5-alpha	DH5α derivative; high efficiency competent cells used for transformation	N/A	New England Biolabs
BW25113/pIJ790	Host for REDIRECT PCR targeting system	cam^r	Datsenko and Wanner 2000 Gust et al. 2003a,b
ET12567/pUZ8002	Non-methylating host (*dam*$^-$ *dcm*$^-$ *hsdM*$^-$); carries the pUZ8002 plasmid that encodes the machinery for the conjugal transfer of DNA into *Streptomyces*	cam^r, tet^r, kan^r	MacNeil et al. 1992 Kieser et al. 2000
***Streptomyces scabies* Strains**			
Δ*txtA*/pRLDB51-1	*S. scabies* strain containing a deletion of the *txtA* gene and carrying the *scab79591* overexpression plasmid pRLDB51-1	$thio^r$, $apra^r$	Bignell et al. 2010b
Δ*cfl*	Δ*txtA*/pRLDB51-1 derivative containing a deletion of the *cfl* (*scab79671*) gene	$thio^r$, $apra^r$, hyg^r	This study
Δ*oxr*	Δ*txtA*/pRLDB51-1 derivative containing a deletion of the *oxr* (*scab79681*) gene	$thio^r$, $apra^r$, hyg^r	This study
Δ*sdr*	Δ*txtA*/pRLDB51-1 derivative containing a deletion of the *sdr* (*scab79721*) gene	$thio^r$, $apra^r$, hyg^r	This study

N/A, not applicable

Table. 2.2: Plasmid and cosmid vectors used in this study

Plasmid or Cosmid	Description	Antibiotic Resistance	Reference or Source
pIJ10700	Template for PCR amplification of the [*hyg+oriT*] cassette used for PCR targeting	hyg^r	Gust et al. 2003b
pIJ10257	Integrates into ΦBT1 *attB* site in *Streptomyces* chromosomes; contains the strong, constitutive promoter *ermE*p* for expression of cloned genes in *Streptomyces*	hyg^r	Hong et al. 2005
pMSAK13	pIJ10257 derivative containing the *neo* gene and promoter sequence cloned into the EcoRV site	hyg^r, kan^r	This study
pGEM® - T Easy	Cloning vector for PCR products	kan^r	Promega
pMSAK13/*cfl*	pMSAK13 derivative containing the *cfl* gene cloned into the NdeI and XhoI sites	hyg^r, kan^r	This study
pMSAK13/*oxr*	pMSAK13 derivative containing the *oxr* gene cloned into the NdeI and XhoI sites	hyg^r, kan^r	This study
pMSAK13/*sdr*	pMSAK13 derivative containing the *sdr* gene cloned into the NdeI and XhoI sites	hyg^r, kan^r	This study
Cosmid 1770	SuperCos1 derivative containing the *S. scabies* CFA-like gene cluster	amp^r, kan^r	Bignell et al. 2010b
1770/Δ*cfl*	Cosmid 1770 derivative in which the *cfl* gene was replaced with the [*hyg+oriT*] disruption cassette	amp^r, kan^r, hyg^r	This study
1770/Δ*oxr*	Cosmid 1770 derivative in which the *oxr* gene was replaced with the [*hyg+oriT*] disruption cassette	amp^r, kan^r, hyg^r	This study
1770/Δ*sdr*	Cosmid 1770 derivative in which the *sdr* gene was replaced with the [*hyg+oriT*] disruption cassette	amp^r, kan^r, hyg^r	This study

Ligation of PCR products was routinely performed using the pGEM®-T Easy vector system according to the manufacturer's instructions (Promega). Prior to ligation, the PCR product was gel-purified and then subjected to A-tailing in order to add A residues to the 3′ ends of the PCR product. The A-tailing reaction consisted of 1 – 7 µl of the purified PCR product, 1× Taq reaction buffer with KCl, 2.5 mM $MgCl_2$, 0.2 mM dATP and 5 U of Taq DNA polymerase (New England Biolabs). The reaction was incubated at 70°C for 30 min, and then 1 – 2 µl was used in the ligation reaction with the pGEM®-T Easy vector.

Ligation reactions were routinely incubated overnight at 4°C, after which 2 – 5 µl was transformed into chemically-competent *E. coli* DH5α cells as described in Section 2.3.4, or into commercially available NEB 5-alpha high efficiency competent *E. coli* cells (Table 1) according to the manufacturer's instructions. When possible, transformants containing the desired cloned inserts were selected by plating onto solid medium containing the appropriate antibiotics as well as X-gal (80 µg/ml) and IPTG (0.5 mM) as described before (Sambrook et al. 1989).

2.2.2 PCR

Polymerase chain reaction (PCR) was used to amplify the *cfl*, *oxr*, *sdr* and *neo* genes for cloning into the pGEM®-T Easy vector. Amplification was conducted in a 50 µl reaction volume in 0.2 ml thin-walled PCR tubes (VWR International) using a Bio-Rad C1000 thermal cycler with a heated lid. Typical reaction conditions consisted of 100 – 200 ng of Cosmid 1770 DNA template, 1× HF buffer, 200 µM of dNTPs, 50 pmol of

forward and reverse primer, 5% v/v DMSO, and 1 U of Phusion DNA polymerase (New England Biolabs). The cycling conditions consisted of an initial denaturation step (98°C for 2 min) followed by 30 – 35 cycles of denaturation (98°C for 30 sec), primer annealing (58 – 60°C for 30 sec) and extension (72°C for 15 sec/kb). This was followed by a final extension step (72°C for 5 min).

PCR was also used to construct the Δ*cfl*, Δ*oxr*, and Δ*sdr* mutant cosmids as part of the Redirect PCR targeting system (Gust et al. 2003a,b). Reactions were conducted in 50 μl volumes and consisted of 50 – 100 ng of pIJ10700 plasmid template, 1× Taq Reaction buffer with KCl, 2 mM $MgCl_2$, 200 μM dNTPs, 50 pmol of forward and reverse primer, 5% v/v DMSO, and 2.5 U of Taq DNA polymerase (Fermentas). The cycling conditions used consisted of an initial denaturation step (95°C for 2 min) followed by 10 cycles of denaturation (95°C for 45 sec), primer annealing (50°C for 45 sec), and extension (72°C for 90 sec). This was followed by an additional 15 cycles of denaturation (95°C for 45 sec), primer annealing (55°C for 45 sec), and extension (72°C for 90 sec). A final extension step (72°C for 5 min) was then conducted.

Additionally, PCR was used to verify the constructed mutant cosmids (1770/Δ*cfl*, 1770/Δ*oxr*, 1770/Δ*sdr*), and to verify the resulting *S. scabies* Δ*cfl*, Δ*oxr* and Δ*sdr* mutant strains. Reactions were performed in 25 μl volumes and consisted of 100 – 200 ng of cosmid or genomic DNA template, 1× Taq Reaction buffer with KCl, 2 mM $MgCl_2$, 200 μM dNTPs, 25 pmol of forward and reverse primer, 5% v/v DMSO, and 1.25 U of Taq DNA polymerase (Fermentas). The cycling conditions used consisted of an initial

denaturation step (95°C for 2 min) followed by 30 cycles of denaturation (95°C for 30 sec), primer annealing (60°C for 30 sec), and extension (72°C for 1 min/kb).

Finally, PCR was used to screen for the presence of the desired insert during construction of the *cfl, oxr,* and *sdr* complementation plasmids. Reactions were performed in 20 μl volumes and consisted of 2 – 5 μl of an overnight culture of *E. coli* containing the *cfl, oxr,* or *sdr* complementation plasmid, 1× HF buffer, 200 μM of dNTPs, 50 pmol of forward and reverse primer, 5% v/v DMSO, and 1 U of Phusion DNA polymerase (New England Biolabs). The cycling conditions consisted of an initial denaturation step (98°C for 2 min) followed by 30-35 cycles of denaturation (98°C for 30 sec), primer annealing (58 – 60°C for 30 sec) and extension (72°C for 15 sec/kb).

All primers used for PCR amplification are listed in Table 2.3.

Table 2.3: Oligonucleotide primers used in this study

Primer Name	Sequence* (5′ → 3′)	Use
DRB651	ATGTCCGTCTTCTCGGTCTTCCAGCAGGTCGCGCA ACGAATTCCGGGGATCCGTCGACC	Redirect primer for Δ*cfl* mutant cosmid construction
DRB652	TCACGACGCGCTCTCCTCGGCCGCCGCGCGGGAC GCGTCTGTAGGCTGGAGCTGCTTC	Redirect primer for Δ*cfl* mutant cosmid construction
DRB653	GTGAAGTTCGGCTTCGTCATGTTCCCCACCCAGGA CGCCATTCCGGGGATCCGTCGACC	Redirect primer for Δ*oxr* mutant cosmid construction
DRB654	TCAGCGCAGGCGGTCGCCGAGTTGCAGGGCGATC CGGTCTGTAGGCTGGAGCTGCTTC	Redirect primer for Δ*oxr* mutant cosmid construction
DRB655	ATGCAAGACAGGTTCACCGACAAGGTCGCCCTGA TAACCATTCCGGGGATCCGTCGACC	Redirect primer for Δ*sdr* mutant cosmid construction
DRB656	TCAGGCCCGCTCGGAGCGGCCACCGTCGATCGTC ACCACTGTAGGCTGGAGCTGCTTC	Redirect primer for Δ*sdr* mutant cosmid construction
DRB662	GTCGAAGTCGACGAAGTCG	Verification of Δ*cfl* mutant cosmid and strain (junction 2)
Red-Down	CGAAGCAGCTCCAGCCTAC	Verification of Δ*cfl* mutant cosmid and strain (junction 2)
DRB663	AGCCACTTCACCCTGCTGA	Verification of Δ*cfl* mutant cosmid and strain (junction 1)
Red-Up	CTGCAGGTCGACGGATCC	Verification of Δ*cfl* mutant cosmid and strain (junction 1)
DRB664	ATCCCCGTGGACAGGCTCCC	Verification of Δ*oxr* mutant cosmid and strain
DRB665	CTTGCCGGTCTGCCGGTCTG	Verification of Δ*oxr* mutant cosmid and strain
MSA1	ACTTCATGCCGTCGCCCGTG	Verification of Δ*sdr* mutant cosmid and strain
MSA2	AGGGGCGGGTCAAGGACCTC	Verification of Δ*sdr* mutant cosmid and strain
MSA5	GCGC**CATATG**TCCGTCTTCTCGGTCT	PCR amplification of *cfl* for construction of complementation plasmid
MSA7	GCGC**CATATG**CAAGACAGGTTCACCG	PCR amplification of *sdr* for construction of complementation plasmid
MSA9	GCGC**CATATG**AAGTTCGGCTTCGTCA	PCR amplification of *oxr* for construction of complementation plasmid
MSA11	GCGC**GATATC**CCGCCTCGGCCTCTGAGCTA	Amplification of *neo* gene and promoter
MSA12	GCGC**GATATC**ATCCCCGCGCTGGAGGATCA	Amplification of *neo* gene and promoter
MSA13	GCGC**CTCGAG**TCACGACGCGCTCTCCTCG	PCR amplification of *cfl* for construction of

		complementation plasmid
MSA14	GCGC**CTCGAG**TCAGGCCCGCTCGGAGCGG	PCR amplification of *sdr* for construction of complementation plasmid
MSA15	GCGC**CTCGAG**TCAGCGCAGGCGGTCGCCG	PCR amplification of *oxr* for construction of complementation plasmid
ermEp*	GCGATGCTGTTGTGGGC	Sequencing of cloned inserts in pIJ10257/kan
M13F	CGCCAGGGTTTTCCCAGTCACGAC	Sequencing of pGEM®-T Easy inserts
M13R	TCACACAGGAAACAGCTATGAC	Sequencing of pGEM®-T Easy inserts

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

2.2.3 Agarose gel electrophoresis of DNA

This procedure was routinely used for size estimation and/or purification of PCR products and restriction digestion products that ranged from 0.5 – 10 kb in size. Samples were prepared by adding 2 – 5 μl of 6× DNA loading dye (Fermentas) and were then separated on a 1% w/v agarose gel (Fisher Scientific) in 1× TBE buffer using a voltage of 80 – 130V. The 1KB DNA ladder RTU (GeneDireX) was routinely used as a molecular weight marker for band size estimation. To visualize the DNA, the EZ-Vision® In-Gel stain (Amresco) was added to the molten agarose gel (1× final concentration) prior to gel casting, and the gel was illuminated under UV light after electrophoresis was completed. Alternatively, the gel following electrophoresis was stained for 30 min in a 1× TBE solution containing ethidium bromide, and was then destained for 10 – 15 min in 1× TBE prior to illumination under UV light. The latter procedure was used whenever the DNA was to be extracted and purified from the gel as described in Section 2.2.4. All gel images were acquired using Alpha Innotech Chemi-imager Gel Documentation System with a Fluorchem HD2 upgrade.

2.2.4 Gel purification of DNA

Following electrophoresis, the desired DNA fragments were excised from the agarose gel using a scalpel. The DNA was then extracted from the gel slice using the Wizard® SV Gel and PCR Clean-Up kit (Promega) according to the manufacturer's instructions.

2.2.5 DNA quantification

DNA samples were routinely quantified using an Implen nanophotometer according to the manufacturer's instructions.

2.2.6 DNA sequencing

DNA sequencing was performed by The Centre for Applied Genomics in Toronto. Reactions consisted of 200 – 300 ng of highly pure plasmid template and 5 pmol of primer. Primers used for sequencing are listed in Table 2.3.

2.3 General *E. coli* procedures

2.3.1 Propagation and maintenance of *E. coli* strains

Strains were routinely grown at 37°C unless otherwise indicated. Liquid cultures were grown with shaking (200-250 rpm) in Difco™ LB Lennox broth (BD Biosciences), low salt LB broth (1% w/v tryptone; 0.5% w/v yeast extract; 0.25% w/v NaCl), SOB (Sambrook et al. 1989) or SOC (New England Biolabs), while solid cultures were grown on LB (Lennox or low salt) medium containing 1.5% w/v agar (BD Biosciences). When necessary, the solid or liquid growth medium was supplemented with ampicillin (100 μg/ml; Amresco), kanamycin (50 μg/ml; Calbiochem), hygromycin B (100 μg/ml; Calbiochem) or chloramphenicol (25 μg/ml; MP Biomedicals, LLC). Hygromycin B was used exclusively with the low salt LB medium as the activity of this antibiotic is negatively affected by the salt concentration in the LB Lennox medium. All strains were maintained at - 80°C as frozen stocks in 20% v/v glycerol.

2.3.2 Preparation of *E. coli* glycerol stocks

E. coli strains were cultured overnight in 2 – 5 ml of LB or low salt LB liquid medium, with or without antibiotics. One ml of the overnight culture was placed into a sterile 1.5 ml microcentrifuge tube and was centrifuged at high speed (14000 × *g*) at room temperature for 2 min. The supernatant was discarded, and the cell pellet was re-suspended in 0.5 – 1 ml of sterile 20% v/v glycerol. The tube was then stored at -80°C.

2.3.3 Preparation of chemically competent *E. coli* cells

An overnight culture was prepared by inoculating cells from an agar plate or a glycerol stock into 2 – 5 ml of LB broth (containing the appropriate antibiotics, if necessary) and then incubating at 37°C with shaking for 16 – 24 hrs. The overnight culture (500 μl) was then subcultured into 50 ml of LB broth (± antibiotics), and the flask was incubated at 37°C with shaking until the OD_{600} = 0.2, after which $MgCl_2$ was added to a final concentration of 20 mM. The culture was incubated for an additional for 1 – 2 hrs or until the culture OD_{600} = 0.4 – 0.6. The culture was immediately placed on ice and left to chill for 2 hrs, after which it was centrifuged at 2559 × *g* for 5 min at 4°C. The cell pellet was re-suspended in 10 ml of a freshly-prepared, ice-cold Ca^{2+} Mn^{2+} solution (100 mM $CaCl_2.2H_2O$, 70 mM $MnCl_2.4H_2O$, and 40 mM $NaOAC.3H_2O$; pH = 5.5) that had been filter-sterilized. The cells were kept on ice for an additional 45 min and were then centrifuged as described above. The pellet was re-suspended in 1 – 2 ml of the ice-cold Ca^{2+} Mn^{2+} solution containing 20% v/v glycerol, after which the suspension was quickly

aliquoted (100 μl per aliquot) into sterile, pre-chilled 0.2 ml PCR tubes. Finally, the cells were flash-frozen in a dry ice- ethanol bath and stored at - 80° C.

2.3.4 Transformation of DNA into chemically-competent *E. coli* cells

Plasmid or cosmid DNA (1 – 5 μl) was routinely mixed with 50 – 100 μl of chemically-competent cells, and the mixture was left on ice for 30 min. The cells were then heat-shocked by incubating at 37°C for 5 min, after which they were placed back onto ice for 1 – 2 min. LB or SOC liquid medium was next added to bring the volume up to 1 ml, and the cells were incubated at 37°C for 1 hr with shaking at 250 rpm. The cells were then plated onto LB agar medium containing the proper antibiotics, and the plates were incubated overnight at 37°C.

2.3.5 Preparation of electrocompetent *E. coli* cells

Electrocompetent cells of BW25113/pIJ790 were prepared by inoculating a single colony from an LB agar plate containing chloramphenicol into 2 – 5 ml of LB broth containing chloramphenicol, and then incubating overnight with shaking at 28°C. The overnight culture (500 μl) was then subcultured into 50 ml of SOB medium containing chloramphenicol, and the culture was incubated at 28°C for 3 – 4 hrs or until the OD_{600} = 0.4 – 0.6. The cells were immediately transferred to pre-chilled centrifuge tubes and were pelleted by centrifugation at 2559 × *g* for 5 min at 4°C. After decanting the supernatant, the cells were washed twice with ice-cold 10% v/v glycerol (50 ml for the first wash, 25

ml for the second wash) and were resuspended in ~100 µl of 10% glycerol. The cells were then used immediately for electroporation.

Electrocompetent cells of the BW25113/pIJ790 strain containing Cosmid 1770 (used for construction of the Δ*cfl*, Δ*oxr*, and Δ*sdr* mutant cosmids by PCR targeting) were prepared as described above, except that the growth medium used contained chloramphenicol and kanamycin. Furthermore, L-arabinose (10mM final concentration) was added to the 50 ml LB culture to induce expression of the λRed recombinase enzymes encoded on the pIJ790 plasmid.

2.3.6 Electroporation of DNA into electrocompetent *E. coli* cells

Electroporation was used to introduce the Cosmid 1770 into the BW25113/pIJ790 strain, and was also used to introduce PCR products into the resulting BW25113/pIJ790/Cosmid 1770 strain during PCR targeting. A DNA solution (1 – 5 µl) prepared using sterile water was gently mixed with 50 µl of electrocompetent cells, and the mixture was transferred into a sterile, pre-chilled electroporation cuvette with a 1 mm gap (VWR). Electroporation was then conducted using a Bio-Rad GenePulser Xcell instrument with the following settings: 1.8 kV voltage, 200 Ω resistance, 25 µF capacitance. Immediately after shocking the cells, ice-cold LB or SOC medium was added to bring the volume up to 1 ml, and the cells were incubated at 37°C for 1 hr with shaking. Finally, the cells were plated onto LB agar or low salt LB agar medium containing the appropriate antibiotics, and the plates were incubated overnight at 37°C.

2.3.7 Isolation of plasmid and cosmid DNA from *E. coli*

Plasmid and cosmid DNA was routinely isolated from overnight liquid cultures of *E. coli* using the alkali lysis method described in Sambrook et al. (1989). When highly pure DNA was required, the EZ-10 Spin Column Plasmid DNA kit (Bio Basic Inc.) was used for DNA isolation as described by the manufacturer.

2.4 General *Streptomyces* procedures

2.4.1 Propagation and maintenance of *Streptomyces scabies* strains

Strains of *S. scabies* were routinely grown at 28°C in TSB (BD Biosciences) or SFMB (Kieser et al. 2000) liquid media, or on ISP-4 (BD Biosciences), OBA (Johnson et al. 2007), SFM, or Difco NA (BD Biosciences) solid media. Unless otherwise indicated, liquid cultures were grown in 50 ml or 125 ml Erlenmeyer flasks containing a stainless steel spring, and were shaken at 200 rpm. When necessary, the growth medium was supplemented with hygromycin B (50 μg/ml), apramycin (50 μg/ml; Sigma Aldrich), nalidixic acid (50 μg/ml; Fisher Scientific), kanamycin (50 μg/ml), or thiostrepton (25 μg/ml; Sigma Life Science). Strains were maintained at - 80°C as frozen spore stocks in 20% v/v glycerol or as frozen mycelial stocks in 5% v/v DMSO.

2.4.2 Preparation of *Streptomyces* spore stocks

Strains were cultured on a single ISP-4 or OBA plate (+ antibiotics) for 7 – 10 days or until the gray spore-associated pigment was visible. The spores were then scraped from the plate using a sterile inoculation loop, and were placed into a sterile 1.5

ml microcentrifuge tube. Next, sterile 40% v/v glycerol (0.5 – 1 ml) was added to the tube, and the contents were vortexed for 1 min to resuspend the spores. The tube was then stored at - 80°C.

2.4.3 Preparation of *Streptomyces* mycelial stocks

Strains were grown in 25 ml of TSB in a 125 ml spring flask for ~48 hrs or until the culture was dense. Aliquots of the culture (950 µl) were then transferred to sterile 1.5 ml microcentrifuge tubes containing 50 µl of 100% DMSO (Fisher Scientific). The contents of each tube were mixed by vortexing, and the tubes were stored at - 80°C.

2.4.4 Introduction of DNA into *S. scabies* by intergeneric conjugation with *E. coli*

This was conducted essentially as described by Kieser et al. (2000) with some modifications. Briefly, the *E. coli* ET12567/pUZ8002 strain, containing the plasmid or cosmid to be transferred, was cultured overnight in 2 – 5 ml of LB broth containing the appropriate antibiotics. The overnight culture (500 µl) was subcultured into 50 ml of LB broth (+ antibiotics) and was grown at 37°C until the OD_{600} reached 0.4 – 0.6. The cells were pelleted by centrifugation at $2559 \times g$ at ambient temperature, and were washed twice with LB broth lacking antibiotics. Finally, the cells were resuspended in 500 µl of LB broth.

S. scabies strains were grown on ISP-4 or OBA plates for 7 – 10 days or until well sporulated. The spores were collected by adding 5 – 10 ml of LB broth to the plate and then scraping the spores with a sterile inoculation loop. Next, the spore suspension was

transferred to a sterile 50 ml disposable conical tube, and the spores were pelleted by centrifugation at 2559 × *g* at ambient temperature. The spores were then re-suspended in 500 µl of fresh LB broth.

Intergeneric conjugation was conducted by mixing the *Streptomyces* spore suspension with the *E. coli* cell suspension and then plating the mixture onto two SFM agar plates supplemented with 10 mM $MgCl_2$. The plates were incubated at 28°C for ~24 hrs, after which they were overlaid with a sterile aqueous solution containing nalidixic acid and the appropriate antibiotic to select for the incoming plasmid or cosmid DNA. The plates were then incubated for an additional 7 – 14 days to allow for the emergence of exconjugants.

2.4.5 Construction of *S. scabies* gene deletion mutants

The Δ*cfl*, Δ*oxr* and Δ*sdr* mutant strains were constructed by replacing the target gene in the *S. scabies* chromosome with a DNA cassette [*hyg+oriT*] conferring resistance to hygromycin B. This was accomplished by first constructing mutant cosmids that harboured the [*hyg+oriT*] cassette in place of the target gene using the Redirect PCR targeting system (Gust et al. 2003a,b), and then introducing each mutant cosmid into *S. scabies* by intergeneric conjugation with *E. coli* (Section 2.4.4). Exconjugants that arose which were hyg^r were then patched onto NA containing kanamycin in order to screen for those exconjugants that were kan^s (and therefore had undergone a double cross-over recombination event). Next, up to 6 hyg^r and kan^s exconjugants per mutant strain were streaked for single colonies onto ISP-4 containing hygromycin B and nalidixic acid, and

were incubated for 7- 10 days or until sporulating colonies were observed. Finally, spores from a single colony of each mutant isolate were used to prepare spore stocks for storage as described in Section 2.4.2.

2.4.6 Chromosomal DNA preparation from *Streptomyces*

Strains of *S. scabies* were cultured in 5 ml of TSB medium (± antibiotics) for 48 hrs, after which a 2 ml aliquot of each was transferred to a centrifuge tube and the mycelia were pelleted by centrifugation. The pellets were then used for chromosomal DNA extraction using the One – Tube Bacterial Genomic DNA Kit (Bio Basic Inc.) according to the manufacturer's instructions. The DNA preps were either used immediately for PCR, or were stored at - 20°C.

2.5 Analysis of COR-like metabolite production and bioactivity

2.5.1 Growth of *S. scabies* strains

Seed cultures of *S. scabies* strains were prepared by inoculating 50 µl of a spore stock into 5 ml of TSB medium, or inoculating 1 ml of a mycelial stock into 9 ml of TSB medium, followed by incubation for ~ 24 - 48 hrs or until the culture was dense. A 1% v/v inoculum of each seed culture was then transferred to 5 ml of SFMB in duplicate or triplicate wells in a 6-well plate (VWR). The cultures were incubated for 7 days at ambient temperature (~25°C) with shaking at 125 rpm.

Large-scale production cultures were set up by transferring a 1% v/v inoculum of each seed culture into duplicate 125 ml flasks containing 25 ml of SFMB. The flasks were then incubated at 28°C for 7 days.

