Characterization of the Coronatine-Like Phytotoxins Produced by the Common Scab Pathogen *Streptomyces scabies*

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Submitted 2 September 2014. Accepted 14 November 2014.

Streptomyces scabies is an important causative agent of common scab disease of potato tubers and other root crops. The primary virulence factor produced by this pathogen is a phytotoxic secondary metabolite called thaxtomin A, which is essential for disease development. In addition, the genome of *S. scabies* harbors a virulence-associated biosynthetic gene cluster called the coronafacic acid (CFA)-like gene cluster, which was previously predicted to produce metabolites that resemble the *Pseudomonas syringae* coronatine (COR) phytotoxin. COR consists of CFA linked to an ethylcyclopropyl amino acid called coronamic acid, which is derived from L-*allo*-isoleucine. Using a combination of genetic and chemical analyses, we show that the *S. scabies* CFA-like gene cluster is responsible for producing CFA-L-isoleucine as the major product as well as other minor COR-like metabolites. Production of the metabolites was shown to require the *cfl* gene, which is located within the CFA-like gene cluster and encodes an enzyme involved in ligating CFA to its amino acid partner. CFA-L-isoleucine purified from *S. scabies* cultures was shown to exhibit bioactivity similar to that of COR, though it was found to be less toxic than COR. This is the first report demonstrating the production of coronafacoyl phytotoxins by *S. scabies*, which is the most prevalent scab-causing pathogen in North America.

Members of the genus *Streptomyces* are gram-positive filamentous saprophytic bacteria that are highly abundant in soil environments. Among the notable features of these organisms are their complex developmental life cycle, their large genome sizes, and their ability to produce numerous secondary metabolites with diverse biological activities. Although more than 500 species of *Streptomyces* have been identified, only a small number (approximately 15) have been shown to be pathogenic to plants (Bignell et al. 2014). The most important plant disease caused by *Streptomyces* spp. is potato common scab, which is named after the brown corky lesions that form on the tuber surface. Such lesions can be superficial, raised, or deep-pitted, and their development and severity is dependent on factors such as environmental

conditions, pathogen virulence and load, and the affected potato cultivar (Dees and Wanner 2012; Loria et al. 1997). Scab-causing *Streptomyces* spp. affect the underground structures of the potato plant (*Solanum tuberosum* L.) such as stems, stolons, and tubers, and they can also infect other economically important root crops such as carrot, beet, parsnip, and radish under field conditions (Dees and Wanner 2012). Furthermore, under controlled conditions, such species can infect the fibrous roots of various monocot and dicot plants, causing root and shoot stunting and necrosis of root meristems (Loria et al. 2008).

Among the best-characterized scab-causing pathogens is *Streptomyces scabies* (syn. *S. scabiei*), which was first identified in 1890 by Roland Thaxter (Thaxter 1891). *S. scabies* has a worldwide distribution and is the predominant scab-causing pathogen in North America (Wanner 2009). The primary virulence factor produced by this organism is a group of related phytotoxic secondary metabolites called the thaxtomins, of which thaxtomin A is the predominant form (King and Calhoun 2009). Thaxtomin A is a nitrated dipeptide that is essential for disease symptom development and functions as a cellulose synthesis inhibitor through an unknown mechanism (Bignell et al. 2014). In addition, *S. scabies* produces the secreted necrogenic protein Nec1, which causes necrosis of potato tuber tissue and is required for the colonization of plant roots by pathogenic *Streptomyces* spp. (Bukhalid and Loria 1997; Joshi et al. 2007), and TomA, which is a secreted tomatinase that is not required for pathogenicity but may function to help suppress plant defense responses (Seipke and Loria 2008). The genome sequence of *S. scabies* strain 87-22 has revealed a number of additional putative virulence loci, including a biosynthetic gene cluster called the coronafacic acid (CFA)-like gene cluster, predicted to produce a molecule resembling the CFA component of the coronatine (COR) phytotoxin produced by the gram-negative plant pathogen *Pseudomonas syringae* (Bignell et al. 2010). COR (Fig. 1) is a key virulence factor that plays several important roles during host infection by *P. syringae*. It facilitates invasion of the pathogen by interfering with stomatal defenses, it promotes bacterial growth and persistence within the plant apoplast, it contributes to disease symptom development, and it induces disease susceptibility in uninfected regions of the plant (Xin and He 2013). Structurally, COR is similar to the L-isoleucine (L-Ile) conjugate of the plant hormone jasmonic acid (JA-L-Ile) and has been demonstrated to function as a molecular mimic of JA-L-Ile (Katsir et al. 2008b; Melotto et al. 2008). This, in turn, allows COR to stimulate JA signal transduction pathways (Katsir et al. 2008a) leading to suppression of salicylic-acid-mediated defense signaling, which is necessary for host resistance to *P. syringae* infection (Xin and He 2013).

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COR is composed of the polyketide CFA linked via an amide bond to coronamic acid (CMA) (Fig. 1), an ethylcyclopropyl amino acid derived from L-Ile via L-*allo*-Ile (Parry et al. 1991; Worley et al. 2013). The biosynthesis of CFA and CMA involve separate gene clusters in *P. syringae*, and linkage of the two moieties is the final step in COR production and is believed to be catalyzed by the coronafacate ligase (Cfl) enzyme encoded within the CFA gene cluster (Liyanage et al. 1995; Rangaswamy et al. 1997, 1998). Although COR is the predominant product and is reportedly the most toxic, *P. syringae* is also known to produce other COR-like molecules where CFA is conjugated to other amino acids such as L-Ile, L-valine (L-Val), L-*allo*-Ile, L-serine, and L-threonine (Fig. 1) (Mitchell 1984, 1985; Mitchell and Ford 1998; Mitchell and Young 1985). The production of such coronafacoyl-amino acid conjugates in addition to COR is believed to be due a relaxed substrate specificity exhibited by Cfl (Bender et al. 1999). Several of the genes from the *P. syringae* CFA biosynthetic gene cluster are conserved in the *S. scabiei* CFA-like gene cluster, including the gene encoding Cfl (Bignell et al. 2010). Interestingly, the Cfa7 modular polyketide synthase (PKS) homolog in *S. scabiei* has been predicted to contain an enoyl reductase (ER) domain that is absent from the corresponding protein in *P. syringae*, and it was hypothesized that the resulting metabolite in *S. scabiei* would be missing the C-C double bond that is normally present in CFA (Bignell et al. 2010). Furthermore, the *S. scabiei* CFA-like gene cluster contains several genes that are absent from the *P. syringae* CFA cluster, and it has been suggested that some of these may contribute to the production of a novel CFA-like molecule (Bignell et al. 2010). Although, the CMA biosynthetic gene cluster appears to be absent from *S. scabiei*, the conservation of the *cfl* gene suggests that *S. scabiei* may make novel COR-like molecules whereby the CFA-like moiety is linked to different amino acids. Deletion of the *cfa6* PKS gene in the *S. scabiei* CFA-like gene cluster reduced the severity of disease symptoms induced on tobacco seedlings, indicating that the resulting metabolite contributes to seedling infection by this pathogen (Bignell et al. 2010).