2.5.2 Chemical extraction of COR-like metabolites

Small-scale extractions were performed by transferring a 1.5 ml sample of each SFMB culture to a 2 ml microcentrifuge tube and then centrifuging the sample for 2 min at 13,300 × *g* in order to pellet the mycelia. The culture supernatant (1 ml) was then transferred into a clean 2 ml microcentrifuge tubes, and the pH of the supernatant was adjusted to 10 – 11 with NaOH. The supernatant was extracted twice with 0.5 volumes of EtOAc (Fisher Scientific) to remove non-acidic hydrophobic compounds. Next, the aqueous supernatant was adjusted to pH = 1 – 2 with HCl, and was extracted three times with 0.5 volumes of EtOAc to recover the organic acids. The organic extracts for each sample were combined and dried down in a fume hood, and the resulting material was re-dissolved in 100 µl of HPLC – grade 100% MeOH (Sigma Aldrich). Each sample was then filtered using a 13 mm syringe filter (0.2 µm PTFE membrane; VWR International) to remove any undissolved particulates prior to HPLC analysis.

Large-scale extractions were performed as described above except that the entire culture supernatant from a 25 ml SFMB cultures was extracted, and chloroform (Fisher Scientific) was used as the extracting solvent rather than EtOAc. The resulting organic acids were redissolved in 200 µl of HPLC – grade 100% MeOH prior to their use in bioassays.

2.5.3 HPLC analysis

Samples (10 µl volumes) of each culture extract were analyzed by HPLC using an Agilent 1260 Infinity Quaternary LC system with a Poroshell 120 EC-C18 column (4.6 × 50 mm, 2.7 µm particle size; Agilent Technologies Inc.). The initial solvent concentration consisted of ACN (Sigma Aldrich) and water (30:70) with 0.1% v/v formic acid (Sigma Aldrich); after 1.5 min, the concentration was increased linearly to 50:50 ACN:water over a period of 2.5 min. This concentration was held for 1 min, after which the system was returned to the initial solvent conditions (30:70 ACN:water) in 1.5 min using a linear gradient. The column temperature (40°C), flow rate (1 ml/min) and detection wavelength (230 nm) remained constant throughout the analysis. Authentic standards of CFA, CFA-Val and CFA-Ile (provided by Dr. Carol Bender) were included to assist in the identification of the COR-like metabolites. The ChemStation software version B.04.03 (Agilent Technologies Inc.) was used for acquiring and analyzing all of the data.

2.5.4 LC-MS analysis

Samples (10 µl) of each culture extract were analyzed using an Agilent 1100 series HPLC system (Agilent Technologies Inc.) interfaced to a Waters G1946A single quadrupole mass spectrometer (Waters Corporation). Separation was achieved using the same column, solvent gradient system, flow rate and column temperature as described in Section 2.4.3. Detection was by ultraviolet radiation (230 nm) and by negative electrospray ionization MS.

2.5.5 Bioassay for detecting the COR-like metabolites

A potato tuber bioassay was performed as described previously (Bignell et al. 2010b) in order to detect the presence of the COR-like metabolites. Briefly, potato tubers purchased from the supermarket were peeled and surface sterilized for 10 min using a 15% v/v bleach solution (Chlorox), and were then washed twice with sterile water. Next, the tubers were cut into similar size pieces using a sterile knife, and the potato pieces were placed onto Whatman™ filter paper (90 mm diameter) that was pre-wetted with ~2 ml of sterile water in glass Petri dishes. A Whatman™ AA paper disk (6 mm diameter; GE Healthcare Ltd.) was then placed onto each potato piece, and organic acid extracts (25 µl) were pipetted onto each disk. A total of four potato pieces were inoculated per extract, and a pure COR standard (0.1 µg/µl in 100% methanol; Sigma Aldrich) was included as a control. After inoculation, the potato disks were incubated under high humidity for 5 days at ambient temperatures in the dark, after which they were assessed for the presence of tissue hypertrophy.

2.6 Computer-assisted DNA and protein sequence analysis

DNA sequences and gene annotations were visualized using Geneious Pro software version 6.1.2 (Biomatters Ltd.). Protein similarity searches were conducted using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool for proteins (BLASTP) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The Pfam program (http://pfam.sanger.ac.uk/) was used to detect the presence of functional domains within *S. scabies* Cfl Oxr and Sdr protein sequences (Finn et al. 2010). The Cfl protein

sequence alignment was generated using ClustalW, and the Cfl phylogenetic tree was constructed with the maximum likelihood method using the Molecular Evolutionary Genetic Analysis (MEGA) program version 5.10 (Tamura et al. 2011). The significance of the branch order was tested using the bootstrapping method with 1000 repetitions.

CHAPTER 3: RESULTS

3.1 Bioinformatics analysis of Cfl, Oxr and Sdr

The *cfl* gene is thought to play a key role in the biosynthesis of COR and COR-like molecules in different pathovars of *P. syringae*, and homologues of this gene have been found in a number of different bacterial plant pathogens including *P. atrosepticum* (Bell et al. 2004), *Pectobacterium carotovorum* subsp. *brasiliense* (Accession # JQ771052.1), *Pseudomonas amygdale* (Accession # WP_004666804), *Dickeya* spp. (Slawiak and Lojkowska 2009) and in *S. scabies* (Bignell et al. 2010b). Previously, it was noted that the *P. syringae* Cfl protein contains two motifs, SGTTGXXKG and TGD, which are conserved in enzymes that activate carboxylic acids by adenylation (Liyanage et al. 1995). An alignment of the Cfl proteins from the different organisms indicated that the two motifs are absolutely conserved among the proteins with the exception of the *S. scabies* Cfl, which has a slightly varied version of each motif (SGTTGXXKS and SGD; Fig. 3.1). In addition, Pfam analysis indicated that all of the Cfl homologues contain an AMP-binding enzyme domain (PF00501) and an AMP-binding enzyme C-terminal domain (PF13193). It has been proposed that the *P. syringae* Cfl catalyzes the adenylation of CFA and the ligation of the CFA-adenylate to CMA and other amino acids (Bender et al. 1999a,b), and a similar activity could be postulated for the Cfl homologue in *S. scabies* and in the other plant pathogens. The relationship between the different Cfl homologues was determined by constructing a phylogenetic tree, and as shown in Fig. 3.2, the homologues from *Pseudomonas*, *Pectobacterium* and *Dickeya* spp. (all Gram-negative organisms) appear to be more closely related to each other than to the

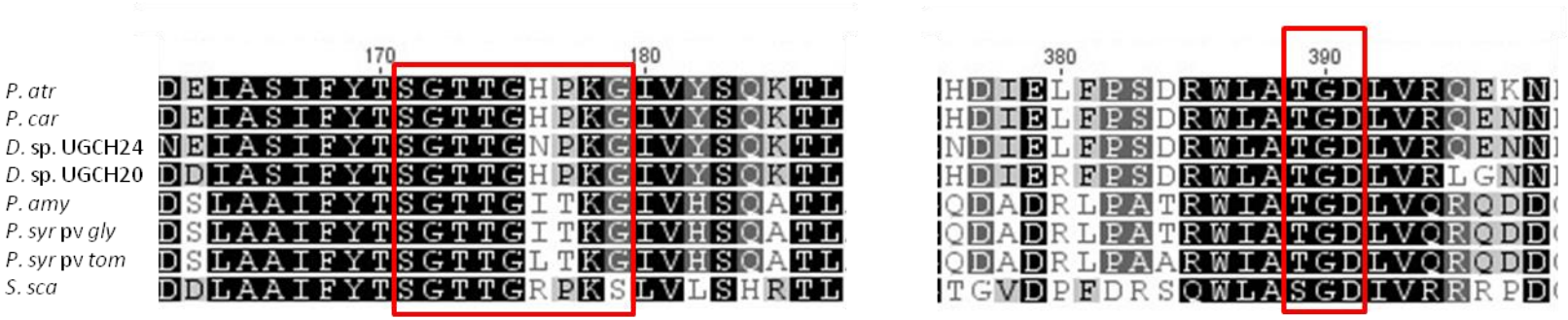
170
180
380
390
P. atr
P. car
D. sp. UGCH24
D. sp. UGCH20
P. amy
P. syr pv gly
P. syr pv tom
S. sca

Figure 3.1: Partial alignment of the Cfl amino acid sequence from different plant pathogenic bacteria. Black indicates 100% amino acid similarity at a given position, while dark gray indicates 80-99% similarity, light gray indicates 60-79% similarity, and white indicates < 60% similarity. The % similarity at each amino acid position was determined using the Blosum62 score matrix with a threshold value of 1. The conserved SGTTGXXKG and TGD motifs are highlighted by the red boxes. *P. atr, Pectobacterium atrosepticum; P. car, Pectobacterium carotovorum* subsp. *brasiliense; D.* sp. UGCH24, *Dickeya* sp. UGCH24; *D.* sp. UGCH20, *Dickeya* sp. UGCH20; *P. amy, Pseudomonas amygdale; P. syr* pv *gly, Pseudomonas syringae* pv *glycinea; P. syr* pv *tom, Pseudomonas syringae* pv *tomato; S. sca, Streptomyces scabies.*

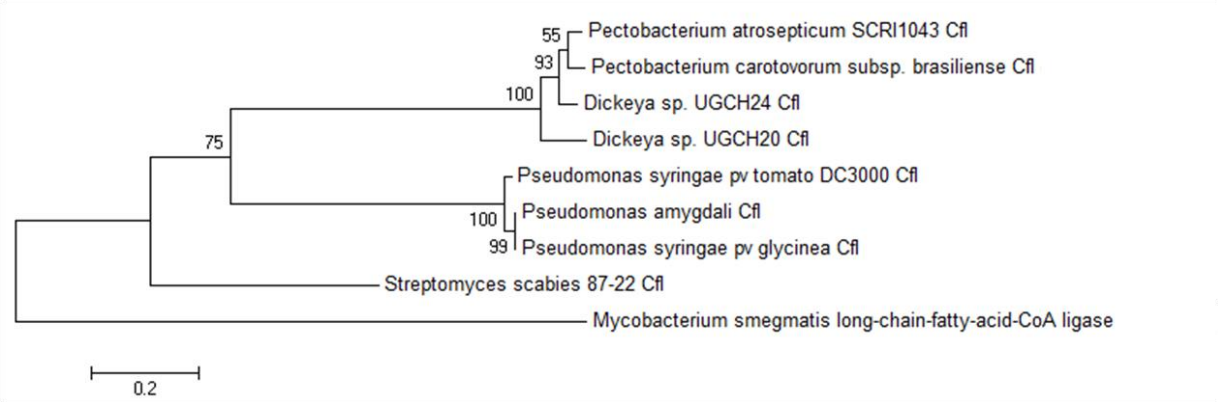

Figure 3.2: Phylogenetic relationships of coronafacate ligase proteins from different bacterial plant pathogens. Bootstrap values are shown for the branch points supported > 50% out of 1000 repetitions. The scale bar indicates the number of amino acid substitutions per site. The protein sequence for a long-chain fatty acid CoA ligase from *Mycobacterium smegmatis* was included as an out-group. Accession numbers for the sequences used are as follows: *Pectobacterium atrosepticum* SCRI1043 Cfl (YP_048726); *Pectobacterium carotovorum* subsp. *brasiliense* Cfl (JQ771052.1); *Dickeya* sp. UGCH20 Cfl (GQ865578.1); *Dickeya* sp. UGCH24 Cfl (GQ865579.1); *Pseudomonas syringae* pv. *tomato* DC3000 Cfl (NP_794429); *Pseudomonas amygdali* Cfl (WP_004666804); *Pseudomonas syringae* pv. *glycinea* Cfl (AAC43314); *Mycobacterium smegmatis* long-chain fatty acid CoA ligase (WP_003891532).

Cfl homologue from *S. scabies* (a Gram-positive organism).

BlastP analysis indicated that Oxr is most similar to an F_{420}-dependent oxidoreductase family protein from the sweet potato pathogen *S. ipomoeae*, and Pfam analysis identified a luciferase-like monooxygenase domain within the protein sequence (Table 3.1), which is consistent with the fact that several luciferase-like monooxygenase family proteins are known to be F_{420}-dependent enzymes (Aufhammer et al. 2004, 2005; Bashiri et al. 2008). The enzyme cofactor F_{420} is a deazaflavin derivative of FMN and is produced mainly by archaeal methanogens and actinomycetes such as *Streptomyces* spp. and *Mycobacterium* spp. (Taylor et al. 2013). In *Streptomyces* spp., F_{420}-dependent oxidoreductases are known or predicted to be involved in the biosynthesis of secondary metabolites such as chlortetracycline (Nakano et al. 2004) and tomaymycin (Li et al. 2009). Chemical transformations that have been attributed to these enzymes include the reduction of ketones, imines and carbon-carbon double bonds (Taylor et al. 2013).

The Sdr protein is most closely related to a predicted 3-oxoacyl-[acyl-carrier protein] reductase from *Streptomyces* spp. PAMC26508, and a domain search revealed the presence of a short chain dehydrogenase domain (Table 3.1). Short chain dehydrogenases/reductases (SDRs) are NAD or NADP dependent oxidoreductases that catalyze carbonyl-hydroxyl oxidoreductions, decarboxylations, epimerizations, dehalogenations, and C=C and C=N reduction reactions (Kavanagh et al. 2008). An analysis of the Sdr amino acid sequence revealed the presence of a TGxxxGxG cofactor binding motif and a YxxxK active site motif, which along with the polypeptide chain length (260 amino acids) places the protein into the 'classical' subfamily of SDRs

Protein	Closest homologue in database (% Identity/Similarity; Source Organism)	Domains Present (E-value)	Predicted Function
Oxr	F_{420}-dependent oxidoreductase family protein (87/93; *Streptomyces ipomoeae*)	Luciferase-like monooxygenase (2.6e-51)	F_{420}-dependent oxidoreductase
Sdr	3-oxoacyl-[acyl-carrier protein] reductase (60/75; *Streptomyces* sp. PAMC26508)	Short chain dehydrogenase (6.5e-31)	Short chain dehydrogenase/reductase

Table 3.1: Closest homologue, predicted protein domains, and predicted function of the Oxr and Sdr proteins encoded in the *S. scabies* CFA-like gene cluster.

(Kavanagh et al. 2008). Such enzymes are most often involved in the NAD(P)(H)-dependent oxidoreduction of hydroxyl/keto groups in many different small molecules, including secondary metabolites (Kavanagh et al. 2008).

3.2 Construction of the *S. scabies cfl, oxr* and *sdr* gene deletion mutants

To examine the role of each gene in the biosynthesis of the COR-like metabolites, mutant strains of *S. scabies* were constructed such that each target gene was replaced with a DNA cassette conferring resistance to the antibiotic hygromycin B. This was accomplished by first using the Redirect PCR targeting system as described previously (Gust et al. 2003a,b) to construct mutant cosmids containing a [*hyg*+*oriT*] cassette in place of the target genes (Fig. 3.3). The hygromycin resistance gene (*hyg*) on the cassette allowed for selection of the desired mutant clones, while the *oriT* sequence allowed for the mutant cosmids to be introduced into *S. scabies* by conjugation with *E. coli*. The mutant cosmids were constructed such that the *hyg* gene was oriented in the same direction as the original target gene in order to avoid possible polar effects on the expression of downstream genes in the CFA-like gene cluster (Fig. 3.3). A total of two mutant cosmids were obtained for each of the target genes, and PCR was used to confirm that the successful replacement of the target gene in each cosmid had taken place (Fig. 3.3 and 3.4).

To generate the *S. scabies* mutant strains, each mutant cosmid was conjugated into the Δ*txtA*/pRLDB51-1 strain, which produces high levels of the COR-like metabolites due

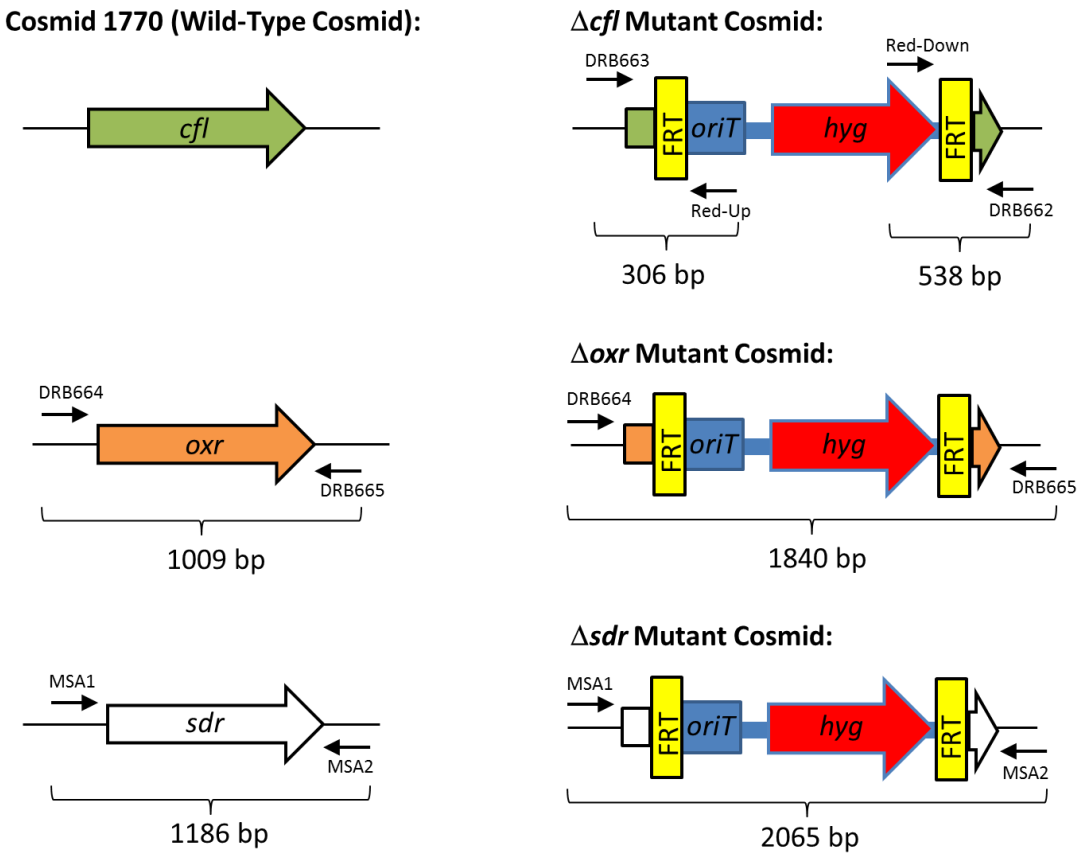

Figure 3.3: Diagram illustrating the construction of the *S. scabies* mutant cosmids and the strategy used for cosmid verification by PCR. The primers used for cosmid verification are represented by the small arrows, and the expected product sizes are indicated. Note that the primer pairs used for verification of the Δ*cfl* mutant cosmids do not generate a product when the wild-type cosmid is used as a template. *hyg*, hygromycin B resistance gene; *oriT*, origin of transfer; FRT, Flip recombinase recognition sites. Diagram not drawn to scale.

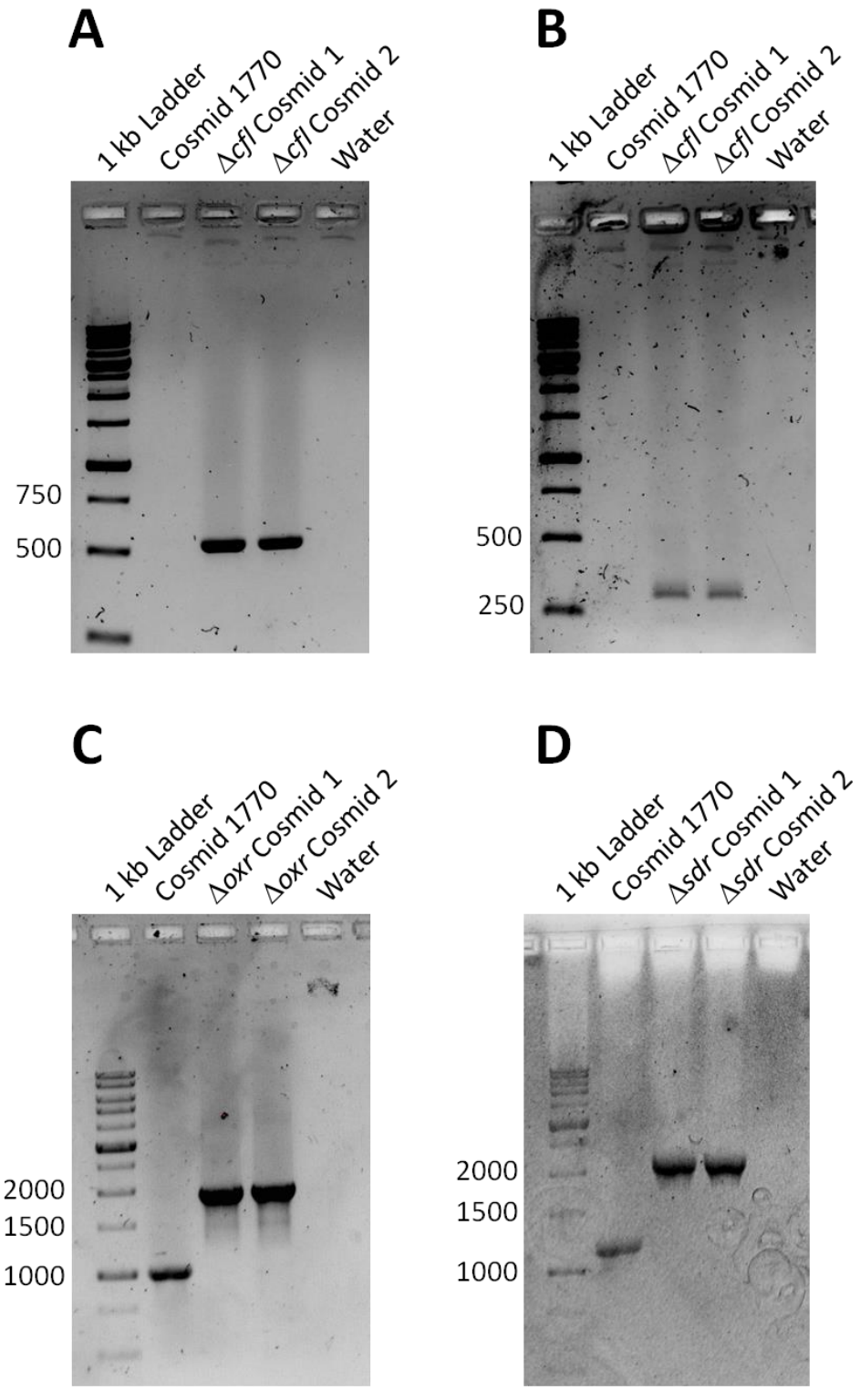
A
1 kb Ladder
Cosmid 1770
Δcfl Cosmid 1
Δcfl Cosmid 2
Water
750
500
B
1 kb Ladder
Cosmid 1770
Δcfl Cosmid 1
Δcfl Cosmid 2
Water
500
250
C
1 kb Ladder
Cosmid 1770
Δoxr Cosmid 1
Δoxr Cosmid 2
Water
2000
1500
1000
D
1 kb Ladder
Cosmid 1770
Δsdr Cosmid 1
Δsdr Cosmid 2
Water
2000
1500
1000

Figure 3.4: Verification of the Δ*cfl*, Δ*oxr*, Δ*sdr* mutant cosmids using PCR. Negative control reactions were conducted using water in place of template DNA. The Red-Down – DRB662 (A) and DRB663 – Red-Up (B) primer pairs were used for verifying the Δ*cfl* mutant cosmids, whereas the DRB664 – DRB665 (C) and MSA1 – MSA2 (D) primer pairs were used for verifying the Δ*oxr* and Δ*sdr* mutant cosmids, respectively. The expected product size for each primer pair and DNA template is indicated in Fig. 3.2. The size (bp) of the marker bands used for PCR product size estimation are shown.

to overexpression of the *scab79591* regulatory gene, and is also unable to produce the thaxtomin A phytotoxin due to deletion of the *txtA* gene (Bignell et al. 2010b). Hyg^r and kan^s exconjugants that arose from each conjugation were chosen for the preparation of spore and mycelial stocks. In total, two Δ*cfl* mutant isolates, four Δ*oxr* mutant isolates and six Δ*sdr* mutant isolates were obtained, and all of the mutant isolates were verified using PCR (Fig. 3.5; data not shown).

3.3 Analysis of COR-like metabolite production in the *S. scabies* deletion mutants

The effect of each gene deletion mutation on COR-like metabolite production was assessed by culturing each mutant in SFMB medium, which supports the production of the metabolites (J. Fyans, unpublished), and then subjecting the culture supernatants to chemical extraction. To detect the presence of the COR-like metabolites, the resulting acidic extracts were subjected to HPLC analysis using an established protocol from the Bignell laboratory (J. Fyans, unpublished data). As shown in Figure 3.6, the Δ*cfl* mutant isolate #1 did not produce detectable levels of the major and minor COR-like metabolites, and similar results were observed for the other Δ*cfl* mutant isolate (data not shown). Instead, both isolates accumulated a metabolite with the same retention time as an authentic CFA standard (Fig. 3.6). The acidic extracts were also examined using LC-MS, and this showed that the accumulated metabolite has the same molecular mass as CFA (208). Furthermore, the metabolite co-migrated with the CFA standard during co-injection experiments (Fig. 3.7). Taken together, this suggests that CFA accumulates in the culture supernatants of the Δ*cfl* mutant.

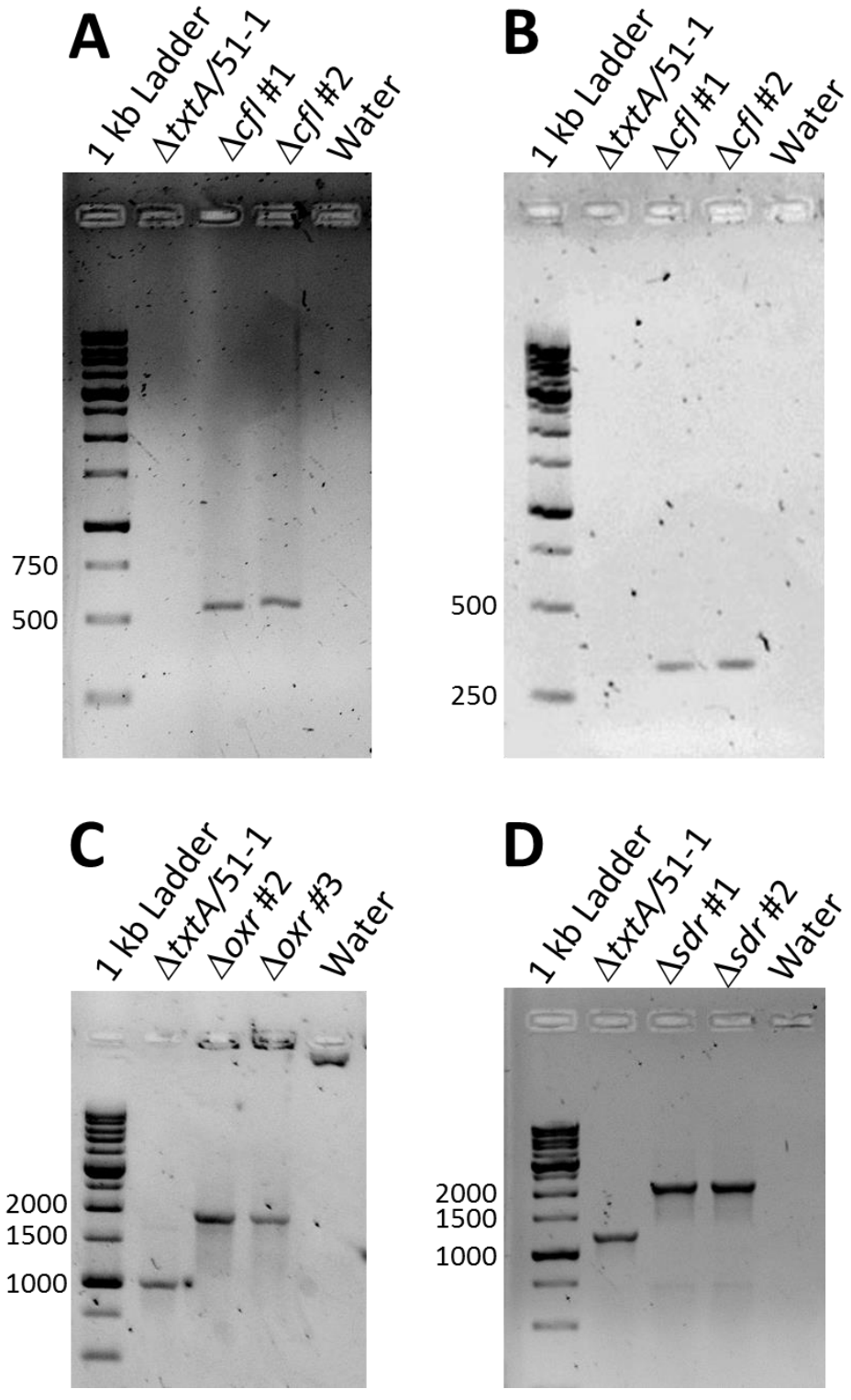
A
1 kb Ladder
ΔtxtA/51-1
Δcfl #1
Δcfl #2
Water
750
500
B
1 kb Ladder
ΔtxtA/51-1
Δcfl #1
Δcfl #2
Water
500
250
C
1 kb Ladder
ΔtxtA/51-1
Δoxr #2
Δoxr #3
Water
2000
1500
1000
D
1 kb Ladder
ΔtxtA/51-1
Δsdr #1
Δsdr #2
Water
2000
1500
1000

Figure 3.5: Verification of the *S. scabies* Δ*cfl,* Δ*oxr,* Δ*sdr* mutant isolates using PCR. Negative control reactions were conducted using water in place of template DNA. The Red-Down – DRB662 (A) and DRB663 – Red-Up (B) primer pairs were used for verifying the Δ*cfl* mutant isolate #1 and #2, the DRB664 – DRB665 primer pair (C) was used for verifying the Δ*oxr* mutant isolate #2 and #3, and the MSA1 – MSA2 primer pair (D) was used for verifying the Δ*sdr* mutant isolate #1 and #2. The expected product size for each primer pair is indicated in Fig. 3.2. The size (bp) of the marker bands used for PCR product size estimation are shown.

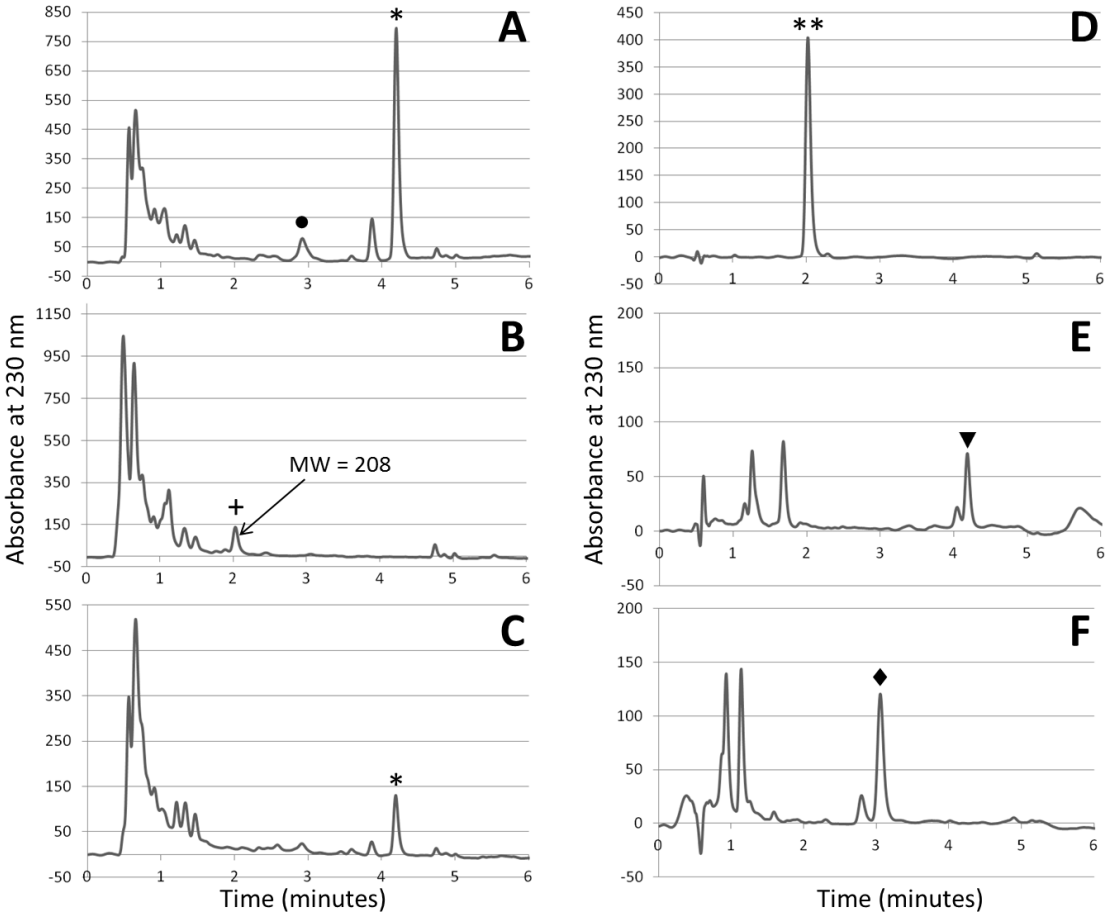

Figure 3.6: HPLC analysis of acidic culture extracts from (A) the *S. scabies* Δ*txtA*/pRLDB51-1 strain, (B) the Δ*cfl* mutant strain (isolate #1), and (C) the Δ*oxr* mutant strain (isolate #2). The peak representing the major COR-like metabolite (CFA-Ile) is indicated with (*), while (●) indicates one of the minor COR-like metabolites (CFA-Val). (+) indicates the metabolite that accumulated in the Δ*cfl* mutant extract, and the molecular mass of the accumulated metabolite, as determined by LC-MS, is indicated. Chromatograms obtained for the authentic standards CFA (D; **), CFA-Ile (E; ▼) and CFA-Val (F; ♦) are also shown.

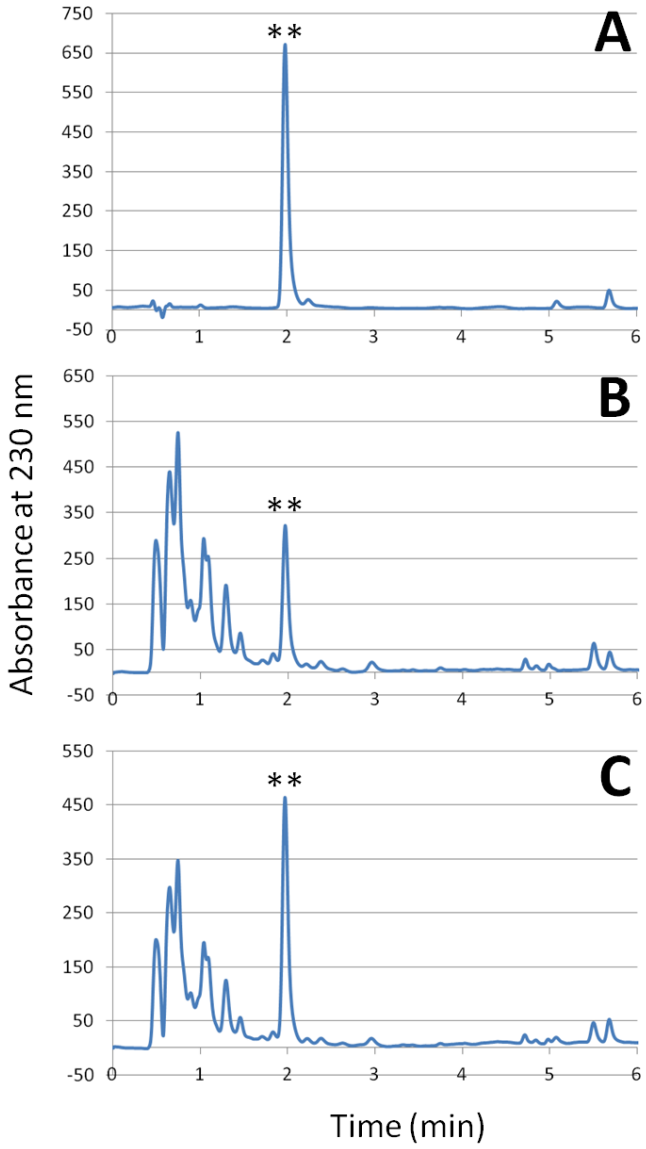

Figure 3.7: Co-injection analysis of the Δ*cfl* mutant acidic extract. An authentic CFA standard (A) and the Δ*cfl* acidic culture extract (B) were analyzed separately by HPLC, after which they were mixed together and analyzed simultaneously (C). The peak representing CFA is indicated by (**).

To confirm that no additional metabolites accumulated in the mutant culture supernatant, the basic organic extracts were also analyzed by HPLC. As shown in Fig. 3.8, no additional metabolites were detected in the basic extract of the Δ*cfl* mutant under the same conditions used for COR-like metabolite detection.

When the Δ*oxr* mutant acidic culture extracts were analyzed by HPLC, it was found that the mutant isolate #2 produced lower levels of the COR-like metabolites as compared to the Δ*txtA*/pRLDB51-1 strain (Fig. 3.6), and similar results were observed for the other three mutant isolates. Production levels of the major COR-like metabolite (CFA-Ile) in the mutant isolates were determined to be 13.5 – 26.4% of that in the Δ*txtA*/pRLDB51-1 strain (Fig. 3.9). Unlike the Δ*cfl* mutant, however, the Δ*oxr* mutant did not appear to accumulate any additional metabolites in either the acidic or basic extracts under the HPLC conditions used (Fig. 3.6 and 3.8).

HPLC analysis of the Δ*sdr* acidic culture extracts showed that production of the CFA-Ile and CFA-Val COR-like metabolites was also significantly reduced in this mutant as compared to the Δ*txtA*/pRLDB51-1 strain (Fig. 3.10). In the case of the CFA-Ile metabolite, production levels in the mutant isolates were 5.5 – 11.7% of that in the Δ*txtA*/pRLDB51-1 strain (Fig. 3.11). Furthermore, the chromatograms for the Δ*sdr* mutant acidic extracts showed three additional peaks that were not present in the Δ*txtA*/pRLDB51-1 chromatogram (Fig. 3.10). Using LC-MS, the molecular mass of the corresponding metabolites was determined to be 309 and 323 (Fig. 3.10), which is equivalent to two mass units larger than the molecular mass of CFA-Val (307) and

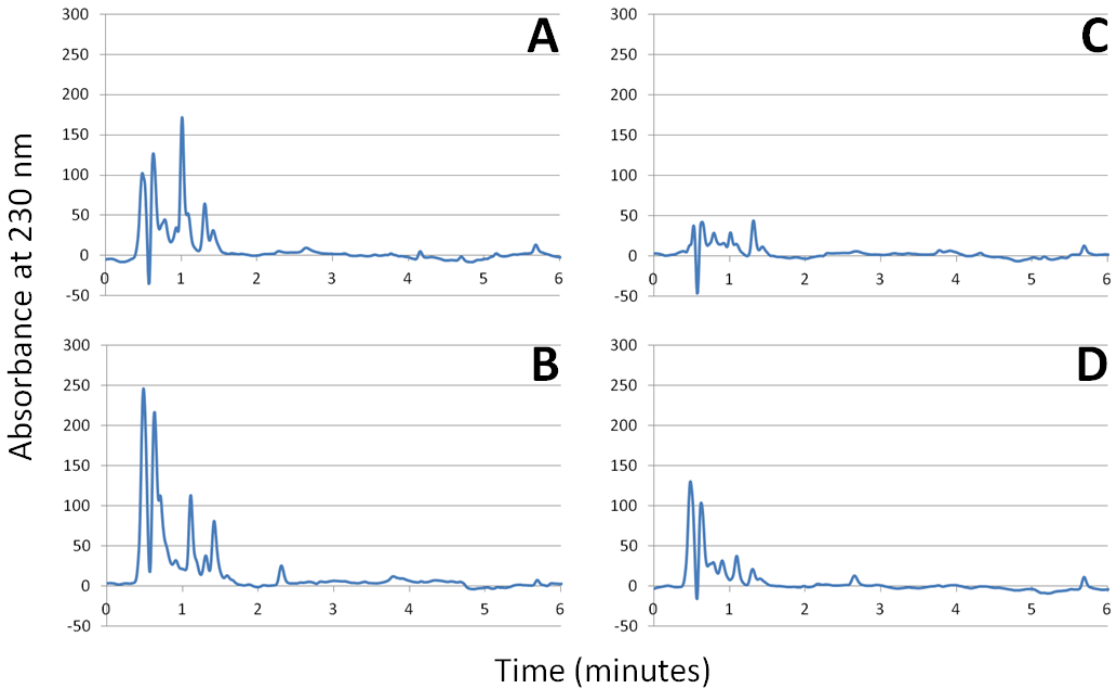

Figure 3.8: HPLC analysis of basic culture extracts from (A) the *S. scabies* Δ*txtA*/pRLDB51-1 strain, (B) the Δ*sdr* mutant strain (isolate #1), (C) the Δ*oxr* mutant strain (isolate #2) and (D) the Δ*cfl* mutant strain (isolate #1).

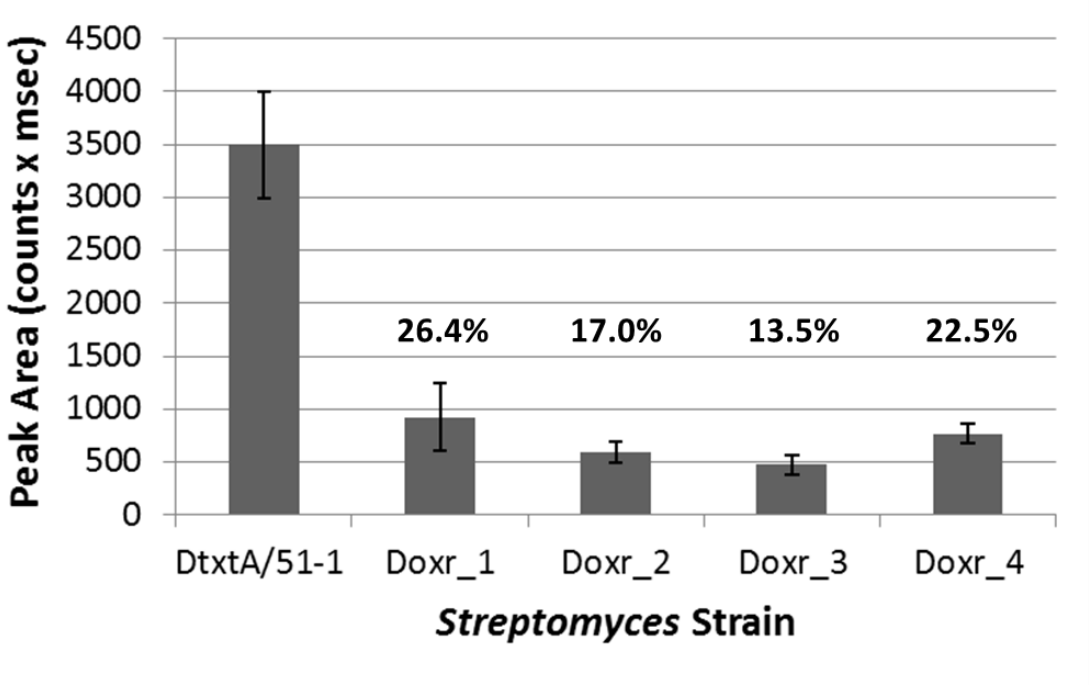

Figure 3.9: Bar graph showing the relative production levels of the major COR-like metabolite (CFA-Ile) in the Δ*txtA*/pRLDB51-1 strain and the four Δ*oxr* mutant isolates. Each bar represents the mean CFA-Ile peak area from triplicate cultures for each strain, while the error bars represent the standard deviation from the mean. The percent production in each mutant isolate relative to the Δ*txtA*/pRLDB51-1 strain is shown above the bars.

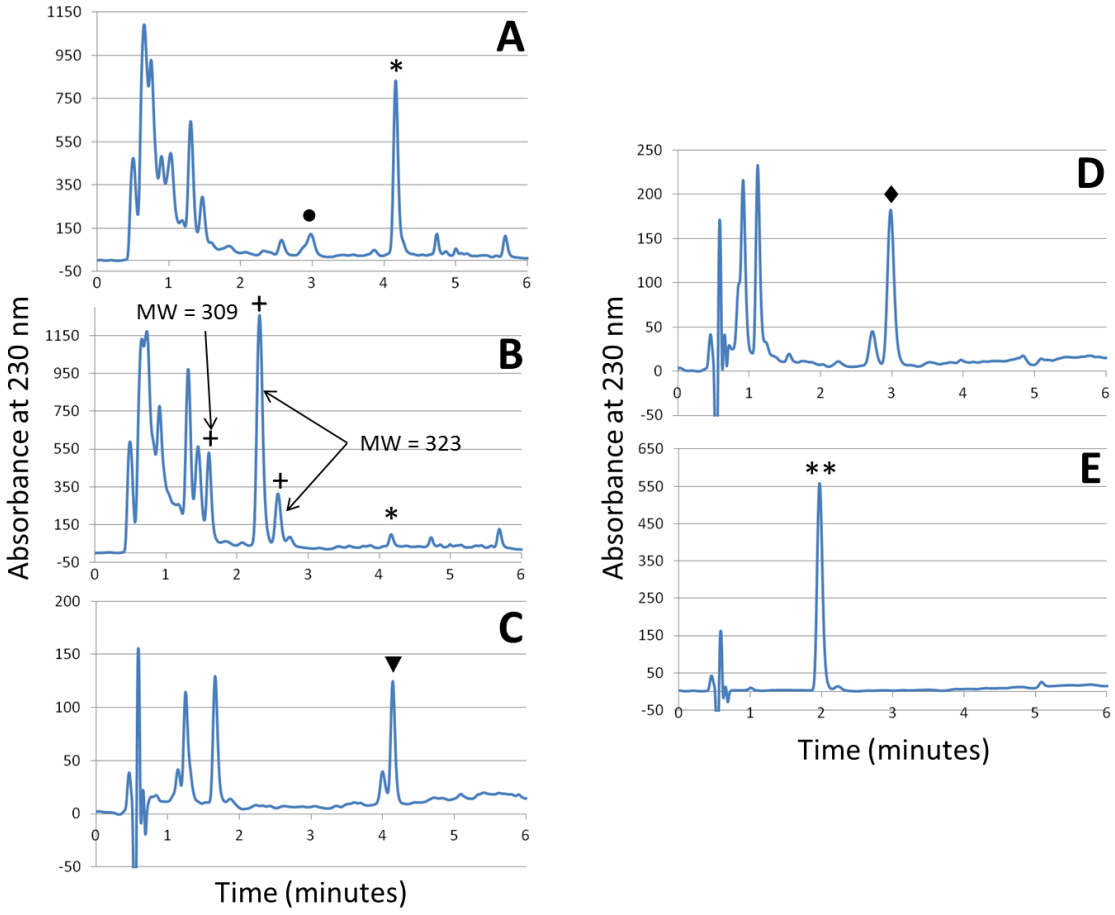

Figure 3.10: HPLC analysis of acidic culture extracts from (A) the *S. scabies* Δ*txtA*/pRLDB51-1 strain and (B) the Δ*sdr* mutant strain (isolate #1). The peak representing the main *S. scabies* COR-like metabolite (CFA-Ile) is indicated with (*), while (•) indicates one of the minor COR-like metabolites (CFA-Val). (+) indicates the metabolites that accumulated in the Δ*sdr* mutant extract, and the molecular weights of the accumulated metabolites, as determined by LC-MS, are indicated. Chromatograms obtained for the authentic standards CFA-Ile (C; ▼), CFA-Val (D; ♦) and CFA (E; **) are also shown.

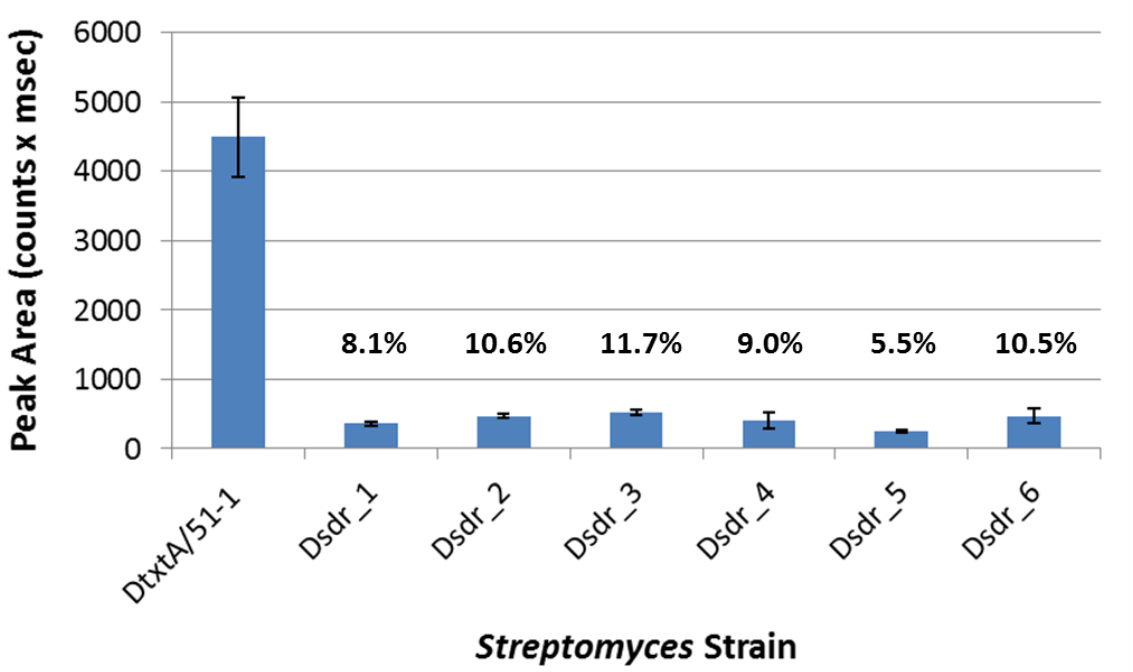

Figure 3.11: Bar graph showing the relative production levels of the major COR-like metabolite (CFA-Ile) in the Δ*txtA*/pRLDB51-1 strain and the Δ*sdr* mutant isolates. Each bar represents the mean CFA-Ile peak area from duplicate cultures for each strain, while the error bars represent the standard deviation from the mean. The percent production in each mutant isolate relative to the Δ*txtA*/pRLDB51-1 strain is shown above the bars.

CFA-Ile (321), respectively. When the basic organic extracts were analyzed by HPLC, only one very small peak was evident in the Δ*sdr* extract and not in the Δ*txtA*/pRLDB51-1 extract, and the retention time of this peak was identical to that of the major peak observed in the acidic extract for the mutant (Fig. 3.6 and 3.8).