In this study, we used a combination of genetic and chemical methods to characterize the metabolites produced by the *S. scabiei* CFA-like gene cluster. Our results demonstrate that the CFA-like gene cluster is responsible for producing a single

primary product that was determined to be the known COR-like molecule CFA-L-Ile (Fig. 1), and that other minor COR-like metabolites are also produced. Biosynthesis of the COR-like metabolites was shown to require the *cfl* gene, which is involved in the ligation of CFA to L-Ile and at least one other amino acid. Furthermore, bioassays performed in this study show that CFA-L-Ile produced by *S. scabiei* exhibits effects similar to those of the COR phytotoxin against different plant hosts.

RESULTS

Chemical extraction and bioactivity of the *S. scabiei* COR-like metabolite.

Previously, it was noted that *S. scabiei* oat bran broth (OBB) culture supernatants were able to induce hypertrophy of potato tuber tissue in a similar manner as COR (Bignell et al. 2010). This, together with the conservation of several CFA biosynthetic genes in *S. scabiei* and *P. syringae*, suggested that the *S. scabiei* COR-like metabolite likely shares similar chemical features with COR. With this in mind, we adapted an existing protocol used for COR extraction (Palmer and Bender 1993) in order to extract the COR-like metabolite from *S. scabiei* OBB and soy flour mannitol broth (SFMB) culture supernatants. The protocol (see Materials and Methods) consisted of a two-step extraction with ethyl acetate or chloroform under basic and acidic conditions, the latter of which is selective for movement of COR and related molecules from the aqueous to the organic phase (Palmer and Bender 1993). Basic and acidic organic extracts from the culture supernatants of wild-type *S. scabiei* 87-22, the $\Delta txtA$ mutant (thaxtomin nonproducer), the $\Delta cfa6$ mutant (COR-like metabolite nonproducer), the $\Delta txtA/50-1a$ strain (thaxtomin nonproducer; contains the integrated vector pRLDB50-1a), the $\Delta txtA/51-1$ strain (thaxtomin nonproducer and COR-like metabolite over-producer), and the $\Delta txtA/\Delta cfa6$ mutant (thaxtomin and COR-like metabolite nonproducer) (Table 1) were prepared, and each was assessed for the ability to elicit tissue hypertrophy in a potato tuber slice bioassay. The $\Delta txtA$ mutant background was included in the analysis in order to account for any potential effects of thaxtomin A in the bioassays. The $\Delta txtA/51-1$ strain harbors an integrative plasmid that overexpresses the *scab7959I*-positive activator of the CFA-like biosynthetic gene cluster (Table 1) and, as such, this strain was expected to function as an overproducer of the COR-like metabolite. Significant tissue hypertrophy was observed upon treatment with the $\Delta txtA$ and $\Delta txtA/51-1$ SFMB acidic culture extracts (Fig. 2A), and some hypertrophy was also observed with the OBB acidic culture extracts for each strain (data not shown). A small amount of tissue hypertrophy also occurred upon treatment with the 87-22 and $\Delta txtA/50-1a$ SFMB acidic culture extracts, whereas no hypertrophy was observed with the $\Delta cfa6$ and $\Delta txtA/\Delta cfa6$ SFMB acidic culture extracts (Fig. 2A), which is consistent with the inability of these mutants to produce the COR-like metabolite due to deletion of the *cfa6* PKS gene (Bignell et al. 2010). In all cases, the basic culture extract exhibited no bioactivity (data not shown), which suggests that the COR-like metabolite is only present within the organic acid fraction of the *S. scabiei* culture supernatant.

We also assessed the $\Delta txtA/51-1$ SFMB acidic culture extracts in a radish seedling bioassay in order to determine whether or not the *S. scabiei* COR-like metabolite can cause stunting of plant growth, as previously reported for COR (Feys et al. 1994; Gnanamanickam et al. 1982; Uppalapati et al. 2005). The acidic culture extract (undiluted and 10-fold diluted) from the $\Delta txtA/51-1$ strain caused significant stunting of the radish seedlings as compared with the mock (water)-treated plants (Fig. 2B), with the undiluted extract causing stunting

Fig. 1. Structure of coronatine (COR, 1), coronafacic acid (CFA, 2), *N*-coronafacoyl-L-isoleucine (CFA-L-Ile, 3), and *N*-coronafacoyl-L-valine (CFA-L-Val, 4) produced by pathovars of *Pseudomonas syringae*.

similar to that of the COR control. Significant stunting was also observed with the undiluted $\Delta txtA/\Delta cfa6$ extract, although not as severe as with the undiluted $\Delta txtA/51-1$ extract (Fig. 2B). Organic extracts from uninoculated SFMB had no significant effect on growth of the radish seedlings.

Together, these results indicate that the developed extraction protocol supports the successful isolation of the COR-like metabolite from *S. scabiei* cultures. Moreover, our results further demonstrate that the COR-like metabolite possesses bioactive properties similar to those of COR.