3.4 Bioactivity of the *S. scabies* mutant culture extracts

A potato tuber bioassay was performed to assess whether the *S. scabies* mutant acidic extracts were able to induce tissue hypertrophy, an effect that has been attributed to the *S. scabies* COR-like metabolites (Bignell et al. 2014). As shown in Figure 3.12, the Δ*cfl* mutant extracts were found to exhibit no bioactivity against the potato tuber tissue, which is consistent with the inability of this mutant to produce the COR-like metabolites (Fig. 3.6). On the other hand, the Δ*oxr* mutant extracts exhibited some tissue hypertrophy inducing activity, but it was not as effective as the Δ*txtA*/pRLDB51-1 extract (Fig. 3.12). Finally, the Δ*sdr* mutant extracts were found to have the same effect on the tuber tissue as the Δ*txtA*/pRLDB51-1 organic acid extract (Fig. 3.12).

3.5 Complementation of the mutant *S. scabies* strains

Genetic complementation was performed to confirm that the observed effects on COR-like metabolite production in the constructed mutants were due exclusively to deletion of the target genes. The plasmid vector pIJ10257 (Table 2.2) is a *Streptomyces* expression plasmid that uses the strong, constitutive promoter, *ermE*p*, and integrates into the ΦBT1 *attB* site in *Streptomyces* chromosomes. As the selectable marker

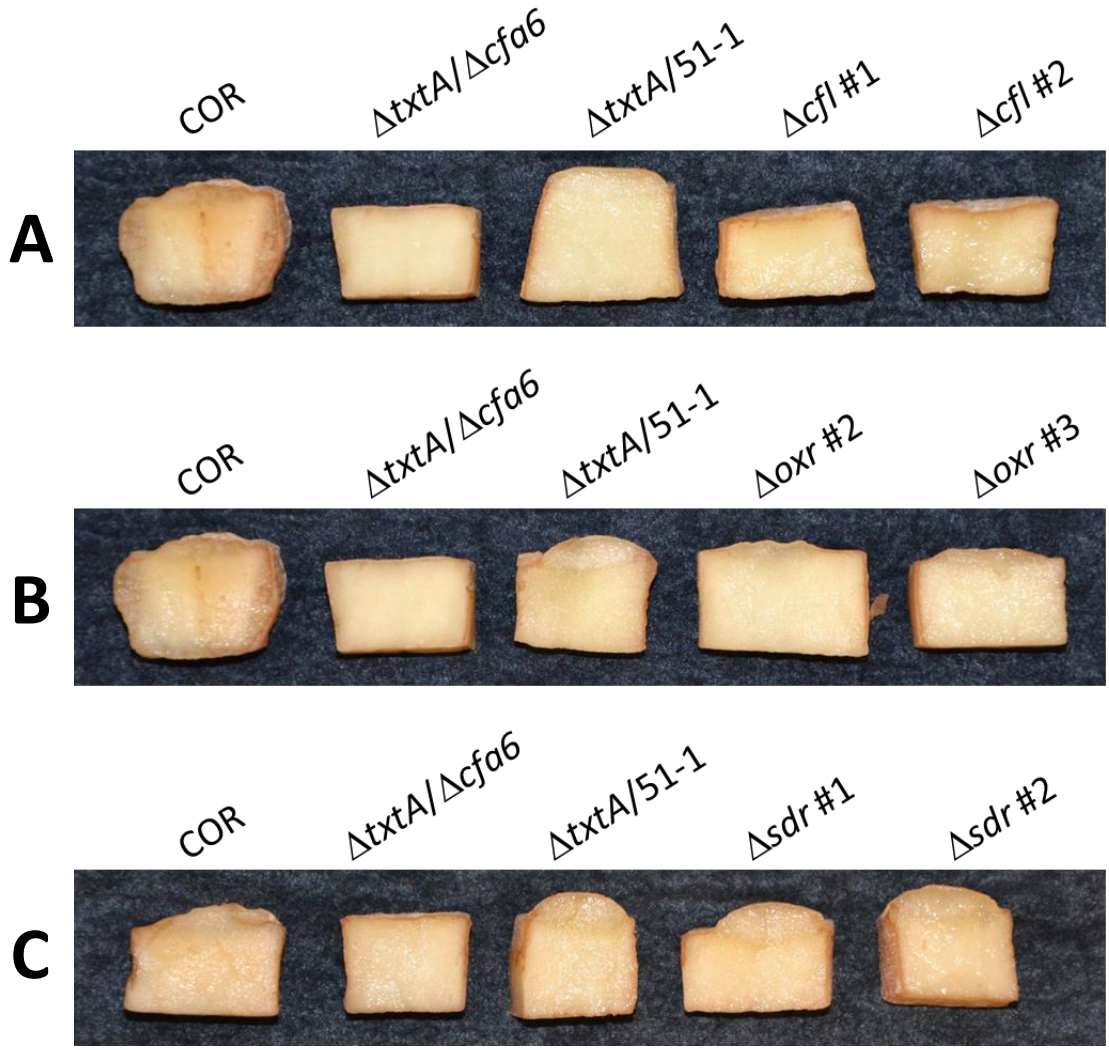

Figure 3.12: Bioactivity of the *S. scabies* Δ*cfl* (isolates #1 and 2) (A), Δ*oxr* (isolates #2 and 3) (B) and Δ*sdr* (isolates #1 and 2) (C) organic acid extracts on potato tuber tissue. Pure, authentic COR and extract from the Δ*txtA*/pRLDB51-1 strain served as positive controls, whereas extract from a COR-like metabolite non-producing strain (Δ*txtA*/Δ*cfa6*) served as a negative control.

for this plasmid (*hyg*) is the same as that used to construct the *S. scabies* gene deletion mutants, a modified version of the plasmid was needed so that it could be used for the mutant complementation experiments. The *neo* gene, conferring resistance to kanamycin, and its promoter were PCR amplified using Cosmid 1770 as the template and using the primers MSA11 and MSA12 (Table 2.3), and the resulting product was cloned into the pGEM® - T Easy plasmid. Next, the cloned PCR product was released from pGEM® - T Easy after digestion with EcoRV, and the DNA fragment was gel-purified and ligated into similarly digested and FastAP-treated pIJ10257. The ligation was transformed into *E. coli*, and transformants were selected for hyg^r and kan^r. The resulting plasmid, pMSAK13, was prepared from an overnight *E. coli* culture and was verified by restriction digestion with EcoRV (Fig. 3.13).

To construct the complementation plasmids, the *cfl*, *oxr* and *sdr* genes were each PCR amplified using gene-specific primers (Table 2.3) and using Cosmid 1770 as the template, and the resulting products were cloned into pGEM-T EASY. After sequencing of each clone to confirm that no PCR-induced mutations were present, the clones were digested with NdeI and XhoI to release the inserts, which were then gel-purified. The pMSAK13 plasmid was digested with the same restriction enzymes and was gel-purified, after which ligation reactions were set up using the digested plasmid and the *cfl*, *oxr* and *sdr* DNA fragments. After transforming the ligation reactions into *E. coli*, the resulting kan^r colonies were screened by PCR for the presence of the cloned inserts (data not shown). Positive clones were subsequently purified and digested with NdeI and XhoI to

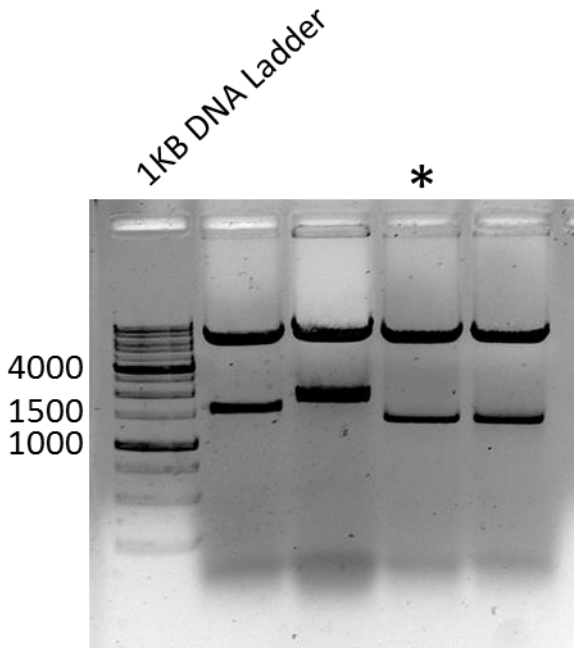

Figure 3.13: Verification of pIJ10257 + *neo* clones using restriction digestion. The plasmid clones were digested with EcoRV to release the 1365 bp *neo* insert, after which they were analyzed by agarose gel electrophoresis. The clone marked with (*) gave the expected insert size and was renamed pMSAK13. The size (bp) of the marker bands used for band size estimation are shown.

confirm the presence of the cloned inserts (Fig. 3.14), and were also sequenced to confirm the correct orientation of each insert in the pMSAK13 plasmid. The complementation plasmids (pMSAK13/*cfl*, pMSAK13/*oxr*, pMSAK13/*sdr*), along with the pMSAK13 vector, were then conjugated into the corresponding *S. scabies* mutant strain.

The resulting kan^r and hyg^r mutant transconjugants that arose were subsequently cultured in SFMB, and the culture supernatants were extracted and analyzed using HPLC and bioassays as before. As shown in Fig. 3.15 and 3.16, introduction of the *cfl* gene back into the Δ*cfl* (isolate #2) strain resulted in restoration of COR-like metabolite production and bioactivity as compared to the Δ*cfl* strain containing only the pMSAK13 plasmid vector alone. Restoration of COR-like metabolite production and bioactivity was also observed when *oxr* was re-introduced into the Δ*oxr* mutant (isolate #2) (Fig. 3.17 and 3.18) and when *sdr* was re-introduced into the Δ*sdr* mutant (isolate #1) (Fig. 3.19). Together, these results indicate that the observed phenotypes of the constructed mutants are due to the gene deletions that were made in each strain.

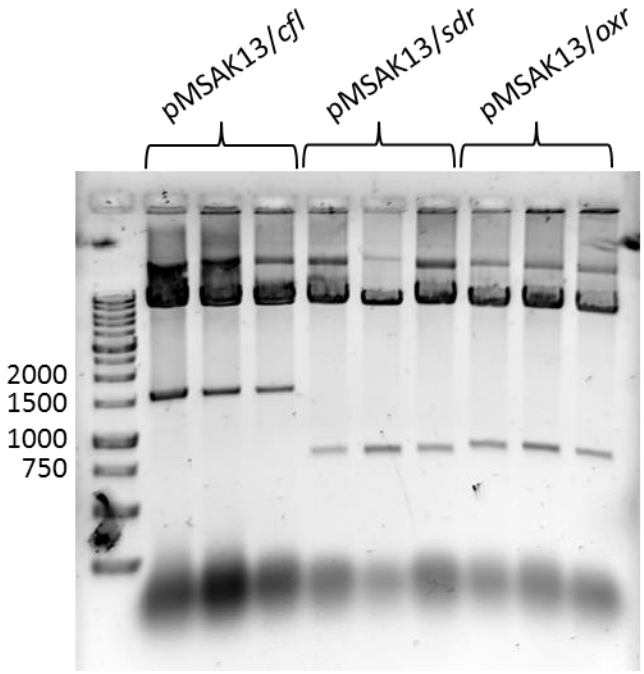

Figure 3.14: Verification of the *cfl, oxr* and *sdr* complementation plasmids by restriction digestion. Plasmids were digested with NdeI and XhoI to release the cloned inserts, and the resulting products were analyzed by agarose gel electrophoresis. The expected insert sizes were 1548 bp (*cfl*), 783 bp (*sdr*) and 831 bp (*oxr*). The sizes (bp) of the marker bands used for product size estimation are shown.

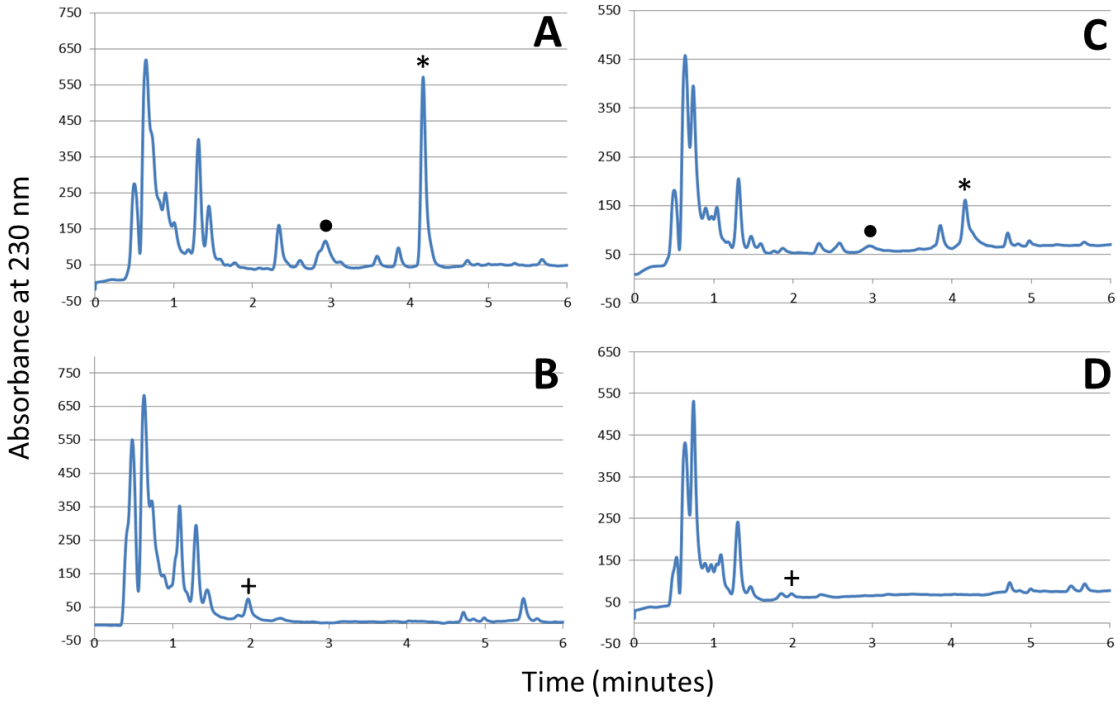

Figure 3.15: HPLC analysis of acidic culture extracts from (A) the *S. scabies* Δ*txtA*/pRLDB51-1 strain, (B) the Δ*cfl* mutant isolate #2, (C) the Δ*cfl* mutant isolate #2 containing pMSAK13/*cfl*, and (D) the Δ*cfl* mutant isolate #2 containing pMSAK13. The peak representing the major *S. scabies* COR-like metabolite (CFA-Ile) is indicated with (*), while (•) indicates the minor COR-like metabolite (CFA-Val). (+) indicates the CFA metabolite that accumulated in the Δ*cfl* mutant extract.

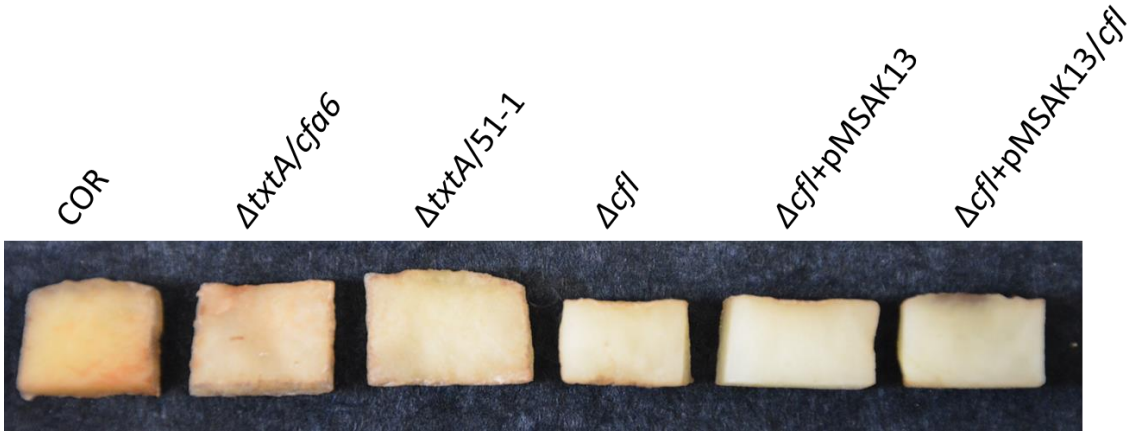

Figure 3.16: Bioactivity of the complemented *S. scabies* Δ*cfl* mutant culture extract on potato tuber tissue. Acidic culture extracts were prepared from the Δ*cfl* mutant isolate #2, the Δ*cfl* mutant isolate #2 containing the pMSAK13 plasmid, and the Δ*cfl* mutant isolate #2 containing pMSAK13/*cfl*. Pure, authentic COR and extract from the Δ*txtA*/pRLDB51-1 strain served as positive controls, whereas extract from a COR-like metabolite non-producing strain (Δ*txtA*/Δ*cfa6*) served as a negative control.

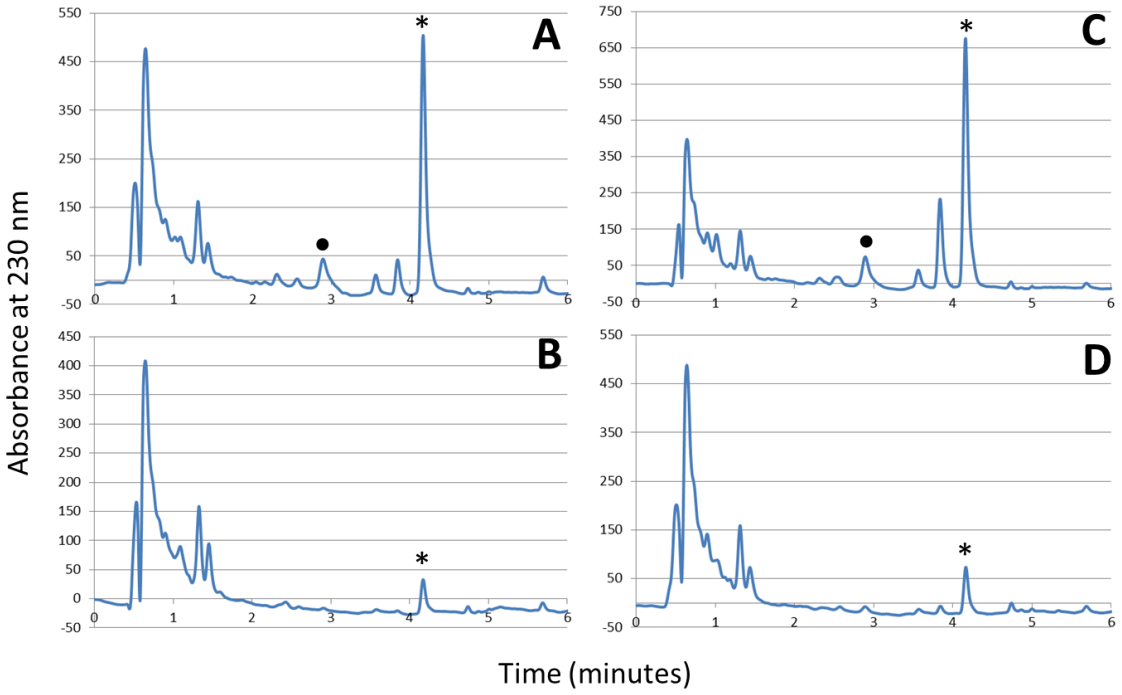

Figure 3.17: HPLC analysis of acidic culture extracts from (A) the *S. scabies* Δ*txtA*/pRLDB51-1 strain, (B) the Δ*oxr* mutant isolate #2, (C) the Δ*oxr* mutant isolate #2 containing pMSAK13/*oxr*, and (D) the Δ*oxr* mutant isolate #2 containing pMSAK13. The peak representing the major *S. scabies* COR-like metabolite (CFA-Ile) is indicated with (*), while (•) indicates the minor COR-like metabolite (CFA-Val).

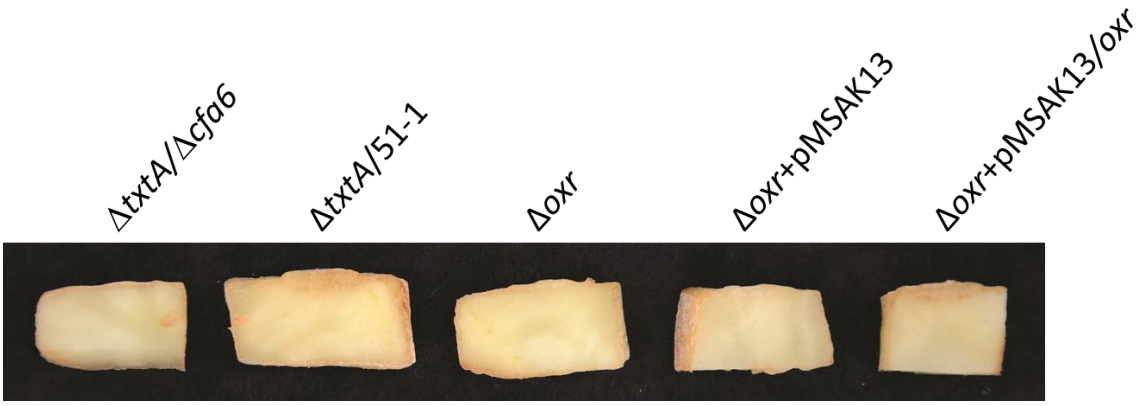

Figure 3.18: Bioactivity of the complemented *S. scabies* Δ*oxr* mutant culture extract on potato tuber tissue. Acidic culture extracts were prepared from the Δ*oxr* mutant isolate #2, the Δ*oxr* mutant isolate #2 containing the pMSAK13 plasmid, and the Δ*oxr* mutant isolate #2 containing pMSAK13/*oxr*. Extract from the Δ*txtA*/pRLDB51-1 strain served as a positive control, whereas extract from a COR-like metabolite non-producing strain (Δ*txtA*/Δ*cfa6*) served as a negative control.

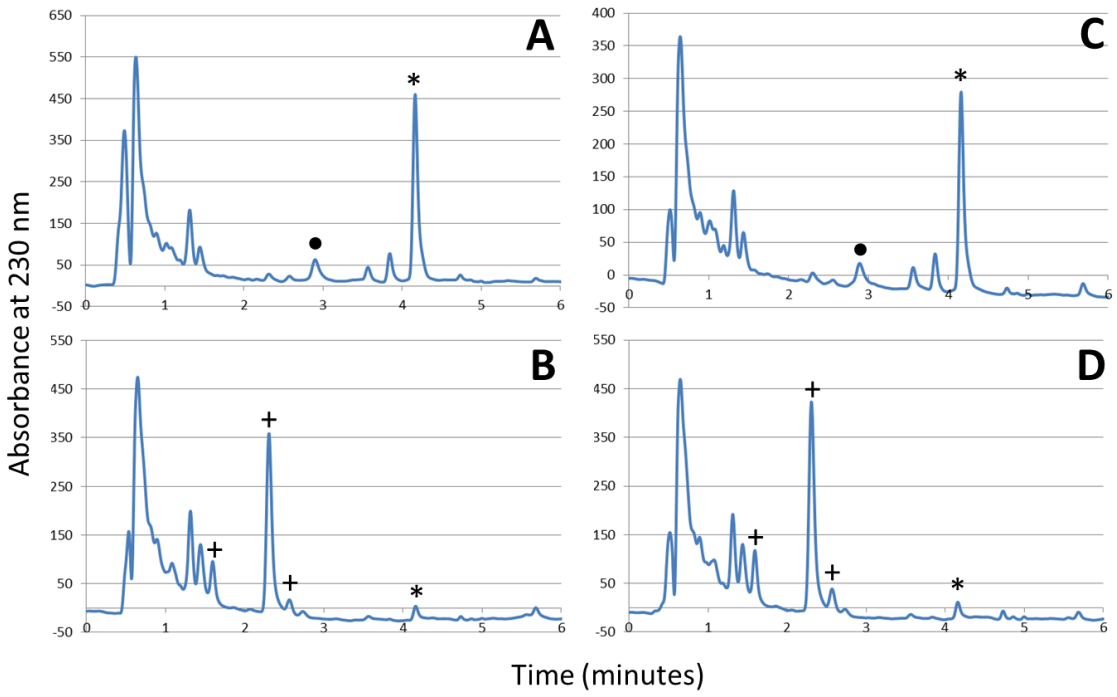

Figure 3.19: HPLC analysis of acidic culture extracts from (A) the *S. scabies* Δ*txtA*/pRLDB51-1 strain, (B) the Δ*sdr* mutant isolate #1, (C) the Δ*sdr* mutant isolate #1 containing pMSAK13/*sdr*, and (D) the Δ*sdr* mutant isolate #1 containing pMSAK13. The peak representing the major *S. scabies* COR-like metabolite (CFA-Ile) is indicated with (*), while (•) indicates the minor COR-like metabolite (CFA-Val). (+) indicates the metabolites that accumulated in the Δ*sdr* mutant extracts.

CHAPTER 4: DISCUSSION

4.1 Functional analysis of the *S. scabies cfl, oxr* and *sdr* genes

S. scabies is the best characterized and most widely distributed pathogen that causes CS disease of potato. Among the virulence factors that contribute to the pathogenic phenotype of this organism are the COR-like metabolites, which may function as plant immune suppressors and may also contribute to disease symptom development. The CFA-like biosynthetic gene cluster, which is responsible for the synthesis of the COR-like metabolites, consists of nine genes that are homologous to genes in the *P. syringae* CFA biosynthetic gene cluster (Fig. 1.4). Among these is the *cfl* gene, which in *P. syringae* is believed to catalyze the adenylation of CFA and the ligation of the CFA-adenylate to CMA and other amino acids to form COR and COR-like molecules, respectively (Fig. 1.7). The *S. scabies* CFA-like gene cluster also contains six genes that have no homologues in the *P. syringae* CFA gene cluster. These include the *oxr* and *sdr* genes, both of which were previously proposed to function as tailoring enzymes during biosynthesis of the CFA backbone (Bignell et al. 2010b). In this study, the role of *cfl*, *oxr* and *sdr* in the production of the *S. scabies* COR-like metabolites was examined by constructing deletion mutants for each gene and then analyzing the effect of each deletion on metabolite biosynthesis and bioactivity.