S. scabiei can produce multiple COR-like metabolites.

To further characterize the *S. scabiei* COR-like metabolite, a high-performance liquid chromatography (HPLC) separation and detection method was developed in order to identify the metabolite in the SFMB acidic culture extracts. Authentic standards of COR, CFA-L-Ile, and CFA-L-Val were included to assist in the identification of the COR-like metabolite and to monitor the robustness of the HPLC method that was developed. Due to the presence of impurities in the CFA-L-Ile and CFA-L-Val standards used, the peaks corresponding to these metabolites were firstly determined by liquid chromatography mass spectrometry (LC-MS) (data not shown). Under the running conditions used, the retention time of each standard was determined to be 3.35 min for COR (Fig. 3i), 4.12 min for CFA-L-Ile (Fig. 3ii), and 2.98 min for CFA-L-Val (Fig. 3iii).

Comparative metabolic profiling using the developed HPLC protocol revealed that the wild-type *S. scabiei* 87-22 SFMB acidic organic extract did not contain any unique compounds compared with the $\Delta cfa6$ mutant extract (Fig. 3iv and vi). However, a comparison of the extracts derived from the *S. scabiei* $\Delta txtA$ (Fig. 3v) and $\Delta txtA/\Delta cfa6$ (Fig. 3vii) strains revealed a unique peak in the $\Delta txtA$ extract with a retention time

of 4.12 min, the same as that of the CFA-L-Ile standard (Fig. 3ii). A peak with the same retention time was also identified in the acidic extracts from the *S. scabiei* $\Delta txtA/50-1a$ (Fig. 3viii) and $\Delta txtA/51-1$ (Fig. 3ix) strains, with the latter strain producing much higher levels of the corresponding metabolite. LC-MS analysis of the extracts provided a low-resolution mass of 321.2 for the metabolite, consistent with the low-resolution mass obtained for the CFA-L-Ile standard. The $\Delta txtA/51-1$ acidic culture extract also contained four minor peaks that were absent from the other extracts and which had retention times of 2.3, 2.9, 3.6, and 3.9 min (Fig. 3ix). Consistent with the results of the potato tuber slice bioassay, no detectable peaks corresponding to potential COR-like metabolites were present in any of the basic culture extracts (data not shown).

Production of the *S. scabiei* COR-like metabolites is highest in media containing soy flour.

We next analyzed the production of the *S. scabiei* COR-like metabolites under different growth conditions in order to determine the optimum conditions for large-scale metabolite production and purification. Studies using OBB and SFMB cultures grown at different temperatures (25 and 28°C) and incubation times (2 to 21 days) revealed that incubation at 25°C for 7 days was best for production of the metabolites in these media (data not shown). In addition, 11 different growth media (including SFMB and OBB) were tested, and quantitative analysis of the primary COR-like metabolite using HPLC revealed that production was best in the three media—SFMB, SFMB medium prepared using “full fat” soy flour rather than defatted soy flour (ffSFMB), and soy medium—that contain soy flour as a component (Fig. 4). Production was determined to be highest in ffSFMB. However, use of this medium was found to complicate the extraction procedure due to the formation of

Table 1. Bacterial strains, plasmids, and cosmids used in this study

Strain, plasmid, or cosmid	Description	Resistance ^a	Reference or source
<i>Streptomyces scabiei</i> strains			
87-22	<i>Streptomyces scabiei</i> wild-type strain	n/a	Loria et al. 1995
$\Delta cfa6$	87-22 derivative harboring a deletion of the <i>cfa6</i> gene	Hyg ^R	Bignell et al. 2010
$\Delta txtA$	87-22 derivative harboring a deletion of the <i>txtA</i> gene	Apra ^R	Johnson et al. 2009
$\Delta txtA/\Delta cfa6$	$\Delta txtA$ derivative harboring a deletion of the <i>cfa6</i> gene	Apra ^R , Hyg ^R	Bignell et al. 2010
$\Delta txtA/50-1a$	$\Delta txtA$ derivative harboring the pRLDB50-1a integrative plasmid	Apra ^R , Thio ^R	Bignell et al. 2010
$\Delta txtA/51-1$	$\Delta txtA$ derivative harboring the pRLDB51-1 integrative plasmid	Apra ^R , Thio ^R	Bignell et al. 2010
Δcfl	$\Delta txtA/51-1$ derivative harboring a deletion of the <i>cfl</i> (<i>scab79671</i>) gene	Apra ^R , Thio ^R , Hyg ^R	This study
<i>Escherichia coli</i> strains			
DH5 α	General cloning host	n/a	Gibco-BRL
NEB 5- α	DH5 α derivative, high efficiency competent cells	n/a	New England Biolabs
BW25113	Host for redirect polymerase chain reaction (PCR) targeting system	n/a	Gust et al. 2003a, b
ET12567	<i>dam</i> [−] , <i>dcm</i> [−] , <i>hsdS</i> [−] ; nonmethylating conjugation host	Cml ^R , Tet ^R	MacNeil et al. 1992
Plasmid or cosmid			
pRLDB50-1a	Integrates into ϕ C31 <i>attB</i> site in <i>Streptomyces</i> chromosomes; for strong constitutive expression of genes cloned downstream of <i>ermEp</i> *	Apra ^R , Thio ^R	Bignell et al. 2010
pRLDB51-1	Derivative of pRLDB50-1a; overexpresses the <i>scab79591</i> pathway-specific regulator for COR-like metabolite biosynthesis	Apra ^R , Thio ^R	Bignell et al. 2010
pIJ790	λ -RED expression plasmid	Cml ^R	Gust et al. 2003a
pIJ10700	Template for PCR amplification of the [<i>hyg+oriT</i>] cassette used for Redirect PCR targeting	Hyg ^R	Gust et al. 2003b
Cosmid 1770	SuperCos1 derivative containing the <i>S. scabiei</i> CFA-like biosynthetic gene cluster	Amp ^R , Kan ^R	Bignell et al. 2010
Cosmid 1770/ Δcfl	Cosmid 1770 derivative in which the <i>cfl</i> gene was replaced with the [<i>hyg+oriT</i>] cassette	Amp ^R , Kan ^R , Hyg ^R	This study
pUZ8002	Nontransmissible <i>oriT</i> -mobilizing plasmid	Kan ^R	Kieser et al. 2000
pGEM-T Easy	Cloning vector for PCR products	Amp ^R	Promega
pIJ10257	Integrates into ϕ BT1 <i>attB</i> site in <i>Streptomyces</i> chromosomes; for strong constitutive expression of genes cloned downstream of <i>ermEp</i> *	Hyg ^R	Hong et al. 2005
pMSAK13	pIJ10257 derivative containing the <i>neo</i> gene and promoter inserted into the <i>EcoRV</i> site	Hyg ^R , Kan ^R	This study
pMSAK13/ <i>cfl</i>	pMSAK13 derivative containing <i>cfl</i> cloned into the <i>NdeI</i> and <i>XhoI</i> sites	Hyg ^R , Kan ^R	This study

^a Hyg^R, Apra^R, Thio^R, Cml^R, Tet^R, Amp^R, and Kan^R = hygromycin B, apramycin, thiostrepton, chloramphenicol, tetracycline, ampicillin, and kanamycin resistance, respectively.

a large emulsion layer between the aqueous and organic phases that was difficult to separate. Because this would lead to greater losses during large-scale extraction, it was decided that SFMB was the best culture medium to use for this purpose.

S. scabies produces CFA-L-Ile as the primary COR-like metabolite.

The primary COR-like metabolite was found to have an HPLC retention time and low-resolution mass similar to that of the CFA-L-Ile standard, suggesting that the two metabolites are the same. To confirm this, large-scale extraction and purification of the metabolite was conducted using the $\Delta txtA/51-1$ strain grown in SFMB at 25°C for 7 days. High-resolution electrospray ionization mass spectrometry (HR-ESI-MS) verified that the

mass of the pure metabolite is consistent with the expected mass of CFA-Ile (Table 2). Furthermore, the pure metabolite and the authentic CFA-L-Ile standard were found to coelute in HPLC co-injection experiments (Fig. 5i–iii). Together, these results are consistent with the production of CFA-L-Ile as the major COR-like metabolite in *S. scabies*.

S. scabies produces CFA-D-Valine as a minor COR-like metabolite.

One of the minor predicted COR-like metabolites (retention time = 2.91 min) detected in the $\Delta txtA/51-1$ acidic culture extract (Fig. 3ix) was found to have a retention time similar to that of the authentic CFA-L-Val standard (Fig. 3iii). LC-MS analysis provided a low-resolution mass of 307.2 for this metabolite, consistent with the low-resolution mass obtained for the CFA-L-Val standard. The metabolite was subsequently purified from large-scale cultures of the $\Delta txtA/51-1$ strain, and analysis of the pure metabolite by HR-ESI-MS afforded a mass consistent with that expected for CFA-Val (Table 2). Surprisingly, HPLC co-injection experiments revealed that the retention time of the metabolite and the CFA-L-Val standard were slightly different and, as such, the two compounds did not coelute as expected (Fig. 5iv–vi), indicating that the metabolite is not CFA-L-Val. We surmised from this that the metabolite consists of CFA linked to an isomer of L-Val rather than to L-Val itself.

Further structural characterization of the putative CFA-Val minor COR-like metabolite was restricted by the fact that it is produced in much lower quantities than CFA-L-Ile (Fig. 3ix) and is difficult to purify in large amounts. Therefore, we decided to conduct an amino acid feeding experiment where we supplied the amino acids L-Val, L-norvaline, and D-Val exogenously to SFMB cultures of *S. scabies* $\Delta txtA/51-1$ to see whether this would lead to enhanced production of the cognate CFA-amino acid conjugate. Feeding with L-Val led to reduced production of several of the COR-like metabolites (Fig. 6), including CFA-L-Ile (Fig. 6ii), whereas production of the putative CFA-Val metabolite was unchanged. The addition of L-norvaline also led to reduced production of CFA-L-Ile and other COR-like metabolites, including the predicted CFA-Val metabolite, and a new compound was also observed with a retention time of 3.25 min (Fig. 6iii). Because LC-MS analysis showed this new compound to have a molecular mass of 307.2, it is proposed to be CFA-L-norvaline. In contrast, the addition of D-Val led to reduced production of CFA-L-Ile as well as enhanced production of the putative CFA-Val COR-like metabolite (Fig. 6iv), the mass of which was confirmed by LC-MS analysis. Therefore, based on the feeding results as well as the co-injection and HR-ESI-MS results for the pure metabolite, we propose that the COR-like metabolite with a retention time of 2.91 min is CFA-D-Val.

The remaining three minor COR-like metabolites were purified and analyzed by HR-ESI-MS, and all were found to have a similar mass of approximately 323.203 (Table 2). The identity of these COR-like metabolites will require further investigation; however, the HR-ESI-MS results suggest that they may represent different isomers of the same molecule.

A. S. scabies Δcfl mutant accumulates CFA.

As a further confirmation that *S. scabies* produces coronafacoyl compounds, the *cfl* gene within the CFA-like biosynthetic gene cluster was replaced with a hygromycin B resistance cassette on the *S. scabies* $\Delta txtA/51-1$ chromosome (see Materials and Methods). Acidic culture extracts from the Δcfl mutant were unable to induce tissue hypertrophy in the potato tuber slice assay (Fig. 7A), and the ability of the extracts to cause radish seedling stunting was also significantly reduced (Fig. 2B). HPLC analysis of the extracts confirmed that

Fig. 2. Detection of COR-like metabolite bioactivity using plant bioassays. **A**, Potato tuber slice bioassay showing the induction of tissue hypertrophy by the *Streptomyces scabies* COR-like metabolites. Acidic organic extracts derived from cultures of *S. scabies* strain 87-22 (wild-type), $\Delta txtA$, $\Delta cfa6$, $\Delta txtA/\Delta cfa6$, $\Delta txtA/50-1a$, and $\Delta txtA/51-1$ were dissolved in methanol and then applied to potato tuber slices. Methanol was included as a negative control and COR (0.8 nmol dissolved in methanol) was used as a positive control. A representative tuber slice out of six tuber slices per treatment is shown. **B**, Radish seedling bioassay showing the stunting of radish seedlings by the *S. scabies* COR-like metabolites. Seedlings were mock treated with water or were treated with extract (undiluted and 10-fold diluted) from soy flour mannitol broth (SFMB) cultures of *S. scabies* $\Delta txtA/51-1$, $\Delta txtA/\Delta cfa6$, and Δcfl . Treatment with extract from an uninoculated SFMB culture served as an additional negative control, while treatment with pure COR (100 nmol) served as a positive control. The mean root and shoot length for 10 radish seedlings per treatment is shown, and error bars represent the standard deviation from the mean. Treatments that produced a statistically significant result compared with the mock treatment are indicated by * or ** ($P \leq 0.001$ or 0.01, respectively).