4.1.1 *cfl*

Previously, it was predicted that the Cfl protein in *S. scabies* contributes to COR-like metabolite biosynthesis by functioning in a similar manner to Cfl in *P. syringae* (Bignell et al. 2010b). The bioinformatics analysis performed in this study supports this idea since it showed that the *S. scabies* Cfl contains two motifs that are conserved in the *P. syringae* Cfl and in other predicted Cfl homologues (Fig. 3.1) and which are characteristic of enzymes that activate carboxylic acids by adenylation (Liyanage et al. 1995). Moreover, the Δ*cfl* mutant strains constructed in this study were unable to produce detectable levels of the major and minor COR-like metabolites as determined by HPLC and bioassays, indicating that as in *P. syringae*, Cfl plays an essential role in metabolite biosynthesis in *S. scabies*. On the other hand, the Δ*cfl* mutant accumulated an intermediate that based on the LC-MS results and the co-injection experiment is most likely CFA, whereas it has been reported that a nonpolar *cfl* mutant of *P. syringae* does not accumulate CFA, leading to the suggestion that Cfl may also have a role in CFA biosynthesis in that organism (Rangaswamy et al. 1997). It therefore appears that in *S. scabies*, Cfl is only required for the final step in COR-like metabolite biosynthesis and that unlike its homologue in *P. syringae*, it is not essential for the production of CFA. The fact that the *S. scabies* Δ*cfl* mutant culture extracts did not display any bioactivity in the potato tuber bioassay is consistent with previous findings that the conjugation of CFA to an amino acid is necessary for optimal bioactivity in plants (Uppalapati et al. 2005).

Although the phenotype of the Δ*cfl* mutant was complemented when the *S. scabies cfl* gene was re-introduced into the mutant, the complementation was only partial since the metabolite production levels were less than that observed in the original

Δ*txtA*/pRLDB51-1 strain (Fig. 3.15). One possible explanation for this is that the *cfl* gene is not expressed at an efficient level in the Δ*cfl* mutant due to the fact that the pMSAK13 plasmid, which harbours the *cfl* gene, inserts into the *S. scabies* chromosome at a location that is different from that of the CFA-like gene cluster. Another possibility is that expression of *cfl* from the strong, constitutive *ermE*p* promoter causes adverse effects in the cells, and this in turn may negatively impact the secondary metabolism of the organism. It is also possible that the particular Δ*cfl* mutant strain that was complemented (isolate #2) may have acquired additional genetic changes in its chromosome that prevented the mutant from being fully complemented by the *cfl* gene itself. The complementation of the Δ*cfl* mutant isolate #1 was not attempted in this study, and perhaps this is something that should be conducted in the future.

4.1.2 *oxr*

The *oxr* gene was previously predicted to be involved in COR-like metabolite biosynthesis since the gene is co-transcribed with other known biosynthetic genes in the CFA-like gene cluster (Bignell et al. 2010b). The results of this study support this idea as it was demonstrated that the *oxr* gene is necessary for normal production of the COR-like metabolites. The fact that some metabolite production could still occur in the mutant suggests that a similar enzyme encoded on the *S. scabies* chromosome can partially complement the loss of Oxr. This idea is supported by the identification of an F_{420}-dependent oxidoreductase (SCAB52161) in the predicted *S. scabies* proteome showing 33% identity and 51% similarity to Oxr at the amino acid level.

Unlike the Δ*cfl* mutant, the Δ*oxr* mutant did not accumulate any potential biosynthetic intermediates in the culture supernatant. A possible explanation for this is that the HPLC and/or the chemical extraction protocol used was not favorable for detection of the accumulated intermediate(s). The Δ*oxr* culture extracts were shown to exhibit some bioactivity against potato tuber tissue, though the activity was reduced as compared to the Δ*txtA*/pRLDB51-1 extract. This observation is presumably due to the small amount of COR-like metabolites that was present in the Δ*oxr* culture extracts.

4.1.3 *sdr*

As with the Δ*oxr* mutant, the constructed Δ*sdr* mutant was significantly reduced in production of the COR-like metabolites in comparison to the Δ*txtA*/pRLDB51-1 strain. The observed low level production in the mutant is again likely due to partial complementation by a similar enzyme in *S. scabies*. BlastP analysis revealed the presence of at least two additional predicted SDR-family proteins, SCAB2261 and SCAB12071, which show significant homology to Sdr (39% identity/55% similarity and 42% identity/57% similarity, respectively) and which could possibly account for the observed CFA-Ile production. Interestingly, the Δ*sdr* mutant accumulated three additional metabolites in the culture supernatant, two of which were determined to have a molecular mass of 323, and the other had a molecular mass of 309. Of particular significance is the fact that such masses are exactly two units larger than the molecular mass of CFA-Ile (321) and CFA-Val (307), respectively, supporting the idea that these molecules are associated with the COR-like metabolite biosynthetic pathway. Given that

Sdr is predicted to be a short-chain dehydrogenase/reductase that catalyzes oxidoreduction reactions, it is plausible that the accumulated metabolites represent reduced forms of CFA-Ile and CFA-Val. Furthermore, the two accumulated metabolites with the exact same molecular mass might represent different isomers of the reduced form of CFA-Ile.

It is interesting to note that even though the Δ*sdr* mutant produced only very low levels of the COR-like metabolites, the mutant culture extract induced a similar amount of potato tissue hypertrophy as the Δ*txtA*/pRLDB51-1 extract. This suggests that the accumulated metabolites exhibit the same level of bioactivity as the COR-like metabolites.

4.2 Hypothetic pathway for COR-like metabolite biosynthesis in *S. scabies*

The results of this study show that *cfl*, *oxr* and *sdr* are indeed involved in the biosynthesis of the COR-like metabolites in *S. scabies.* Given that COR-like metabolites are known to also be produced by *P. syringae* in minor amounts, the involvement of *oxr* and *sdr* in metabolite biosynthesis is of particular significance as this is the first report of such genes being implicated in the biosynthesis of these molecules. Furthermore, the study indicates that there may be differences in the function of the Cfl homologues in *P. syringae* and *S. scabies* in terms of their role in metabolite biosynthesis. Based on the previous knowledge of COR/COR-like metabolite biosynthesis in *P. syringae* (Fig. 1.6 and 1.7), together with the results presented here as well as unpublished results from other

members of the Bignell laboratory, we can now propose a hypothetical biosynthetic pathway for the COR-like metabolites in *S. scabies* (Fig. 4.1).

As in *P. syringae,* the pathway is proposed to begin with ∝-ketoglutarate, and this may be converted to **(1)** through the action of the Cfa1 – 8 and SCAB79711 enzymes (Fig. 4.1). Cfa1 – 5 are believed to convert the ∝-ketoglutarate to an intermediate that then serves as the starter unit for polyketide biosynthesis by the Cfa6 and Cfa7 PKS (Fig. 1.6), while Cfa8 and SCAB79711 are thought to produce the ethylmalonyl-CoA extender unit that is needed for polyketide biosynthesis. Oxr, which is a predicted F_{420}-dependent oxidoreductase, may then catalyze the oxidation of **(1)** to form **(2)**, which is proposed to contain the C=C double bond that is found between C5 and C6 of the CFA moiety (Fig. 4.1). The fact that **(1)** lacks the C=C double bond is consistent with our inability to detect an intermediate in the Δ*oxr* culture supernatant. This is because **(1)** would not be expected to absorb UV light at 230 nm (the detection wavelength used for HPLC) due to the absence of a conjugated double bond system. Another enzyme encoded within the CFA-like gene cluster, SCAB79691, is predicted to function as a P450 monooxygenase (Bignell et al. 2010b) and may introduce a hydroxyl group at the C1 position to give **(3)**. This idea is supported by work conducted in the Bignell laboratory showing that SCAB79691 is essential for COR-like metabolite biosynthesis in *S. scabies* (Y. Li, personal communication). Sdr, which is predicted to be a member of the 'classical' subfamily of SDRs that catalyze oxidoreduction of hydroxyl/keto groups, may then

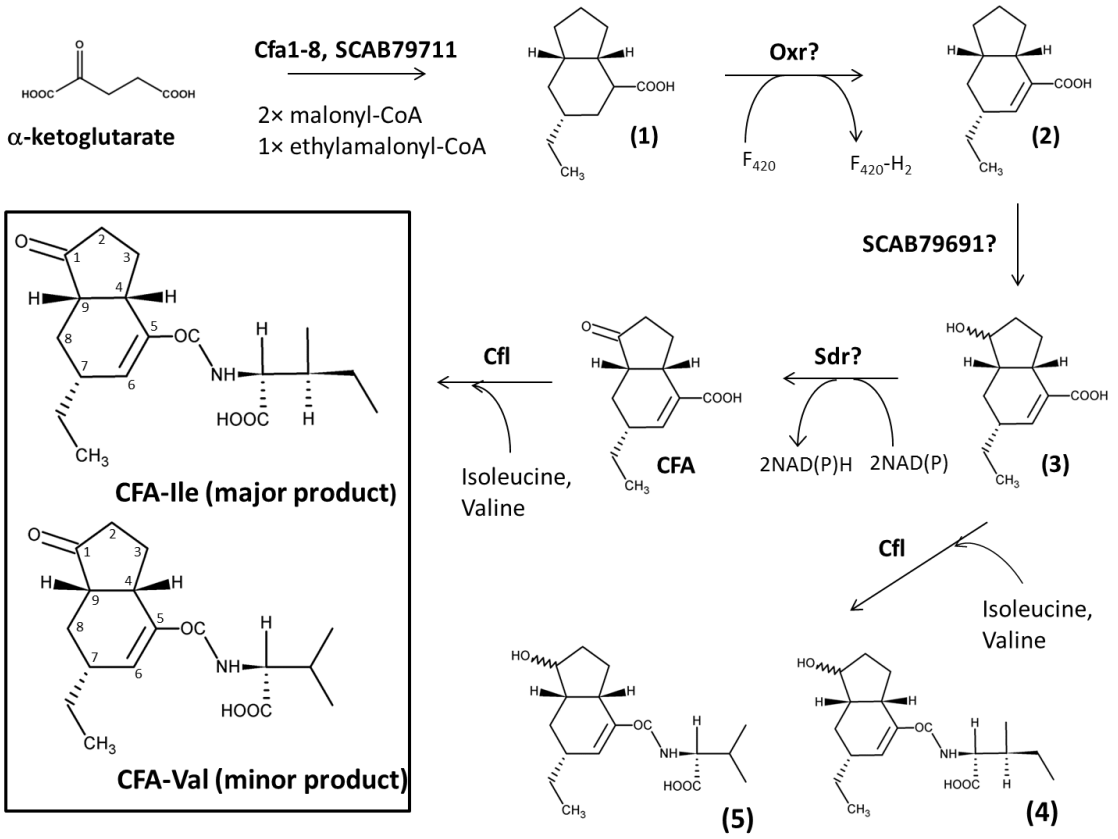

Figure 4.1: Hypothetical biosynthetic pathway for production of the COR-like metabolites in *S. scabies.*

oxidize the hydroxyl group in **(3)** to form CFA, which in turn is linked to isoleucine and valine (and possibly other amino acids) through the action of Cfl to form the COR-like metabolites (Fig. 4.1). Although a metabolite with the same molecular mass as **(3)** was not detected in the Δ*sdr* culture extracts, we did detect metabolites with a molecular mass consistent with **(4)** and **(5)**, which represent reduced forms of CFA-Ile and CFA-Val, respectively. Presumably in the absence of Sdr, the Cfl enzyme is able to adenylate **(3)** and link it to isoleucine and valine to generate the reduced forms of the COR-like metabolites, which based on the bioassay results retain the bioactivity of the natural products.

The hypothetical biosynthetic pathway shown in Figure 4.1 is distinct from that proposed for COR/COR-like molecules in *P. syringae* in that it implicates at least three additional genes (*oxr*, *sdr*, *scab79691*) in the biosynthesis of CFA. A BlastP analysis of the *P. syringae* pv *tomato* DC3000 genome (http://pseudomonas-syringae.org/) revealed that although there are similar proteins to Sdr encoded within the genome, no homologues exist for Oxr or SCAB79691. Therefore, if the pathway shown in Figure 4.1 is correct, then it would appear that *S. scabies* and *P. syringae* utilize alternative biosynthetic pathways for production of similar phytotoxins.

4.3 Concluding remarks and future directions

The research presented in this thesis has generated a number of questions that should be addressed in future studies on the *S. scabies* COR-like metabolites. For example, purification and structural analysis of the metabolites that accumulated in the

Δ*sdr* culture supernatant should be performed in order to confirm the predicted role of Sdr as shown in Figure 4.1. The purified metabolites could also be used in several different plant bioassays in order to better characterize their bioactivity as compared to the COR-like metabolites. This, in turn, would help us to understand the structural requirements for the observed bioactivities of COR and COR-like metabolites. Further analysis of the Δ*oxr* culture supernatants is also needed to determine whether or not the mutant accumulates any biosynthetic intermediates. This could be accomplished by testing different chemical extraction solvents as well as different HPLC detection parameters. If one or more intermediates are detected, then this should be followed by purification and structural elucidation of the intermediate(s). In addition, it would be interesting to study the *S. scabies* Cfl protein in more detail in order to better understand its role in the metabolite biosynthetic pathway. For example, the protein could be overexpressed and purified, and *in vitro* biochemical reactions could be performed in order to confirm that the enzyme does in fact catalyze the adenylation of CFA and the ligation of CFA to isoleucine and valine. *In vitro* assays could also be used to further study the substrate specificity of Cfl by testing the ability of the enzyme to ligate CFA to a variety of different amino acids and determining the relative efficiency of amino acid utilization by the enzyme. Such an experiment could possibly help to explain why CFA-Ile appears to be the predominant product made by *S. scabies in vivo.* Finally, it would be interesting to further investigate the functional conservation of the Cfl homologues from *S. scabies* and *P. syringae* by testing the ability of the *P. syringae* Cfl to complement the *S. scabies* Δ*cfl* mutant and vice versa.

CS disease is an extremely difficult disease to control, and novel control strategies are in need in order to reduce the economic impact of the disease on potato growers worldwide. Research into plant pathogenic *Streptomyces* spp. and the virulence determinants they use to cause CS disease is considered an essential step in the development of new control strategies that have consistent and long-term effectiveness. The research presented here makes a significant contribution to the elucidation of the biosynthetic pathway for the COR-like metabolites, which are thought to contribute to the virulence phenotype of the most widely distributed CS-causing pathogen. The knowledge gained could one day assist in the development of strategies that specifically target the production of this and other *S. scabies* phytotoxins in agricultural settings in order to reduce or eliminate the symptoms associated with the disease.

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CHAPTER 6: APPENDIX

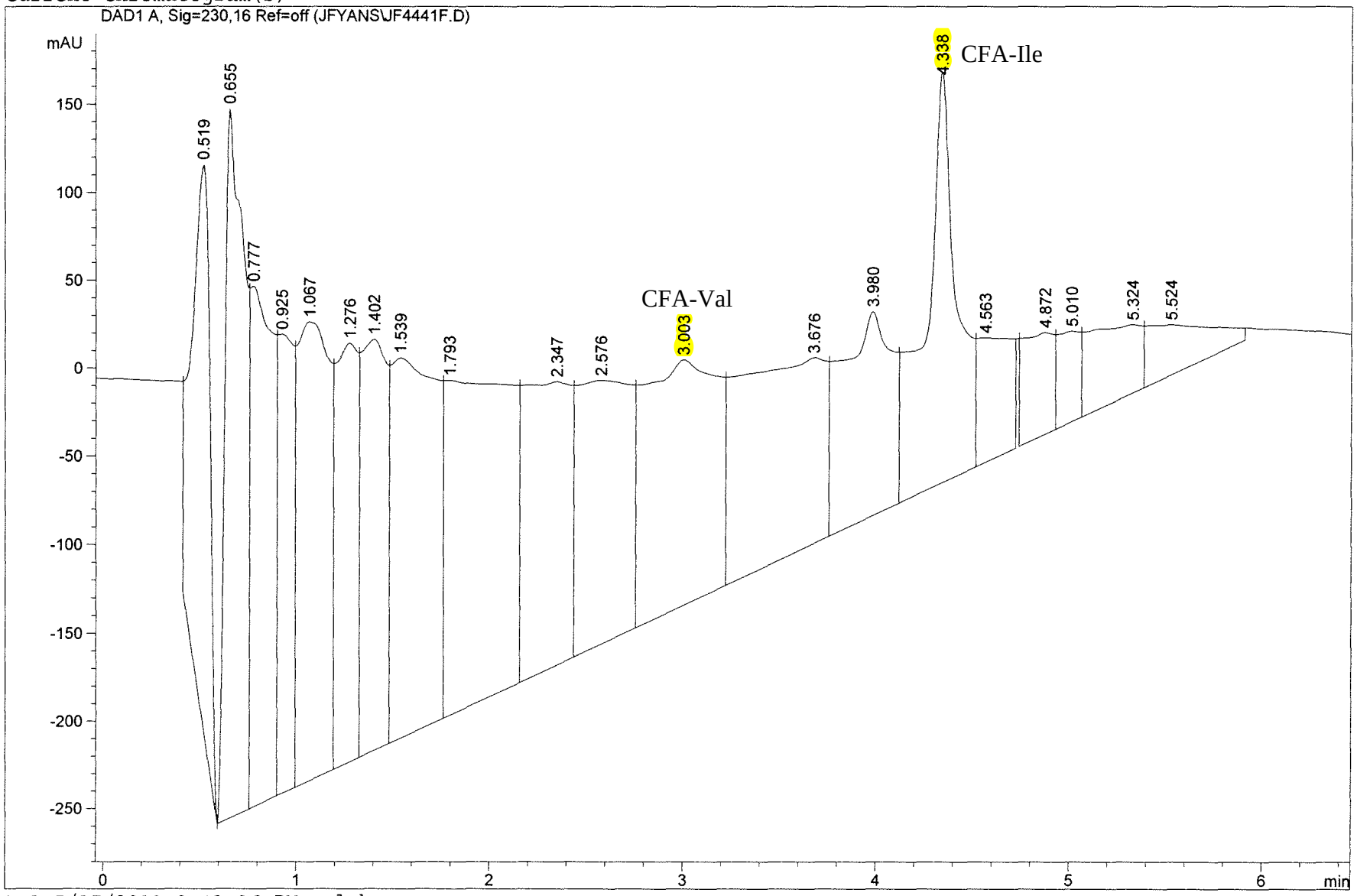
The appendix consists of the raw LC-MS data that were obtained for the acidic culture extracts derived from the *S. scabies* Δ*txtA*/pRLDB51-1, Δ*cfl* #1, Δ*oxr* #2 and Δ*sdr* #2 strains. For each sample analyzed, an absorbance chromatogram (obtained at 230 nm) and a total ion current (TIC) chromatogram was obtained, and the peaks of interest from each chromatogram are indicated. An extracted-ion chromatogram was also obtained for each peak of interest, and the corresponding molecular ion and (m/z) ratio are indicated with yellow highlight.

Print of window 38: Current Chromatogram(s)

Injection Date	: 5/8/2013 1:22:48 PM	Seq. Line	: 2
Sample Name	: DtxtA/51-1	Location	: Vial 2
Acq. Operator	: jfyans	Inj	: 1
Acq. Instrument	: Instrument 1	Inj Volume	: 10 µl
Acq. Method	: D:\Methods\JFYANS.m		
Last changed	: 5/8/2013 1:10:14 PM by jfyans		
Analysis Method	: D:\Methods\EALSBOU.M		
Last changed	: 5/16/2013 5:41:23 PM by ealsbou		

EID/LC-MS

Current Chromatogram(s)

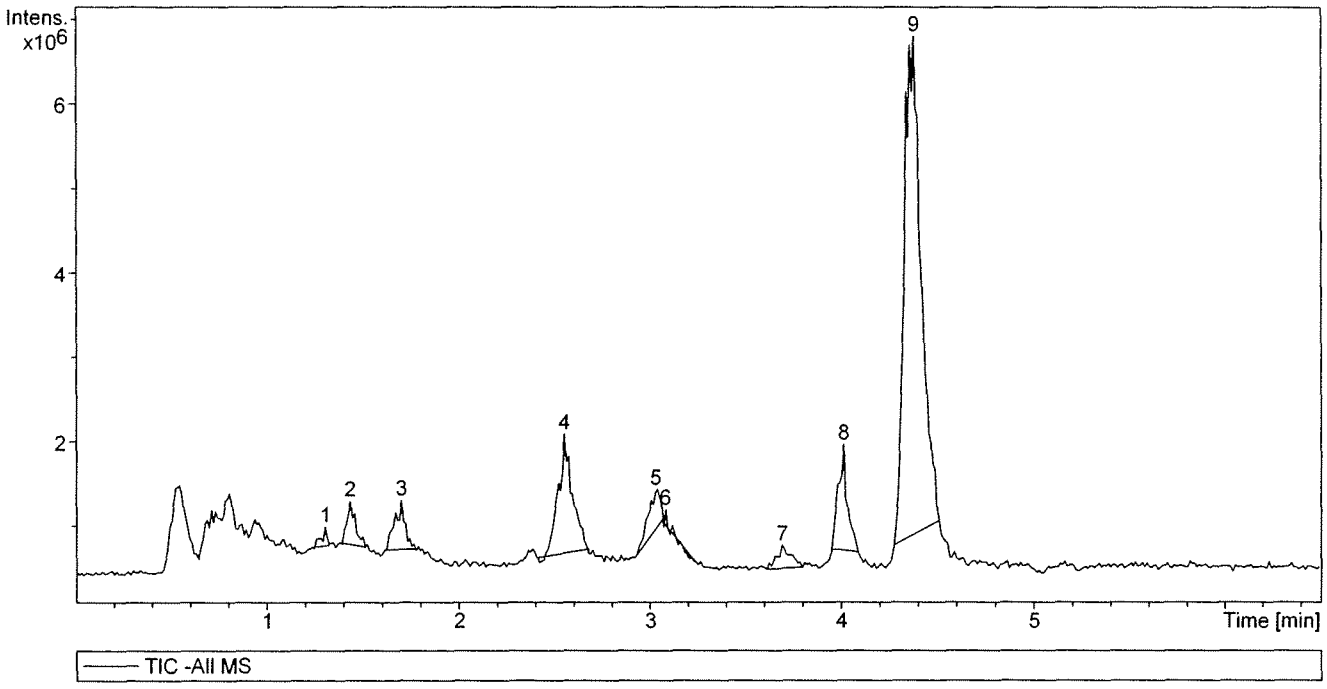


Compound Mass Spectrum Report - MS

Analysis Name: JF4441F.D **Instrument:** LC-MSD-Trap-SL **Print Date:** 05/17/2013 02:53:11 PM
Method: JFYANS.M **Operator:** jfyans **Acq. Date:** 5/8/2013 1:22:36 PM
Sample Name: DtxtA/51-1
Analysis Info:

Acquisition Parameter:

Mass Range Mode	Std/Normal	Trap Drive	39.9	Scan Begin	50 m/z
Ion Polarity	Negative	Octopole RF Amplitude	137.0 Vpp	Scan End	500 m/z
Ion Source Type	ESI	Capillary Exit	-107.9 Volt	Averages	7 Spectra
Dry Temp (Set)	350 °C	Skimmer	-40.0 Volt	Max. Accu Time	200000 µs
Nebulizer (Set)	70.00 psi	Oct 1 DC	-12.00 Volt	ICC Target	10000
Dry Gas (Set)	12.00 l/min	Oct 2 DC	-1.70 Volt	Charge Control	on



Compound List:

#	RT [min]	Range [min]	Height	Area	Area Frac %	
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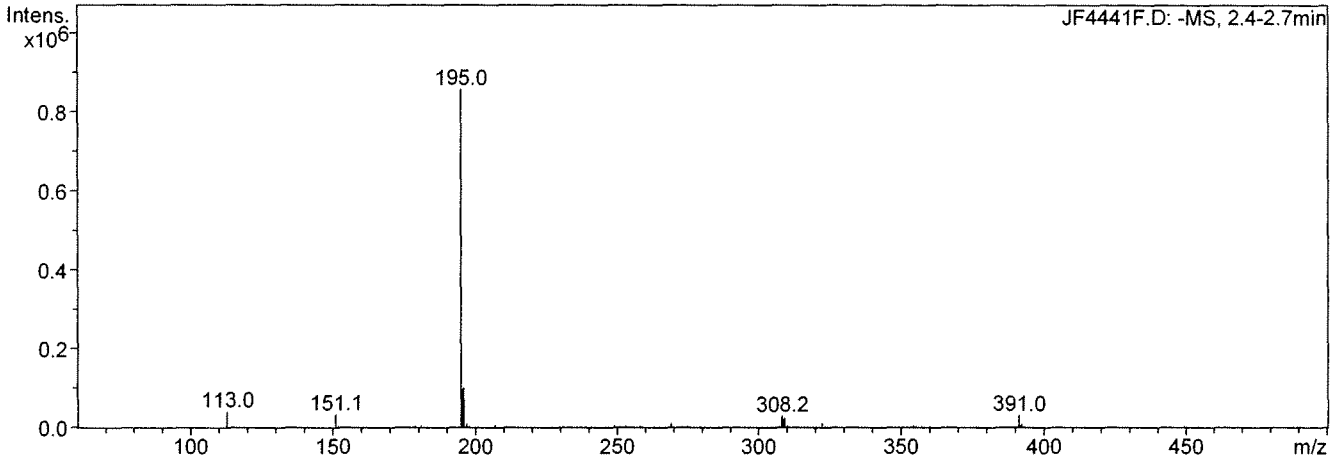
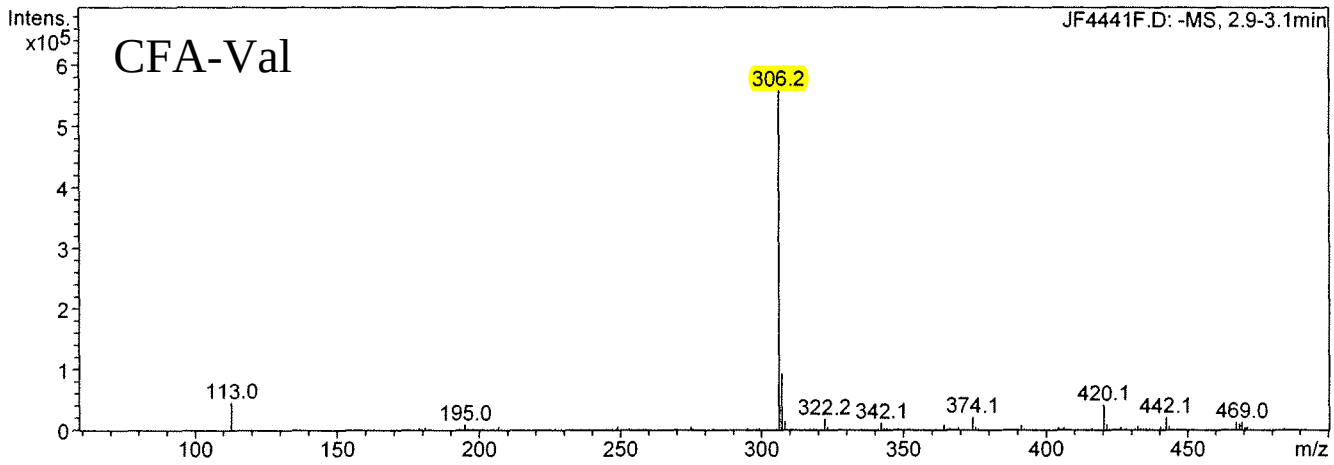
2	1.4	1.4 - 1.5	509703	1627557	3.0	
3	1.7	1.6 - 1.8	577708	2181250	4.0	
4	2.5	2.4 - 2.7	1406277	7187320	13.0	
5	3.0	2.9 - 3.1	431827	2044698	3.7	CFA-Val
6	3.1	3.1 - 3.2	182806	219970	0.4	
7	3.7	3.6 - 3.8	273363	1385935	2.5	
8	4.0	3.9 - 4.1	1253400	4122268	7.5	
9	4.4	4.3 - 4.5	5892838	35920676	65.1	CFA-Ile

Compound Mass Spectrum Report - MS

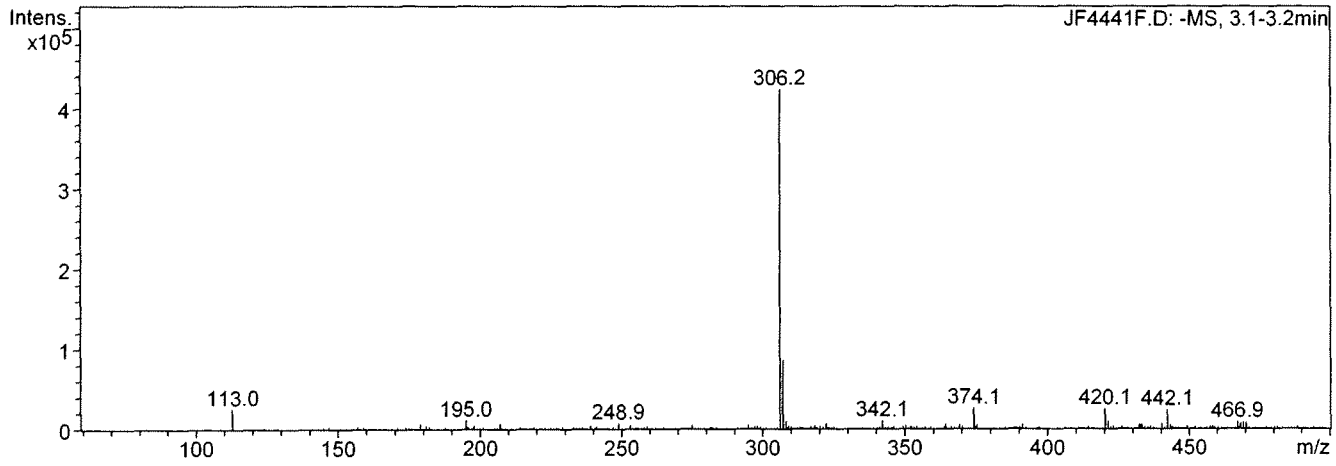
Cmpd 4, 2.5 min



Cmpd 5, 3.0 min

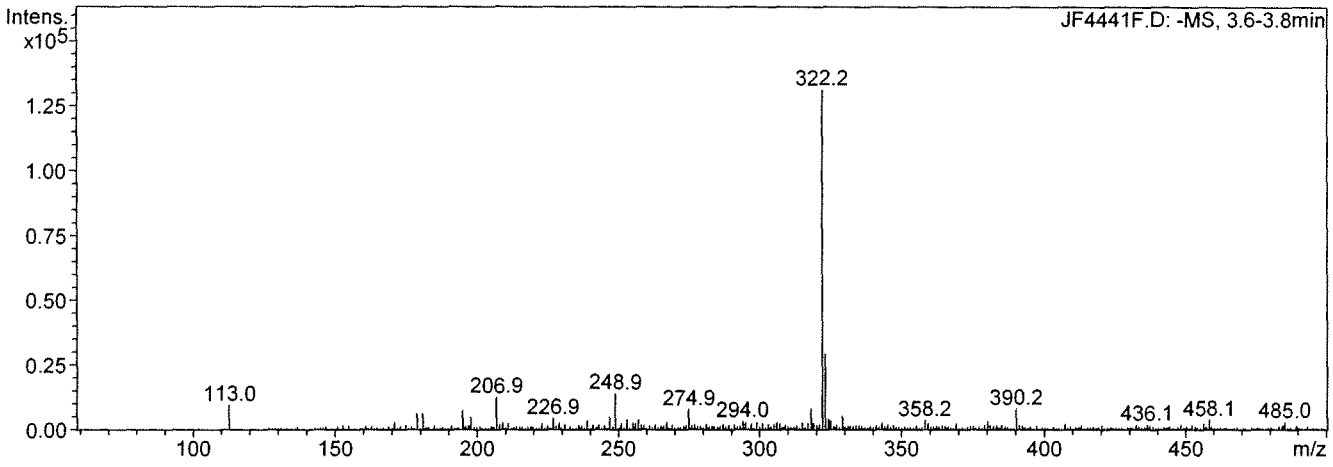


Cmpd 6, 3.1 min

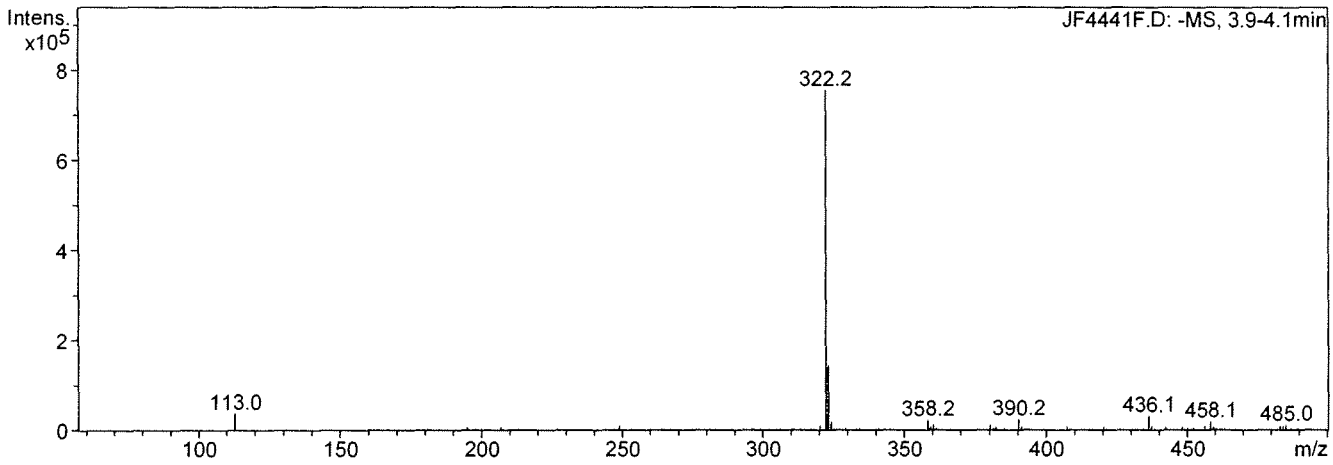


Compound Mass Spectrum Report - MS

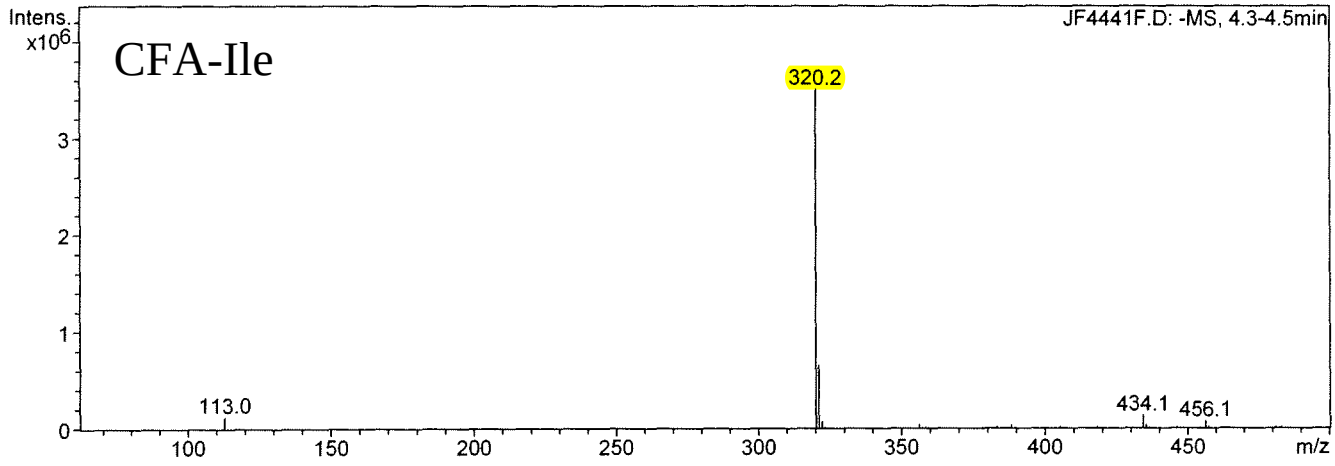
Cmpd 7, 3.7 min



Cmpd 8, 4.0 min



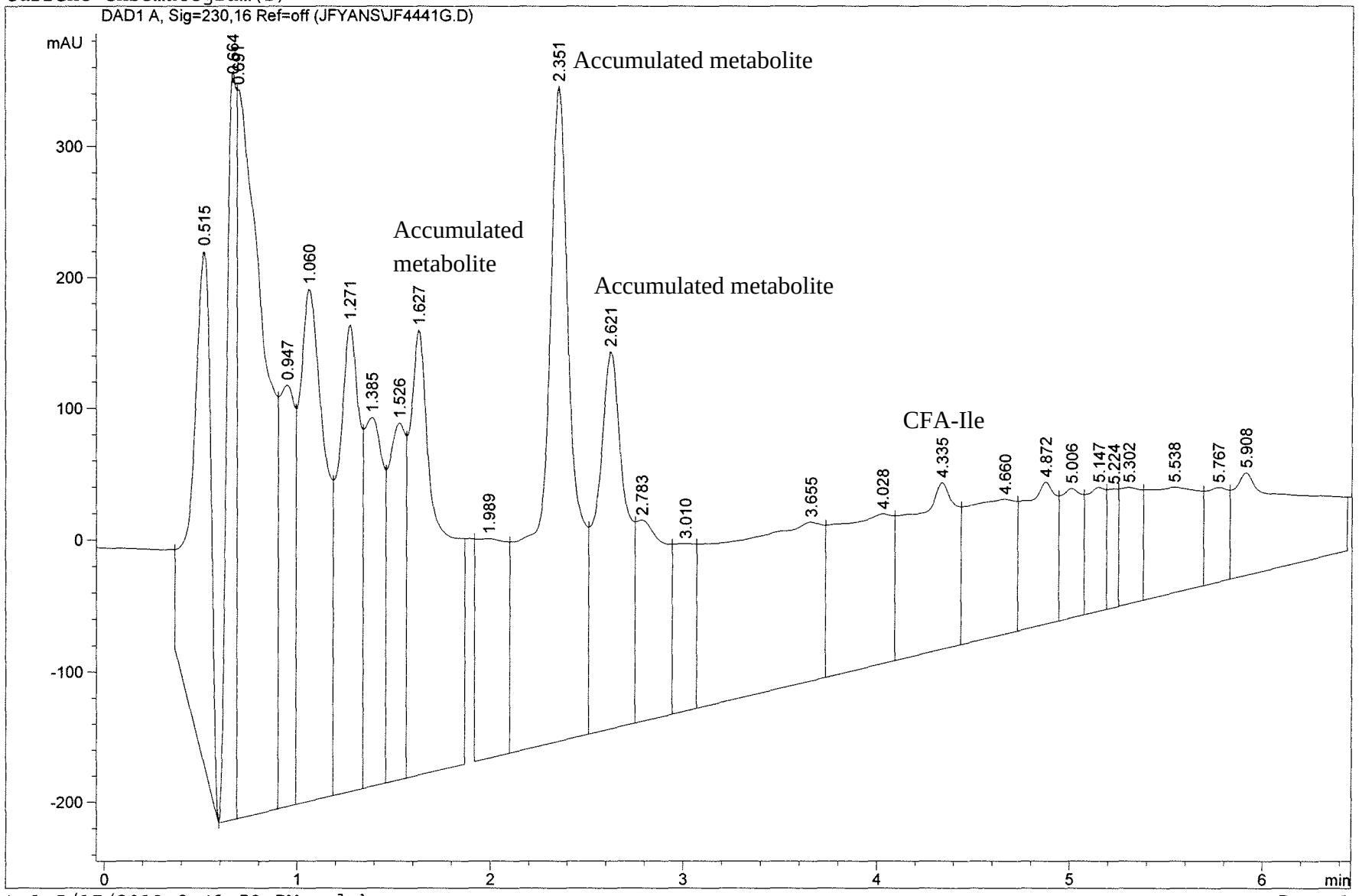
Cmpd 9, 4.4 min



Print of window 38: Current Chromatogram(s)

Injection Date : 5/8/2013 1:33:11 PM Seq. Line : 3
Sample Name : DtxtA/D721 #2 Location : Vial 3
Acq. Operator : jfyans Inj : 1
Acq. Instrument : Instrument 1 Inj Volume : 10 µl
Acq. Method : D:\Methods\JFYANS.m
Last changed : 5/8/2013 1:10:14 PM by jfyans
Analysis Method : D:\Methods\EALSBOU.M
Last changed : 5/16/2013 5:41:23 PM by ealsbou
EID/LC-MS

Current Chromatogram(s)

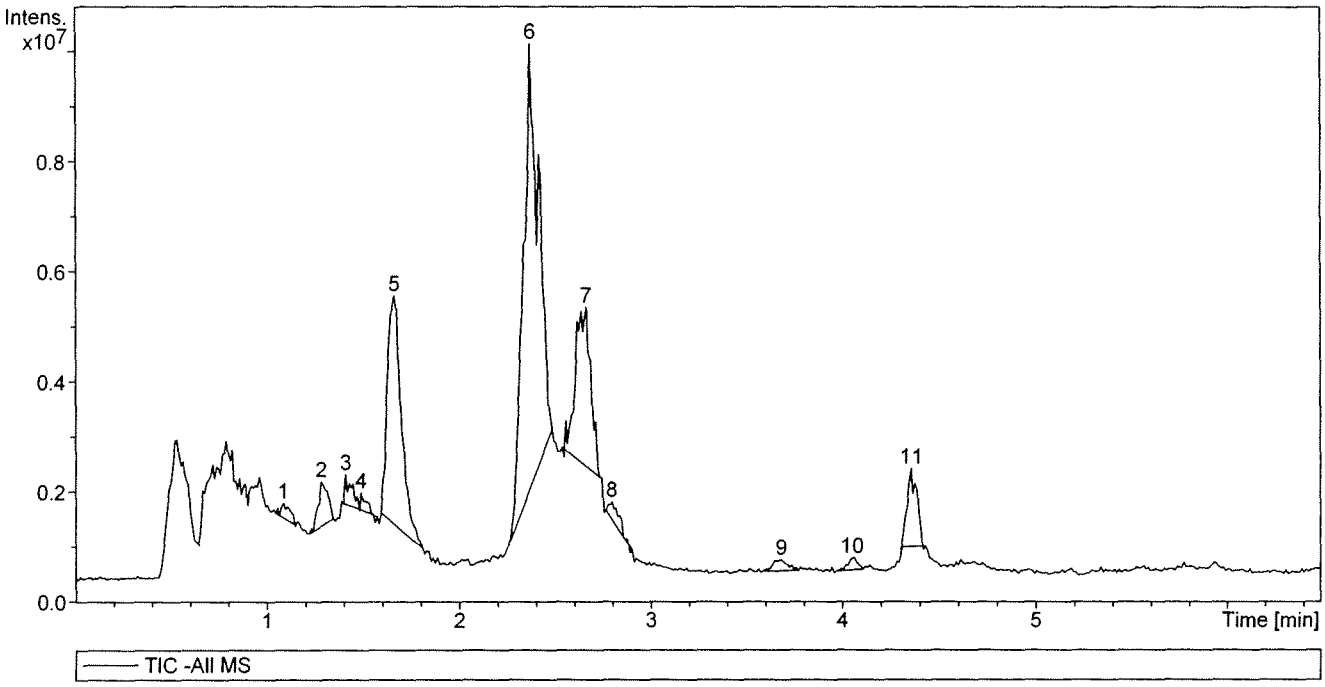


Compound Mass Spectrum Report - MS

Analysis Name: JF4441G.D **Instrument:** LC-MSD-Trap-SL **Print Date:** 05/17/2013 02:55:46 PM
Method: JFYANS.M **Operator:** jfyans **Acq. Date:** 5/8/2013 1:32:59 PM
Sample Name: DtxtA/D721 #2
Analysis Info:

Acquisition Parameter:

Mass Range Mode	Std/Normal	Trap Drive	39.9	Scan Begin	50 m/z
Ion Polarity	Negative	Octopole RF Amplitude	137.0 Vpp	Scan End	500 m/z
Ion Source Type	ESI	Capillary Exit	-107.9 Volt	Averages	7 Spectra
Dry Temp (Set)	350 °C	Skimmer	-40.0 Volt	Max. Accu Time	200000 µs
Nebulizer (Set)	70.00 psi	Oct 1 DC	-12.00 Volt	ICC Target	10000
Dry Gas (Set)	12.00 l/min	Oct 2 DC	-1.70 Volt	Charge Control	on



Compound List:

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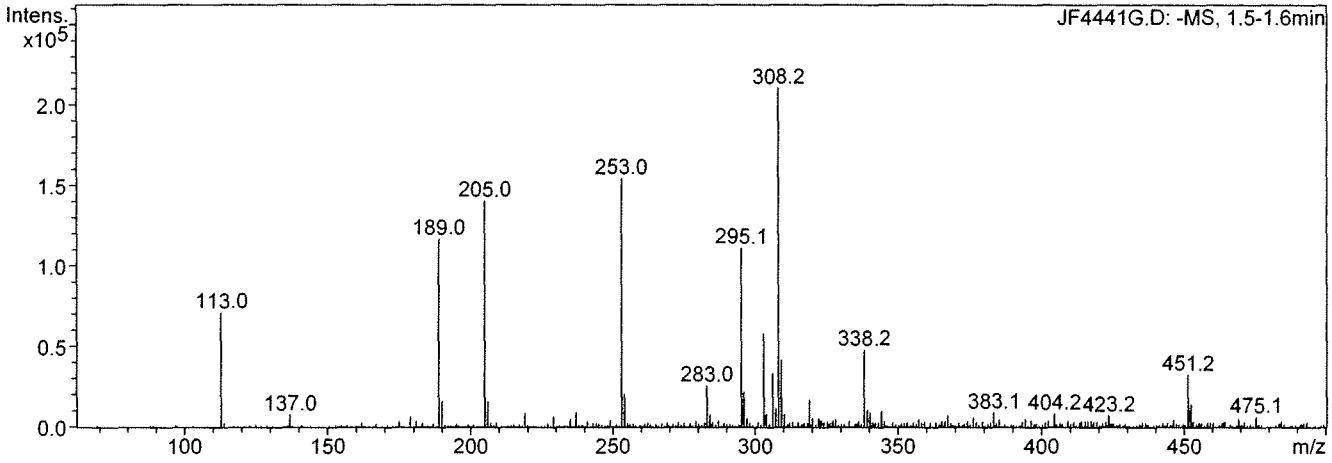
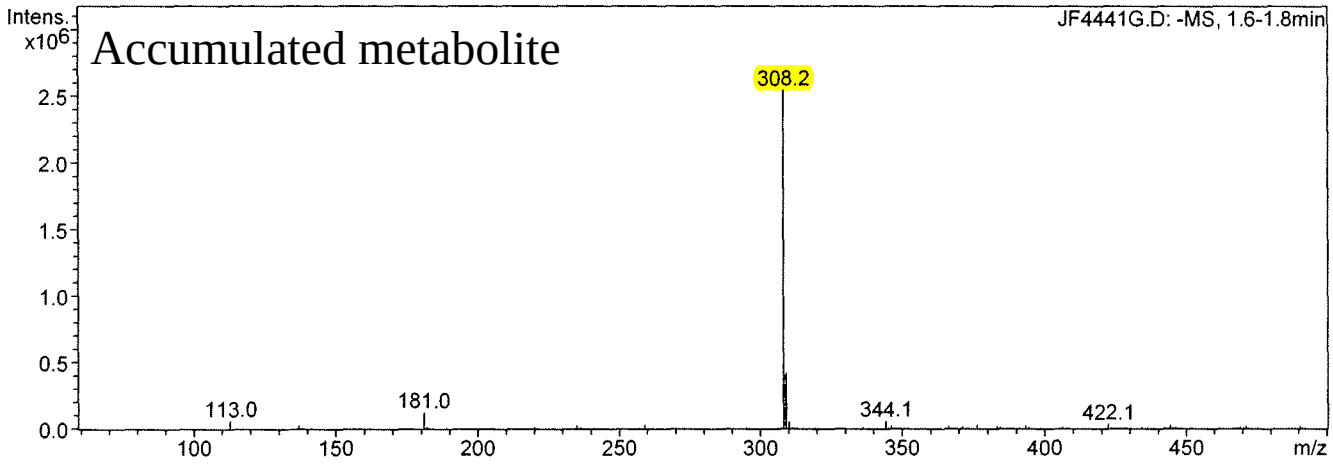
1	1.1	1.0 - 1.1	263476	976633	1.0	
2	1.3	1.2 - 1.3	802668	2782232	2.9	
3	1.4	1.4 - 1.5	533286	1440665	1.5	
4	1.5	1.5 - 1.6	309438	669696	0.7	
5	1.7	1.6 - 1.8	4148095	20798928	21.8	
6	2.4	2.3 - 2.5	8091747	44523668	46.6	Accumulated metabolites
7	2.7	2.5 - 2.7	2895913	15913717	16.7	
8	2.8	2.8 - 2.9	317948	1311143	1.4	
9	3.7	3.6 - 3.8	208849	1180972	1.2	
10	4.0	4.0 - 4.1	223359	808130	0.8	
11	4.4	4.3 - 4.4	1422351	5064599	5.3	CFA-Ile