production of the COR-like metabolites was abolished in the Δcfl mutant (Fig. 7Bii). A peak with a retention time similar to that of an authentic CFA standard was observed in the Δcfl mutant chromatogram (Fig. 7Biii). LC-MS analysis of the Δcfl mutant extract revealed that the accumulated metabolite has a mass of 208, which is consistent with the expected mass for CFA. Furthermore, HPLC co-injection experiments showed that the accumulated metabolite comigrates with the CFA standard (Fig. 7Biv). Production of the COR-like metabolites could be partially restored by reintroduction of the *S. scabiei cfl* gene into the Δcfl mutant on an integrative plasmid (data not shown), indicating that the observed phenotypic effects of the mutant are associated with the *cfl* gene deletion.

Taken together, these results demonstrate that CFA is an intermediate in the biosynthesis of the *S. scabiei* COR-like metabolites and that the *cfl* gene is essential for COR-like metabolite biosynthesis in this organism. Furthermore, our results are consistent with previous findings showing that conjugation of CFA to an amino acid is necessary for bioactivity in plants (Uppalapati et al. 2005).

The pure *S. scabiei* CFA-L-Ile possesses bioactive properties similar to COR.

To further characterize the primary COR-like metabolite produced by *S. scabiei*, we assessed the bioactivity of the pure CFA-L-Ile in the potato tuber slice assay alongside our pure

COR standard. Equimolar amounts (16 nmol) of COR and CFA-L-Ile led to the formation of hypertrophic outgrowths on the potato tissue; however, the level of hypertrophy observed following the application of CFA-L-Ile was less than that observed following the application of COR (Fig. 8A). Similar results were observed using higher and lower (8 and 32 nmol) amounts of each metabolite (data not shown).

The bioactivity of the pure CFA-L-Ile was also tested in our radish seedling bioassay. Three different concentrations of COR and CFA-L-Ile (0.9, 9, and 90 nmol) were used, and the growth of seedlings treated with all three concentrations of COR showed a statistically significant reduction when compared with the solvent (methanol) control plants (Fig. 8B), whereas only seedlings treated with 90 nmol of CFA-L-Ile showed a statistically significant reduction in growth when compared with the solvent (dimethyl sulfoxide [DMSO]) control. Therefore, our results confirm that CFA-L-Ile possesses biological activities against different plant hosts similar to those of COR, though CFA-L-Ile appears to be less toxic than COR in our bioassays.

DISCUSSION

In this study, we have shown that the common scab pathogen *S. scabiei* is capable of producing the coronafacoyl compound CFA-L-Ile as well as other minor COR-like metabolites. This is

Fig. 3. Detection of the COR-like metabolites by analytical high-performance liquid chromatography. Shown are the chromatograms obtained for the standards COR (i), CFA-L-Ile (ii), and CFA-L-Val (iii) and for the soy flour mannitol broth acidic culture extracts of *Streptomyces scabiei* 87-22 (iv), $\Delta txtA$ (v), $\Delta cfa6$ (vi), $\Delta txtA/\Delta cfa6$ (vii), $\Delta txtA/50-1a$ (viii), and $\Delta txtA/51-1$ (ix). Peaks of interest are indicated by * as follows: COR (i), CFA-L-Ile (ii), CFA-L-Val (ii), and the COR-like metabolites produced by *S. scabiei* (v, viii, and ix).

the first report of coronafacoyl phytotoxins being produced by *S. scabies*, which is the oldest and most widely distributed scab-causing pathogen. The best known coronafacoyl phytotoxin is COR, which is an important virulence determinant produced by several pathovars of *P. syringae*. COR elicits a number of different biological activities, including inhibition of plant growth and induction of potato tuber tissue hypertrophy (Feys et al. 1994; Gnanamanickam et al. 1982; Sakai et al. 1979; Uppalapati et al. 2005). Though COR is the primary coronafacoyl compound produced by *P. syringae*, other COR-like metabolites can also be produced in minor amounts (Mitchell 1984, 1985; Mitchell and Ford 1998; Mitchell and Young 1985). Our results demonstrate that the pure CFA-L-Ile produced by *S. scabies* can elicit radish seedling stunting and potato tuber hypertrophy in a manner similar to COR, though it is not as toxic as COR in its bioactivity. This is in agreement with previous findings showing that COR is the most toxic coronafacoyl compound produced by *P. syringae* (Shiraishi et al. 1979; Uppalapati et al. 2005).

Although the production of COR and COR-like molecules has been most extensively studied in *P. syringae*, it is known or suspected that other plant-pathogenic bacteria also produce these phytotoxins. For example, *Xanthomonas campestris* pv. *phormiicola*, which is a pathogen of the New Zealand flax plant (*Phormium tenax* J. R. & G. Forst), has been shown to produce COR, CFA-L-Ile, and CFA-L-Val (Mitchell 1991; Palmer and Bender 1993; Tamura et al. 1992), and coronafacoyl-amino acid conjugates are likely produced by the potato blackleg and soft rot pathogen *Pectobacterium atrosepticum* (syn. *Erwinia carotovora* subsp. *atroseptica*), because this organism contains all of the necessary genes for their production (Bell et al. 2004). As with *S. scabies*, *P. atrosepticum* does not harbor the genes necessary for CMA production (Bell et al. 2004) and, therefore, it is unable to produce COR. However, mutants of the *cfa6* and *cfa7* genes in *P. atrosepticum* as well as a *cfa6* mutant of *S. scabies* were all shown to be reduced in virulence, suggesting that coronafacoyl-amino acid conjugates play an important role in the plant-pathogenic phenotype of these organisms (Bell et al. 2004; Bignell et al. 2010).