Compound Mass Spectrum Report - MS

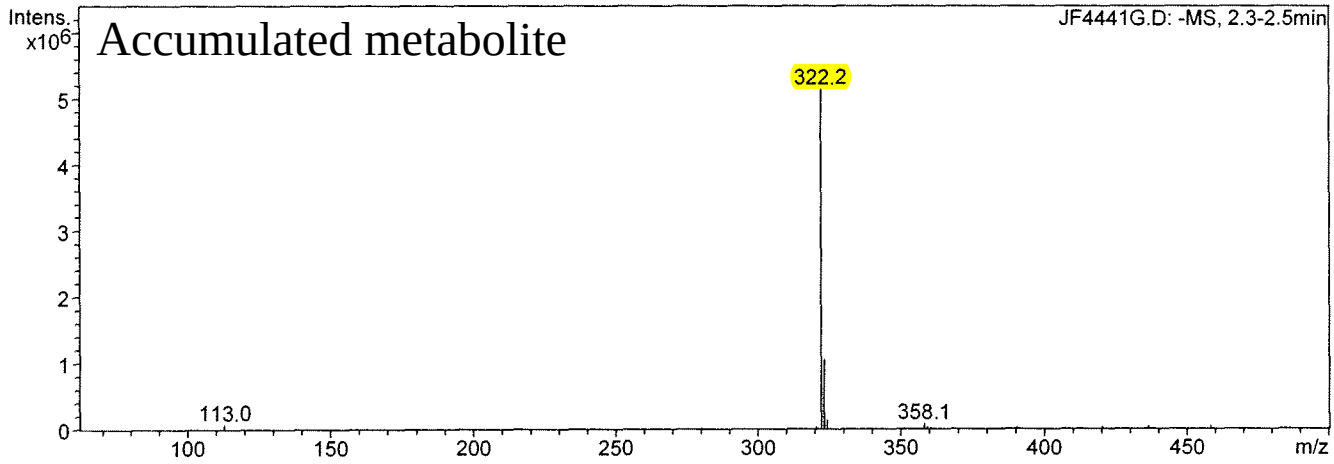
Cmpd 4, 1.5 min



Cmpd 5, 1.7 min

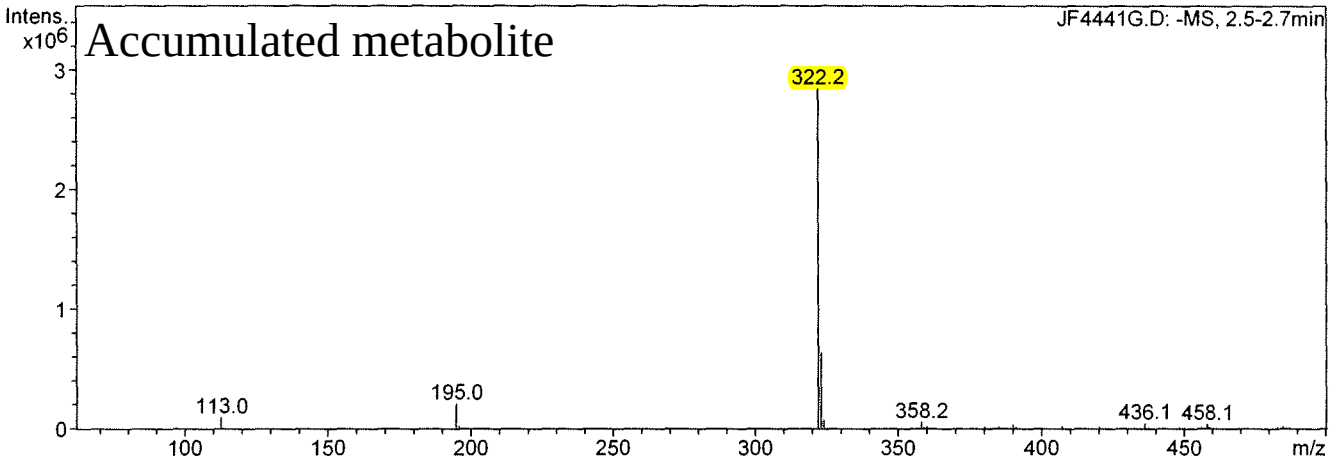


Cmpd 6, 2.4 min

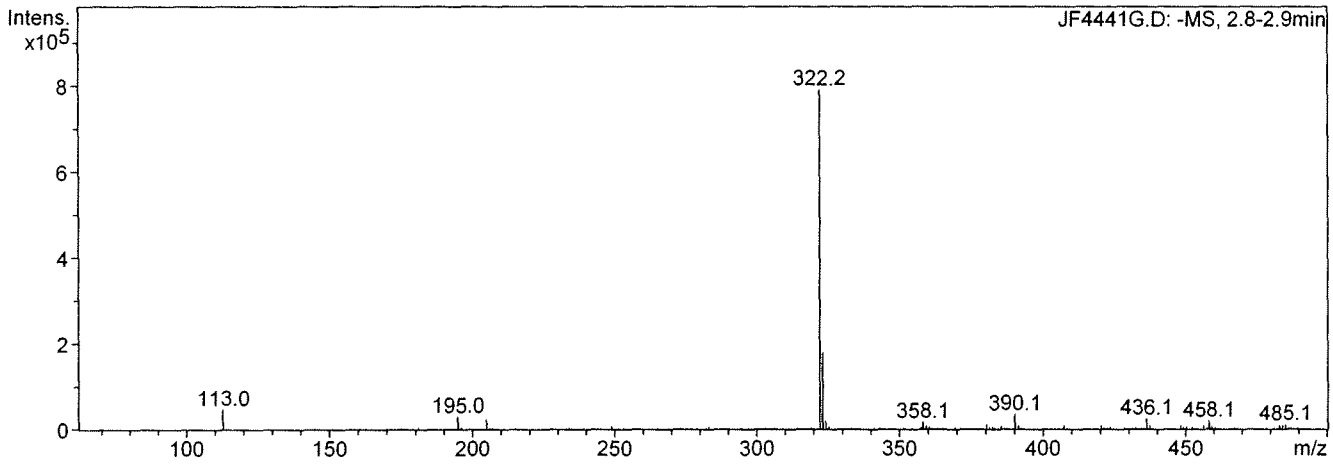


Compound Mass Spectrum Report - MS

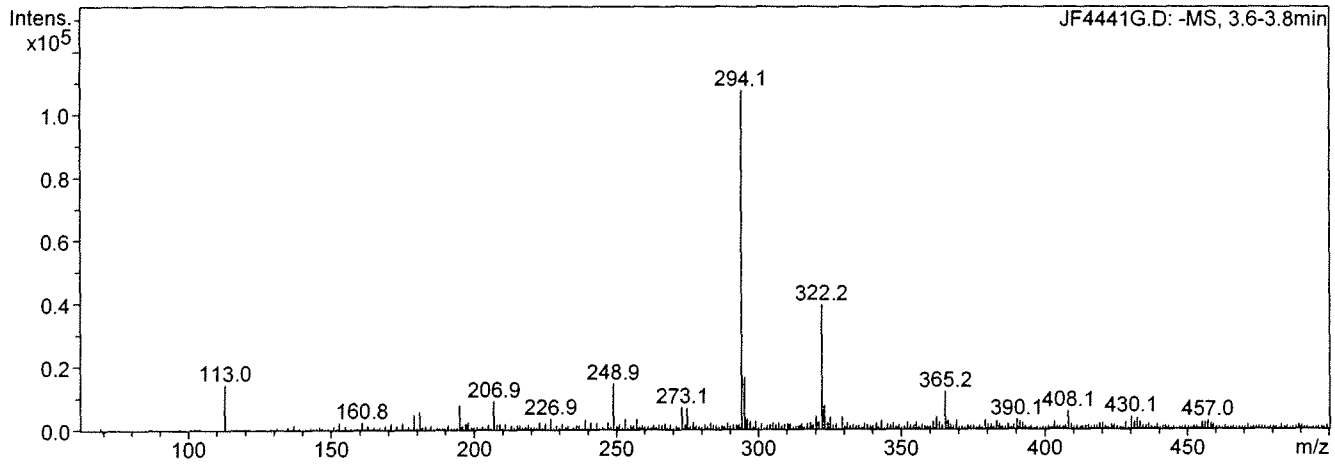
Cmpd 7, 2.7 min



Cmpd 8, 2.8 min

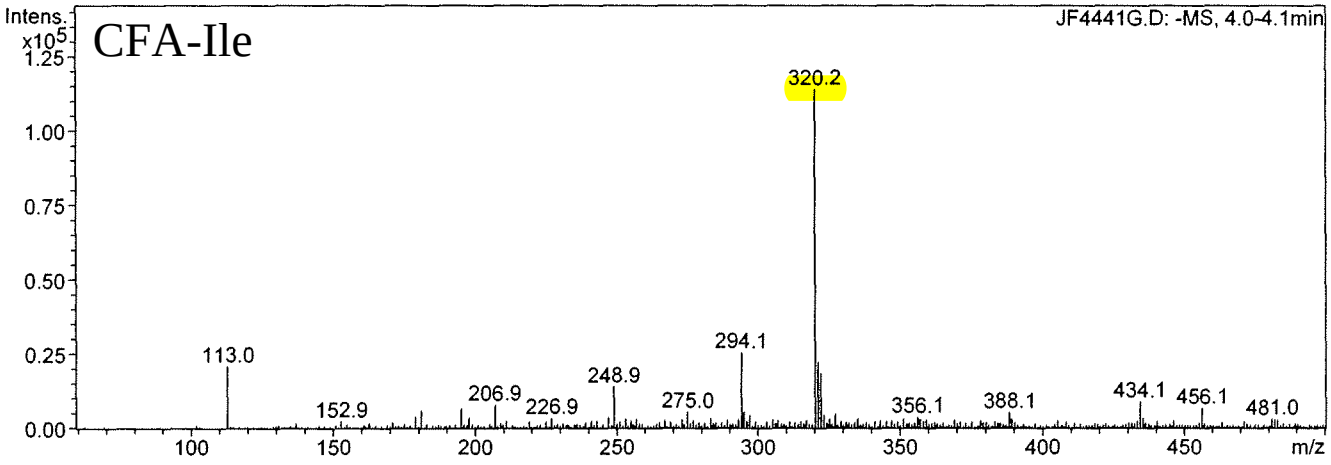


Cmpd 9, 3.7 min

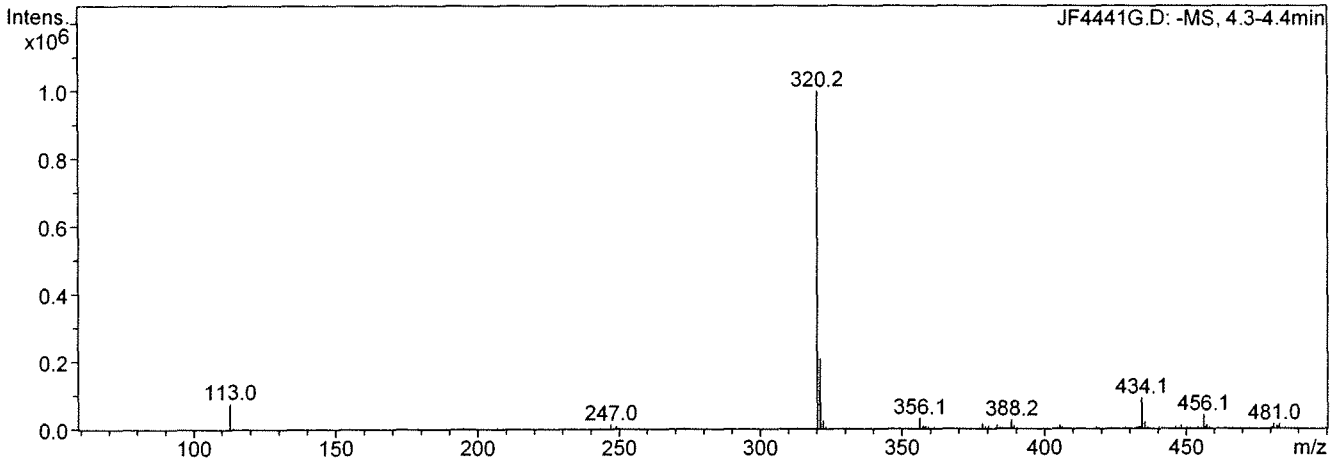


Compound Mass Spectrum Report - MS

Cmpd 10, 4.0 min



Cmpd 11, 4.4 min

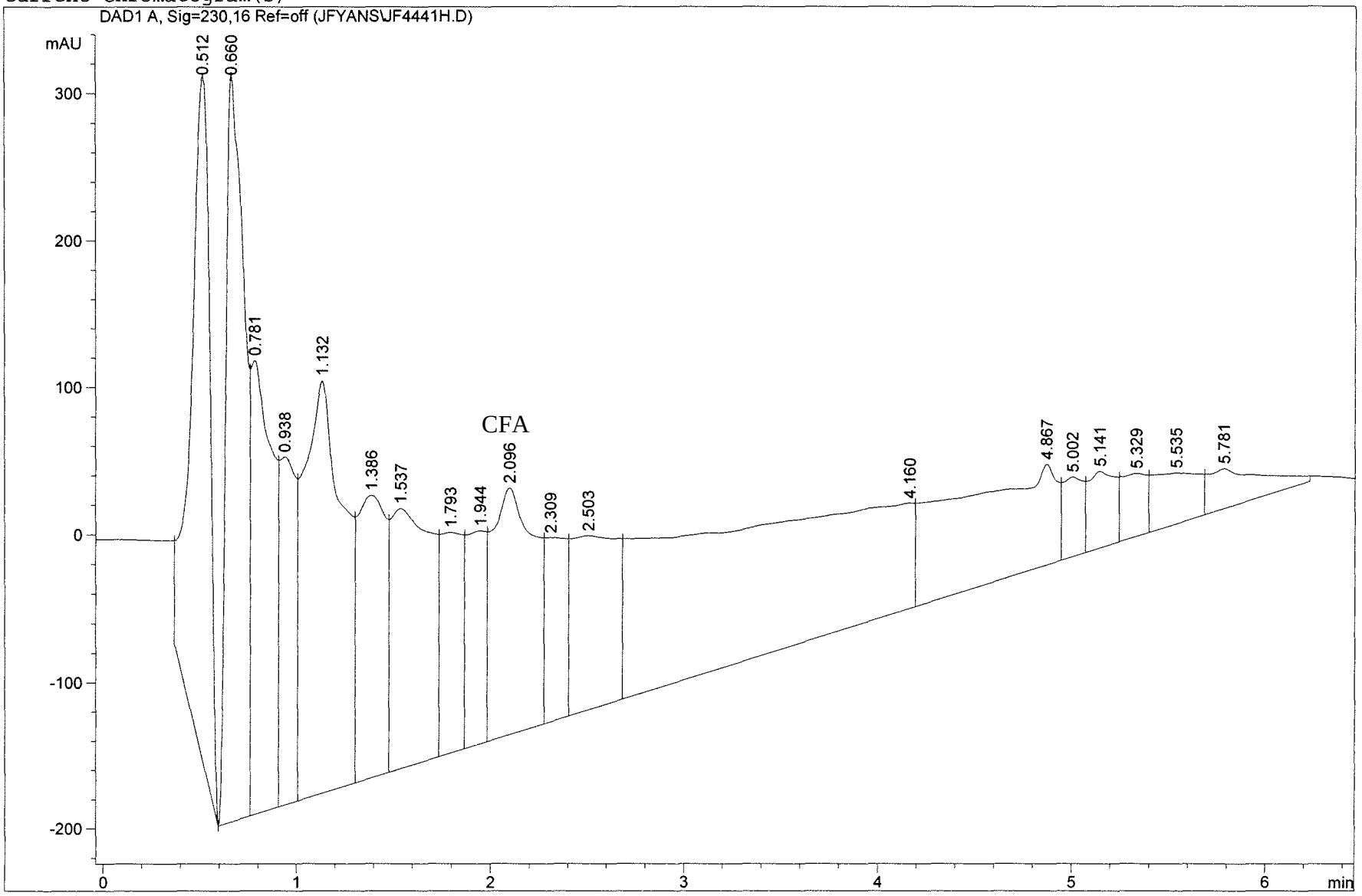


Print of window 38: Current Chromatogram(s)

Injection Date	: 5/8/2013 1:43:35 PM	Seq. Line	: 4
Sample Name	: DtxtA/Dcfl #1	Location	: Vial 4
Acq. Operator	: jfyans	Inj	: 1
Acq. Instrument	: Instrument 1	Inj Volume	: 10 µl
Acq. Method	: D:\Methods\JFYANS.m		
Last changed	: 5/8/2013 1:10:14 PM by jfyans		
Analysis Method	: D:\Methods\EALSBOU.M		
Last changed	: 5/16/2013 5:41:23 PM by ealsbou		

EID/LC-MS

Current Chromatogram(s)

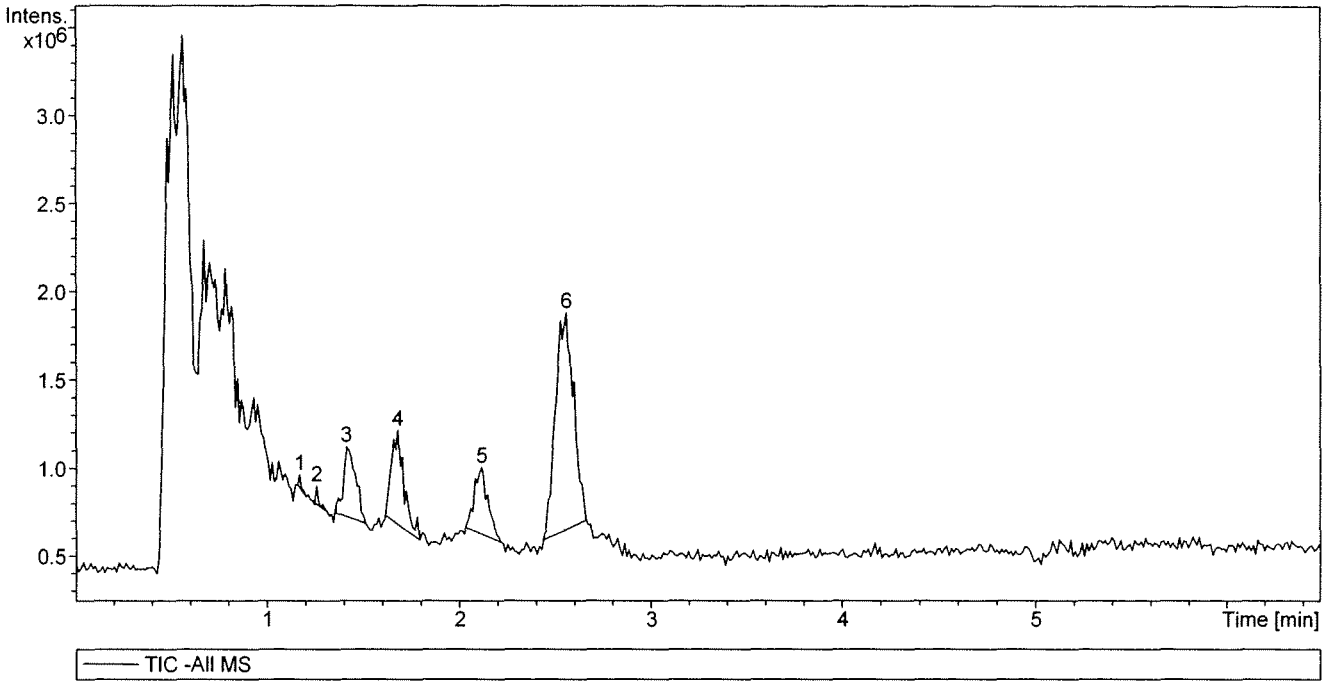


Compound Mass Spectrum Report - MS

Analysis Name: JF4441H.D **Instrument:** LC-MSD-Trap-SL **Print Date:** 05/17/2013 02:58:19 PM
Method: JFYANS.M **Operator:** jfyans **Acq. Date:** 5/8/2013 1:43:22 PM
Sample Name: DtxtA/Dcfl #1
Analysis Info:

Acquisition Parameter:

Mass Range Mode	Std/Normal	Trap Drive	39.9	Scan Begin	50 m/z
Ion Polarity	Negative	Octopole RF Amplitude	137.0 Vpp	Scan End	500 m/z
Ion Source Type	ESI	Capillary Exit	-107.9 Volt	Averages	7 Spectra
Dry Temp (Set)	350 °C	Skimmer	-40.0 Volt	Max. Accu Time	200000 µs
Nebulizer (Set)	70.00 psi	Oct 1 DC	-12.00 Volt	ICC Target	10000
Dry Gas (Set)	12.00 l/min	Oct 2 DC	-1.70 Volt	Charge Control	on

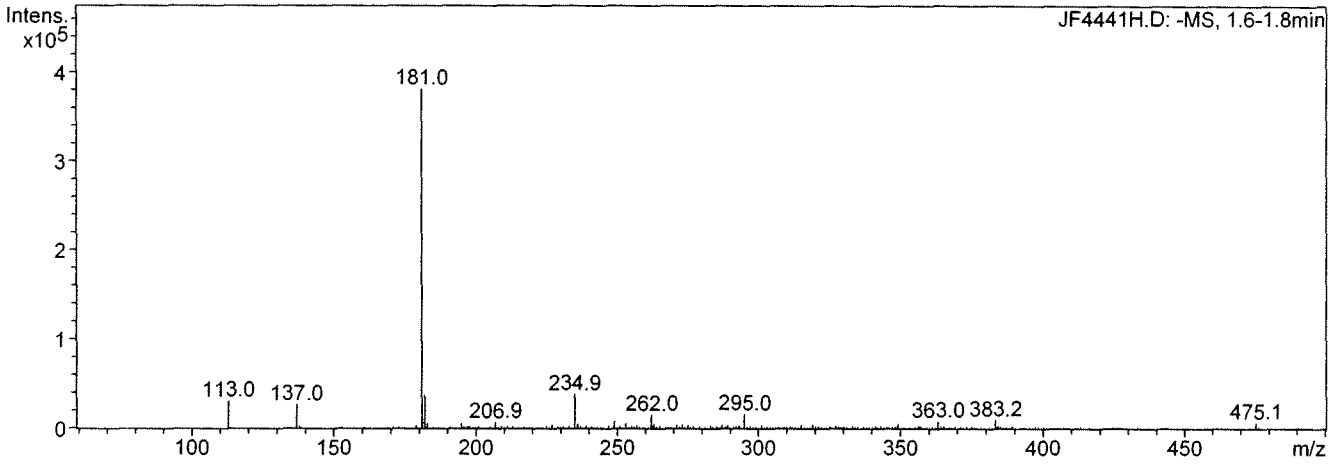


Compound List:

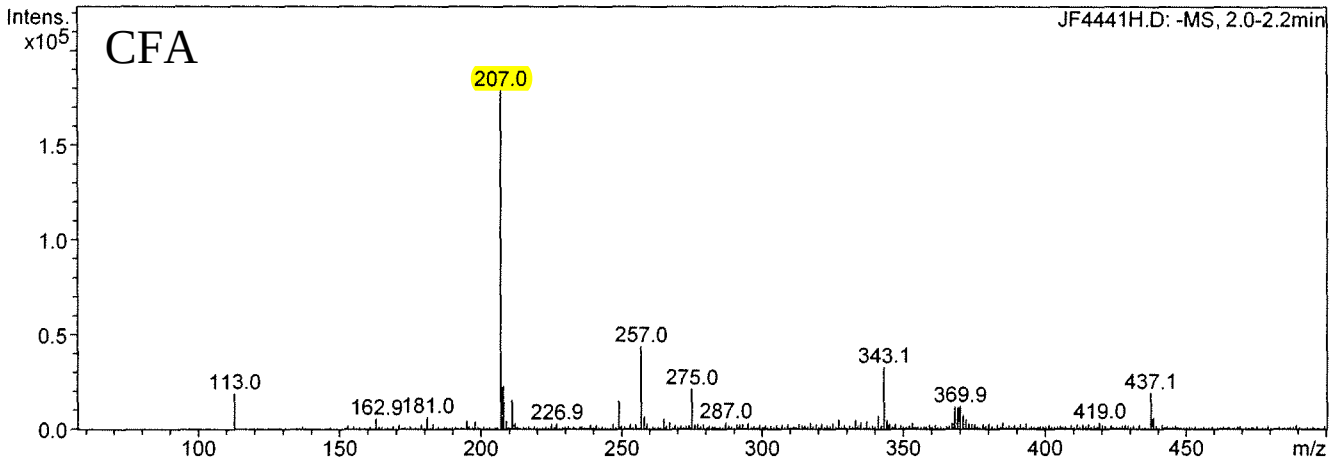
#	RT [min]	Range [min]	Height	Area	Area Frac %	
1	1.2	1.2 - 1.2	71773	71044	0.5	
2	1.3	1.2 - 1.3	101268	109209	0.8	
3	1.4	1.4 - 1.5	395843	1780950	12.5	
4	1.7	1.6 - 1.8	533668	2405894	16.8	
5	2.1	2.0 - 2.2	377177	1840801	12.9	CFA
6	2.6	2.4 - 2.7	1227696	8089657	56.6	

Compound Mass Spectrum Report - MS

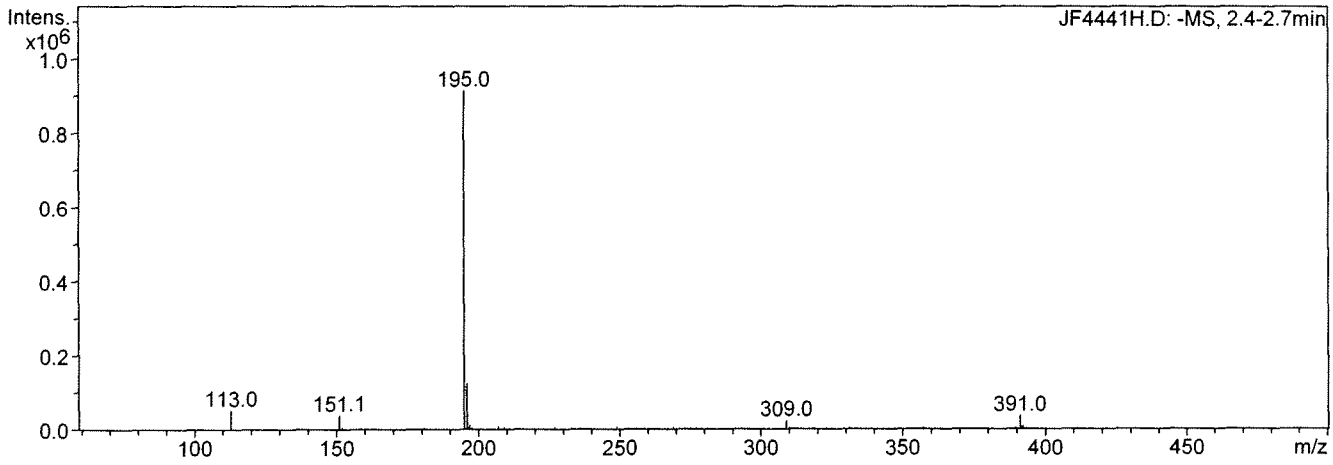
Cmpd 4, 1.7 min



Cmpd 5, 2.1 min



Cmpd 6, 2.6 min

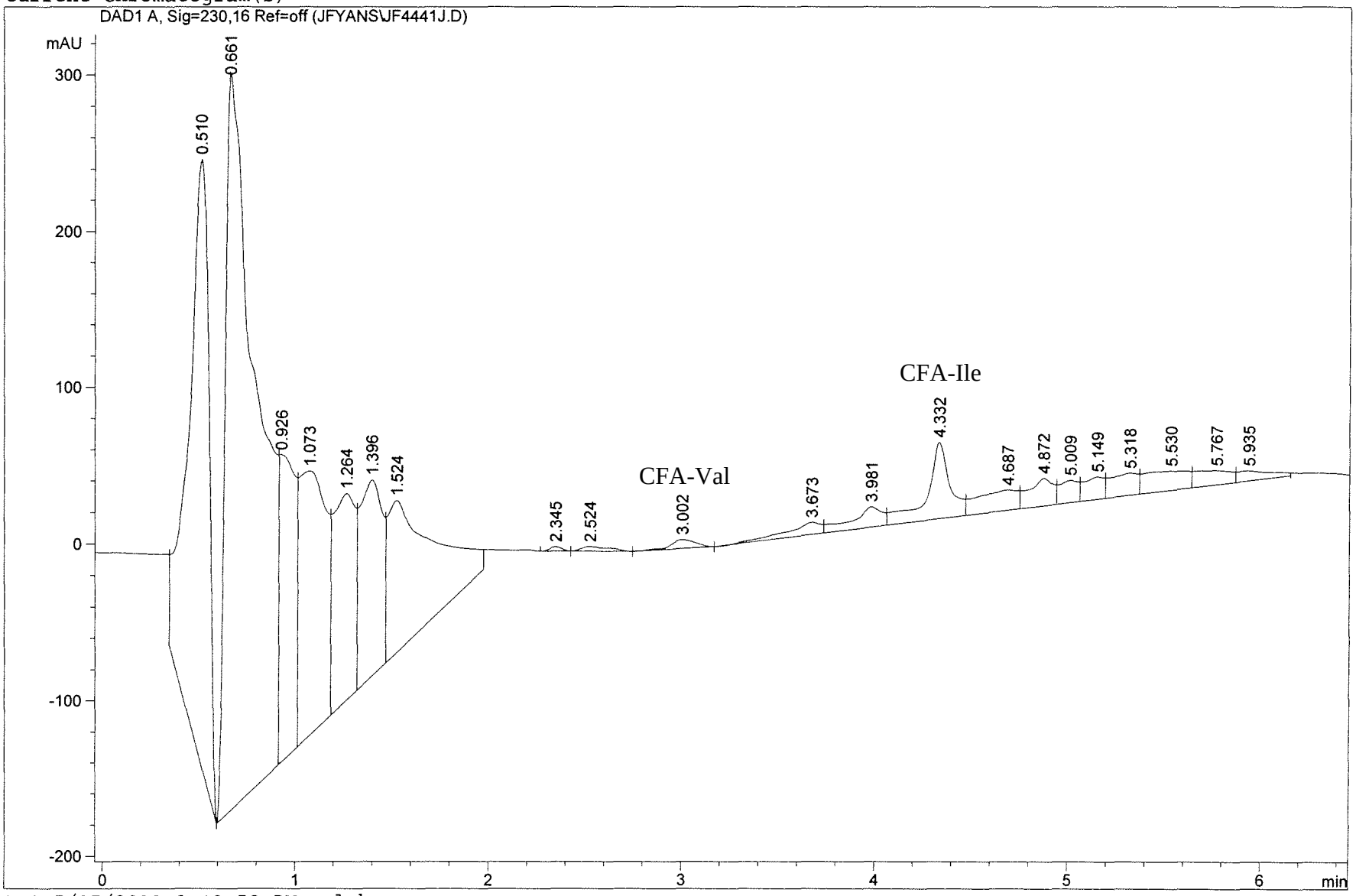


Print of window 38: Current Chromatogram(s)

Injection Date	: 5/8/2013 2:04:24 PM	Seq. Line	: 6
Sample Name	: DtxtA/D681 #2	Location	: Vial 6
Acq. Operator	: jfyans	Inj	: 1
Acq. Instrument	: Instrument 1	Inj Volume	: 10 µl
Acq. Method	: D:\Methods\JFYANS.m		
Last changed	: 5/8/2013 1:10:14 PM by jfyans		
Analysis Method	: D:\Methods\EALSBOU.M		
Last changed	: 5/16/2013 5:41:23 PM by ealsbou		

EID/LC-MS

Current Chromatogram(s)

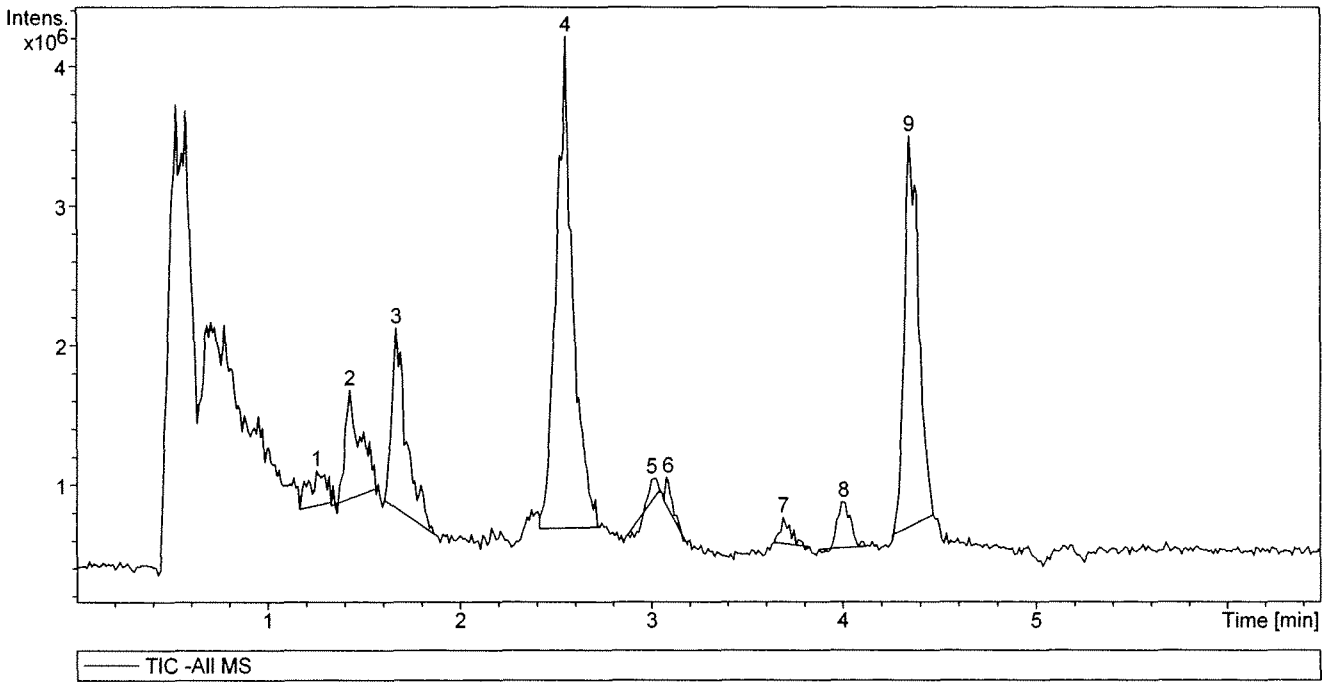


Compound Mass Spectrum Report - MS

Analysis Name: JF4441J.D **Instrument:** LC-MSD-Trap-SL **Print Date:** 05/17/2013 03:02:17 PM
Method: JFYANS.M **Operator:** jfyans **Acq. Date:** 5/8/2013 2:04:12 PM
Sample Name: DtxtA/D681 #2
Analysis Info:

Acquisition Parameter:

Mass Range Mode	Std/Normal	Trap Drive	39.9	Scan Begin	50 m/z
Ion Polarity	Negative	Octopole RF Amplitude	137.0 Vpp	Scan End	500 m/z
Ion Source Type	ESI	Capillary Exit	-107.9 Volt	Averages	7 Spectra
Dry Temp (Set)	350 °C	Skimmer	-40.0 Volt	Max. Accu Time	200000 µs
Nebulizer (Set)	70.00 psi	Oct 1 DC	-12.00 Volt	ICC Target	10000
Dry Gas (Set)	12.00 l/min	Oct 2 DC	-1.70 Volt	Charge Control	on

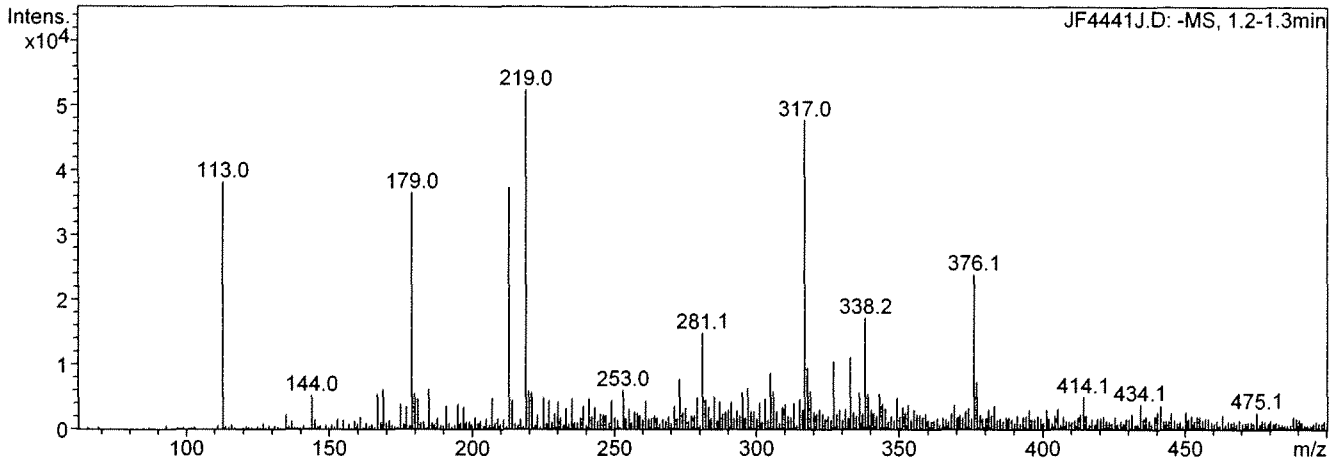


Compound List:

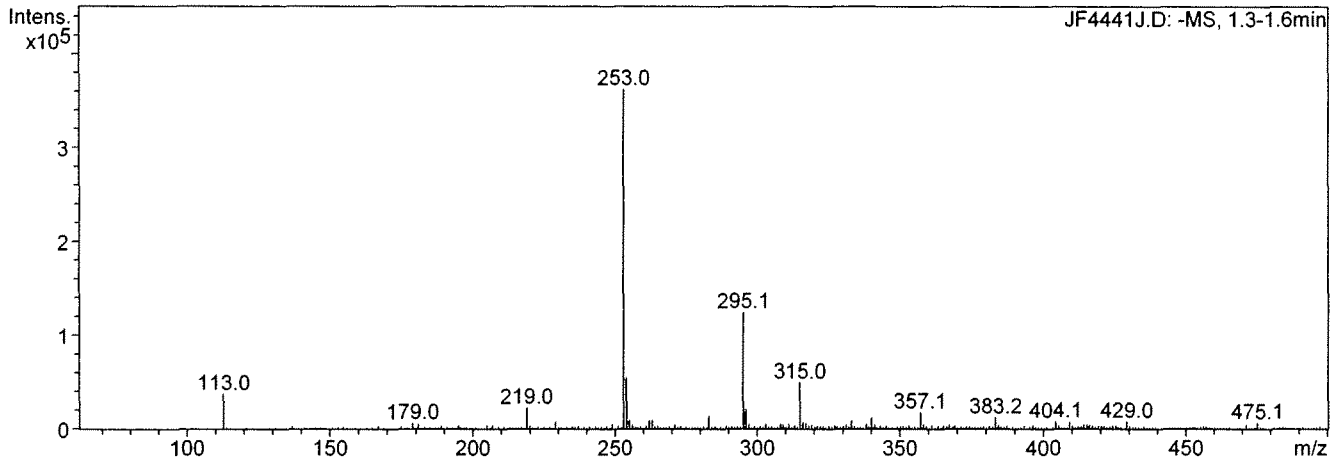
#	RT [min]	Range [min]	Height	Area	Area Frac %	
1	1.3	1.2 - 1.3	247488	1547588	3.0	
2	1.4	1.3 - 1.6	773422	4431781	8.6	
3	1.7	1.6 - 1.9	1286315	6756662	13.1	
4	2.6	2.4 - 2.7	3518483	21180298	41.1	
5	3.0	2.9 - 3.0	154427	452331	0.9	CFA-Val
6	3.1	3.1 - 3.2	196138	539197	1.0	
7	3.7	3.6 - 3.8	187717	731769	1.4	
8	4.0	3.9 - 4.1	330624	1583745	3.1	
9	4.3	4.3 - 4.5	2796720	14303136	27.8	CFA-Ile

Compound Mass Spectrum Report - MS

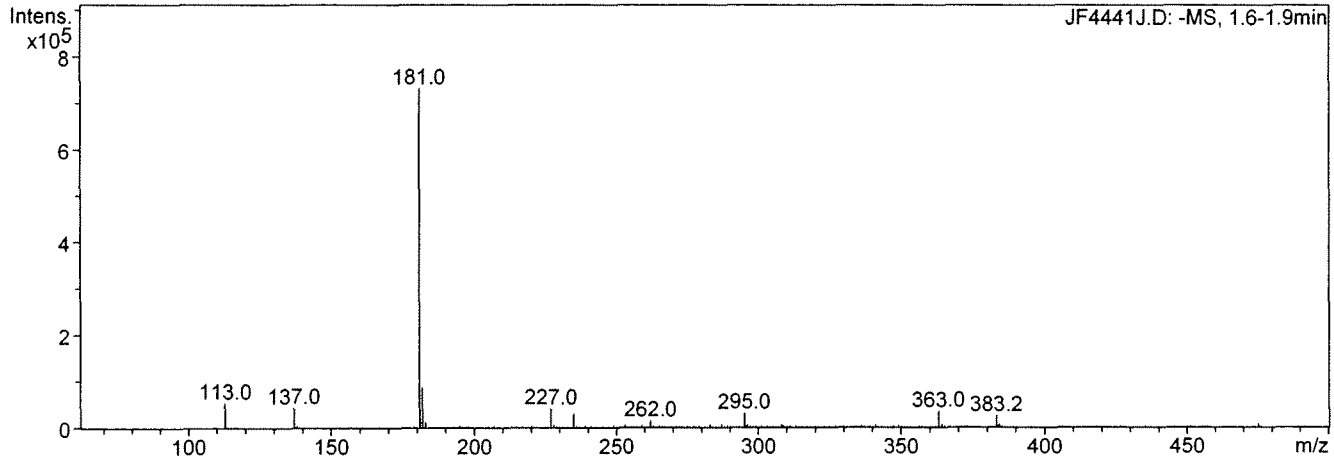
Cmpd 1, 1.3 min



Cmpd 2, 1.4 min

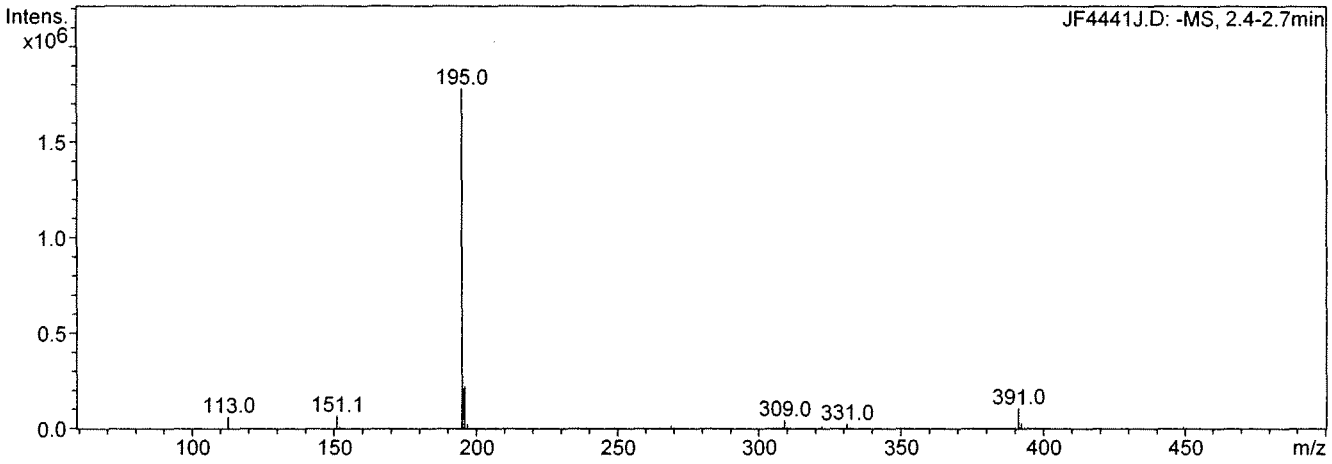


Cmpd 3, 1.7 min

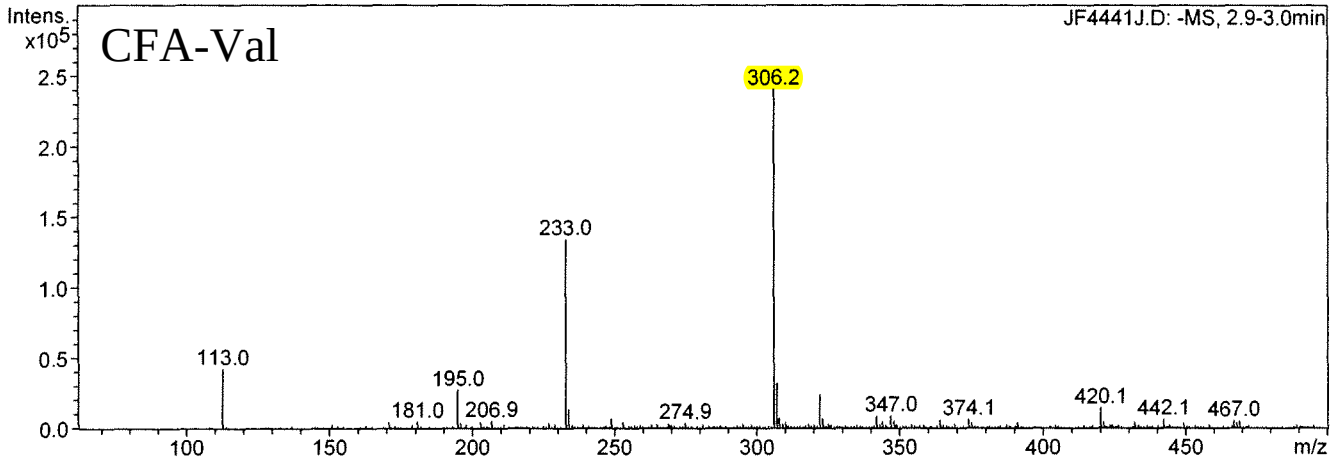


Compound Mass Spectrum Report - MS

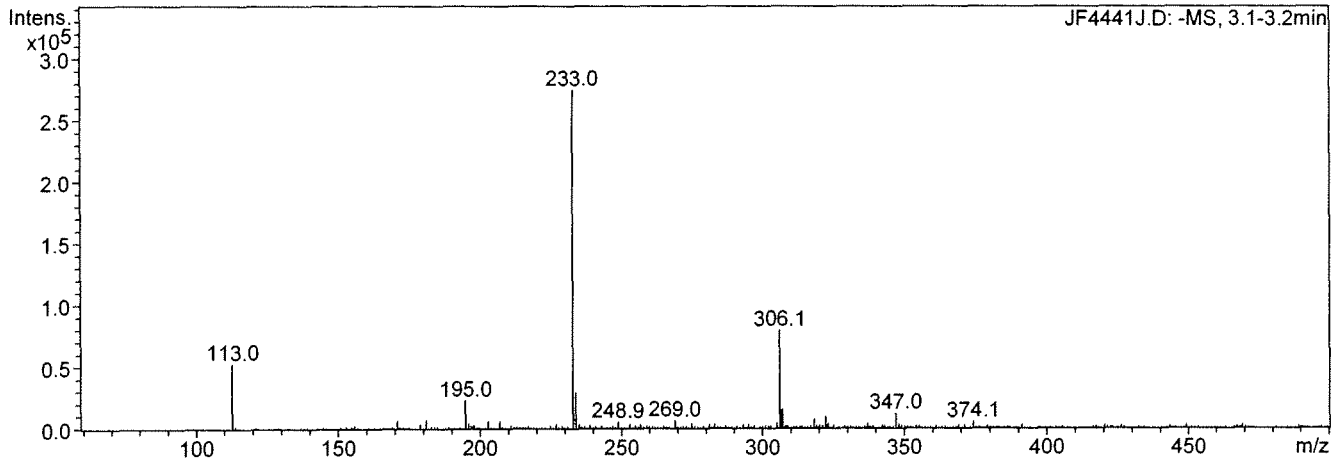
Cmpd 4, 2.6 min



Cmpd 5, 3.0 min

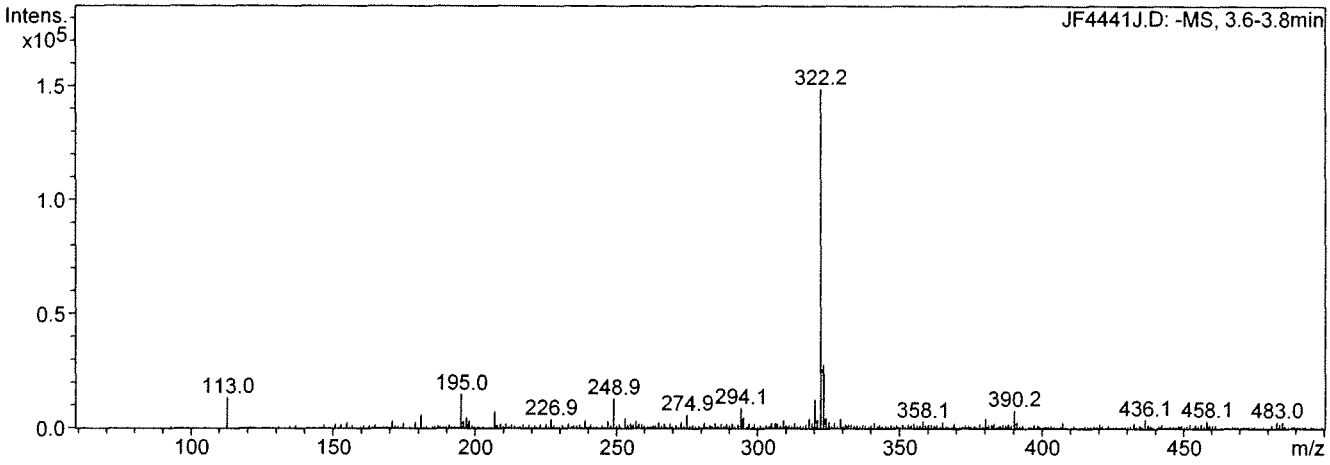


Cmpd 6, 3.1 min

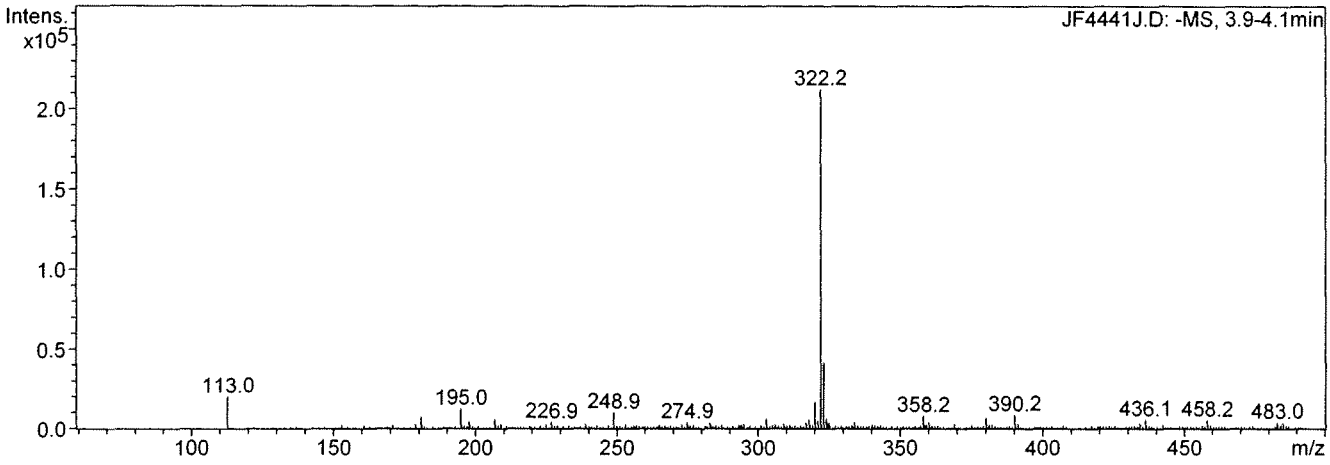


Compound Mass Spectrum Report - MS

Cmpd 7, 3.7 min



Cmpd 8, 4.0 min



Cmpd 9, 4.3 min