Fig. 4. Production of the primary COR-like metabolite by *Streptomyces scabies* $\Delta txtA/51-1$ cultured in different growth media. Cultures were incubated at 25°C for 7 days prior to extraction of the COR-like metabolites from the culture supernatant. Quantification of the primary metabolite was by analytical high-performance liquid chromatography using the ChemStation software. Each bar shows the mean percentage production of the primary metabolite in three cultures compared with production in soy flour mannitol broth (SFMB), with error bars representing standard deviation. ffSFMB = full-fat soy flour mannitol broth, OBB = oat bran broth, PMB = potato mash broth, SA = starch asparagine medium. Production levels that were significantly different from that in SFMB are indicated by * or ** ($P \leq 0.005$ or 0.05, respectively).

Mutational analysis of the *S. scabies* *cfl* gene showed that it is required for ligation of CFA to its amino acid partner, a role that was previously proposed for the Cfl homolog in *Pseudomonas syringae* (Liyanage et al. 1995; Rangaswamy et al. 1997, 1998). The *S. scabies* Δcfl mutant accumulated CFA in the culture supernatant, consistent with the enzyme being involved exclusively in the final step of COR-like metabolite biosynthesis. Interestingly, a *P. syringae* Δcfl mutant was previously shown to be abolished in both COR and CFA production, suggesting that *cfl* in that organism may also play a role in CFA biosynthesis (Rangaswamy et al. 1997). Given that the regulatory systems for controlling metabolite biosynthesis appears to differ between the two organisms (Bender et al. 1999; Bignell et al. 2010), it is also plausible that the regulation of CFA and COR biosynthesis in *P. syringae* may involve a “feed-forward” type mechanism in which small amounts of COR are needed to activate high levels of *cfa* gene expression and metabolite production. The fact that the *S. scabies* Δcfl mutant culture extracts were abolished in bioactivity in both the potato tuber slice assay and the radish seedling bioassay is consistent with previous reports that conjugation of CFA to an amino acid is necessary for bioactivity in plants (Uppalapati et al. 2005). Although the Cfl enzyme from *S. scabies* appears to exhibit relaxed amino acid substrate specificity, our results suggest that it may favor L-Ile as the primary substrate, though we cannot rule out the possibility that the L-Ile levels were higher than that of other amino acids in the microbial growth media used in this study. However, the preference for L-Ile as a substrate makes sense given that L-Ile is structurally related to CMA and is a precursor for CMA biosynthesis in *P. syringae* (Parry et al. 1991; Worley et al. 2013). Furthermore, COR is known to function as a molecular mimic of JA-L-Ile (Katsir et al. 2008b; Melotto et al. 2008), and it logically follows that CFA-L-Ile would also bear a high structural resemblance to JA-L-Ile. Our results also suggest that the *S. scabies* Cfl favors the non-proteinogenic amino acid D-Val over its L-isomer for ligation to CFA, though this needs to be validated. The ligation of D-amino acids to CFA has not been previously reported in *P. syringae* and, although D-amino acids have been reported to be linked to CFA in *X. campestris* pv. *phormiicola*, such coronafacoyl-amino acid conjugates were found to be produced in much lower amounts compared with the corresponding L-amino acid conjugates (Mitchell 1991). It is interesting to note that a putative racemase, SCAB79531, is encoded only five genes upstream of the CFA-like gene cluster, and it is reasonable that such an enzyme could be involved in the conversion of L-Val to D-Val for CFA-D-Val biosynthesis, an idea that we are currently exploring.

The production of CFA by *S. scabies* was unexpected, given that the CFA-like gene cluster contains a number of genes that are absent from the CFA biosynthetic gene cluster in *P. syringae* (Bignell et al. 2010). Three of these genes (*scab79681*, *scab79691*, and *scab79721*) encode enzymes that were

Table 2. High-resolution electrospray ionization mass spectrometry results for the major and minor COR-like metabolites produced by the *Streptomyces scabies* $\Delta txtA/51-1$ strain^a

Retention time (min)	Observed mass (Da)	Expected mass (Da)	Difference (mDa)
2.3	323.2027	n/a	n/a
2.9	307.1693	307.1705 ^b	−1.2
3.6	323.2034	n/a	n/a
3.9	323.2030	n/a	n/a
4.2	321.1870	321.1862 ^c	+0.8

^a Abbreviation: n/a = not applicable.

^b Expected mass for $C_{17}H_{24}NO_4$.

^c Expected mass for $C_{18}H_{26}NO_4$.

previously predicted to modify the CFA backbone in order to produce novel COR-like metabolites, and all three genes were shown to be expressed along with the genes that are conserved in *P. syringae* (Bignell et al. 2010). Furthermore, the *S. scabies* Cfa7 PKS contains a predicted ER domain that is absent from the *P. syringae* Cfa7, and it was predicted that this would result in reduction of the C-C double bond that is normally present in CFA. It is conceivable that the ER domain in the *S. scabies* Cfa7 PKS is nonfunctional or is skipped over during polyketide biosynthesis, a phenomenon that has been reported for other microbial PKSs (Moss et al. 2004). Alternatively, it is possible that the double bond is reduced by the Cfa7 ER domain during polyketide synthesis and is later

reintroduced by a separate enzyme. A candidate enzyme for this is the putative oxidoreductase encoded by the *scab79681* gene because mutational studies have confirmed that the gene is involved in COR-like metabolite biosynthesis (Altowairish 2014).

It is noteworthy that, even though we could detect COR-like metabolite production in the *S. scabies* $\Delta txtA$ and $\Delta txtA/51-1$ strains, production was undetectable in the wild-type *S. scabies* 87-22 strain under the culturing condition used. The $\Delta txtA$ strain is abolished in thaxtomin A production (Johnson et al. 2009), and it is plausible that this leads to an increase in the availability of precursors for COR-like metabolite biosynthesis. The fact that production of the COR-like metabolites was highest

Fig. 5. High-performance liquid chromatography (HPLC) co-injection analysis of the purified COR-like metabolites produced by *Streptomyces scabies*. (i) HPLC chromatogram of the pure major COR-like metabolite predicted to be CFA-Ile, (ii) chromatogram of the CFA-L-Ile standard (ii), and (iii) co-injection experiment results where the CFA-L-Ile standard and the purified major COR-like metabolite were mixed prior to HPLC analysis. (iv) HPLC chromatogram of the pure minor COR-like metabolite predicted to be CFA-Val, (v) chromatogram of the CFA-L-Val standard, and (vi) co-injection experiment results where the CFA-L-Val standard and the purified minor COR-like metabolite were mixed prior to HPLC analysis. Peaks of interest are indicated by *.

in the $\Delta txtA/51-1$ strain is consistent with the fact that this strain overexpresses the *scab79591*-positive activator of the CFA-like biosynthetic gene cluster (Bignell et al. 2010). It was previously shown that expression of the *cfaI* promoter, which drives expression of the CFA biosynthetic genes, is active during

colonization of plant roots by *S. scabiei* 87-22 (Bignell et al. 2010), and it is possible that production of the metabolites by *S. scabiei* 87-22 requires host-derived signals to activate the expression of the biosynthetic genes. Given that production of COR has been found to be highly variable among different strains of *P. syringae* (Hwang et al. 2005; Palmer and Bender 1993; Völksch et al. 1989), it is also conceivable that other strains of *S. scabiei* are able to produce higher levels of the COR-like metabolites, and we are currently investigating this further.

This study provides important insight into the *S. scabiei* COR-like metabolites and establishes a firm ground on which future studies can be built. Methods developed herein for growth, extraction, and activity assessment will undoubtedly prove to be invaluable for addressing a number of key questions regarding the biosynthesis and function of the metabolites. Work is currently in progress to purify the minor COR-like metabolites in sufficient quantities for structural elucidation and bioactivity assessment. Investigations into the function of the unique genes within the CFA-like gene cluster are also currently underway, as well as in vitro studies on the substrate specificity of the Cfl enzyme. Findings stemming from such work not only may prove useful in understanding the molecular mechanisms of plant pathogenicity in *S. scabiei* but also may provide important insight into other microbial pathogens that use similar strategies during plant-microbe interactions and, furthermore, are of general interest to those working in microbial natural product research.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and maintenance.

Bacterial strains used in this study are listed in Table 1. *S. scabiei* strains were routinely cultured on International *Streptomyces* Project medium 4 (BD Biosciences, Mississauga, ON, Canada), oat bran agar (Johnson et al. 2007), soy flour mannitol agar (Kieser et al. 2000), Difco nutrient agar (BD Biosciences), or potato mash agar (5% [wt/vol] instant mashed potato flakes and 2% [wt/vol] agar) solid media. Liquid cultures were grown in trypticase soy broth (TSB; BD Biosciences), SFMB, ffSFMB, OBB, starch asparagine medium (Paradkar and Jensen 1995), soy medium (Paradkar and Jensen 1995), SCM medium (Lambalot and Cane 1992), SGGP medium (Yamamoto et al. 1986), GSPG medium (Romero et al. 1986), prefermentation medium (Butler et al. 1999), MM1 medium (Butler et al. 1999), or potato mash broth. Unless otherwise indicated, growth of *S. scabiei* strains was carried out at 28°C and, when necessary, the growth medium was supplemented with the following antibiotics at the indicated final concentrations: apramycin (100 µg/ml), hygromycin B (50 µg/ml), kanamycin (50 µg/ml), nalidixic acid (50 µg/ml), or thiostrepton (25 µg/ml). *S. scabiei* strains were maintained at -80°C as spore suspensions in 20% (vol/vol) glycerol (Kieser et al. 2000) or as mycelial suspensions in TSB containing 5% (vol/vol) DMSO (Butler et al. 2001). Seed cultures for COR-like metabolite production studies

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Fig. 6. High-performance liquid chromatography analysis of acidic culture extracts following addition of amino acids to cultures of *Streptomyces scabiei* $\Delta txtA/51-1$. (i) Chromatogram of acidic organic extract derived from an unsupplemented *S. scabiei* $\Delta txtA/51-1$ culture. Peak 1 represents the CFA-L-Ile metabolite and peak 2 represents the putative CFA-Val metabolite. Other putative COR-like metabolites are indicated with *. Chromatograms of *S. scabiei* $\Delta txtA/51-1$ acidic organic extracts derived from cultures supplemented with (ii) L-Val, (iii) L-novoline, and (iv) D-Val. The dotted line in chromatograms (ii) to (iv) is identical to (i) and has been included for comparison purposes. The new peak identified in chromatogram (iii) is indicated with ▽. Each chromatogram is a representative of three samples analyzed.

were prepared by inoculating TSB with *S. scabies* spore stocks (0.1% [vol/vol] final concentration) or mycelial stocks (10% [vol/vol] final concentration) and then incubating for 24 to 48 h until dense mycelial growth was obtained. Production cultures were subsequently prepared by inoculating the production medium with seed culture to a final concentration of 1% (vol/vol), then incubating at 25°C for 7 days. Small-scale production cultures (5 ml) were grown in triplicate in Corning six-well tissue culture plates (VWR International, Mississauga, ON, Canada), with shaking at 125 rpm, whereas medium- (25 ml) and large- (1 liter) scale production cultures were grown in 125-ml or 4-liter Erlenmeyer flasks, respectively. *E. coli* strains used in this study are listed in Table 1. Strains were cultured at 28 or 37°C in Luria Bertani (LB) Lennox broth (BD Biosciences) with agitation at 200 rpm or on LB agar. When necessary, the medium was supplemented with the following antibiotics at the indicated final concentrations: ampicillin (100 µg/ml), chloramphenicol (25 µg/ml), kanamycin (50 µg/ml), or hygromycin B (100 µg/ml). For hygromycin B selection, LB medium containing reduced NaCl (2.5 g/liter) was utilized. *Escherichia coli* strains were maintained at -80°C in 20% (vol/vol) glycerol (Sambrook and Russell 2001).

Plasmids, cosmids, and genetic manipulation.

Plasmids and cosmids used or constructed in this study are listed in Table 1. Standard molecular biology procedures were

used for all genetic manipulations (Sambrook and Russell 2001). Genomic DNA was extracted from TSB cultures of *S. scabies* using the One-Tube Bacterial Genomic DNA Kit (Bio Basic Inc., Markham, ON, Canada), as per the manufacturer's directions. Polymerase chain reactions (PCRs) were routinely performed using the Fermentas Taq DNA polymerase (Fisher Scientific, Ottawa, ON, Canada) or the Finnzymes Phusion DNA polymerase (New England Biolabs Canada, Whitby, ON, Canada) according to the manufacturer's instructions, except that DMSO (5% [vol/vol] final concentration) was normally included in the reactions. When necessary, PCR products were cloned into the pGEM-T Easy vector (Table 1) according to the manufacturer's instructions. Sequencing of DNA was performed by The Centre for Applied Genomics (Toronto). All oligonucleotides used for PCR or sequencing are listed in Supplementary Table S1.

Extraction of the COR-like molecules from culture supernatants.

The following organic extraction procedure was adjusted accordingly for "small-scale" extraction using 0.5 to 1 ml of culture supernatant, "medium-scale" extraction using 10 to 30 ml of culture supernatant, or "large-scale" extraction using 1 to 3 liters of culture supernatant. Supernatants were obtained by centrifugation of liquid cultures followed by filtration in the case of large-scale extractions. The pH of the supernatant was

Fig. 7. A *Streptomyces scabies* Δcfl mutant is defective in COR-like metabolite biosynthesis and accumulates CFA. **A**, Bioactivity of the Δcfl mutant acidic culture extracts (isolates 1 and 2) on potato tuber tissue. Pure COR (8 nmol) and culture extract from the $\Delta txtA/51-1$ strain served as positive controls, while culture extract from the $\Delta txtA/\Delta cfa6$ strain served as a negative control. **B**, High-performance liquid chromatography analysis of soy flour mannitol broth acidic culture extracts from the (i) *S. scabies* $\Delta txtA/51-1$ and (ii) Δcfl strains. The CFA-L-Ile peak is indicated by *, while the CFA peak is indicated by **. (iii) Chromatogram of the pure CFA standard and (iv) results of a co-injection experiment using the pure CFA standard and the Δcfl culture extract.

adjusted to approximately 10 to 11 using NaOH, after which the supernatant was extracted twice with 0.5 volume of chloroform or ethyl acetate. The resulting basic organic extract was separated from the aqueous phase by gravity or centrifugation and was either transferred to a fresh tube or discarded. Next, the pH of the aqueous phase was adjusted to approximately 1 to 2 using HCl and extracted three times with 0.5 volume of chloroform or ethyl acetate. The resulting acidic organic extracts were pooled and dried down, and the residual material was dissolved in either 100% (vol/vol) HPLC-grade methanol or 30% (vol/vol) HPLC-grade acetonitrile and filtered using a 0.2- μ m syringe filter (VWR International).

Plant bioassays.

An in vitro potato tuber slice bioassay was performed as described previously (Bignell et al. 2010; Loria et al. 1995) using *S. scabiei* organic culture extracts dissolved in 100% (vol/vol) methanol, or CFA-L-Ile (8, 16, or 32 nmol) purified from *S. scabiei* cultures and dissolved in DMSO. Samples (25 μ l) were applied to sterile Whatman AA paper disks (6 mm in diameter; GE Healthcare Life Sciences, Baie d'Urfe, PQ, Canada) on aseptically excised potato tuber slices. Six tuber slices were used per treatment, with solvent (methanol or DMSO) serving as the negative control and pure COR (Sigma Aldrich Canada, Oakville, ON, Canada) dissolved in 100% (vol/vol) methanol serving as the positive control. Following

5 to 7 days of incubation under high humidity in the dark at room temperature, the tuber slices were dissected and imaged.

An in vitro radish seedling bioassay was performed using radish seed ('Cherry Belle'; Heritage Harvest Seed, Carmen, MB, Canada) that were surface sterilized by treating with 70% (vol/vol) ethanol for 5 min followed by 10% (vol/vol) bleach (Chlorox) and 0.1% (vol/vol) polysorbate 20 for 10 min with gentle agitation. The seed were washed several times with sterile HPLC-grade water, after which they were placed into a sterile deep petri dish containing Whatman number 4 filter paper (90 mm in diameter) moistened with sterile water and nystatin (50 μ g/ml final concentration). The seed were allowed to germinate in the dark at ambient temperature (23 to 24°C) for approximately 24 h, and then they were selected for uniformity. *S. scabiei* organic culture extracts (0.5 ml) obtained from medium-scale extractions were transferred into triplicate wells in six-well tissue culture plates, and the solvent was evaporated off. Sterile water (5 ml) was added to each well, followed by four germinated radish seeds. When the pure COR and CFA-L-Ile metabolites were used in assay, the metabolites were directly added to 5 ml of sterile water in triplicate wells, while solvent (methanol or DMSO) served as the negative control. The plates were incubated with gentle shaking (125 rpm) at ambient temperature for 4 days under a 16-h photoperiod, after which the seedling root and shoot lengths were measured.

HPLC and LC-MS analysis of the COR-like metabolites.

Analytical HPLC detection of the COR-like metabolites in culture extracts was performed using an Agilent 1260 Infinity LC system equipped with a quaternary pump (Agilent Technologies Canada Inc., Mississauga, ON, Canada). Samples were loaded onto a Poroshell 120 EC-C18 column (4.6 by 50 mm, 2.7-micron particle size; Agilent Technologies Canada Inc.) and were eluted using a linear gradient consisting of acetonitrile (A) and H₂O (B) with 0.1% (vol/vol) formic acid at a constant flow rate of 1 ml/min. The initial mobile phase consisted of A/B at 30:70%, and this was maintained for 1.5 min, after which the ratio was increased linearly to A/B at 50:50% over a period of 2.5 min. This ratio was held for 1 min and was then returned to A/B at 30:70% using a linear gradient over 1.5 min. The column temperature was held constant at 40°C, and the metabolites were monitored using a detection wavelength of 230 nm. Authentic standards of COR (Sigma Aldrich) and CFA (purified from *P. syringae* cultures), as well as the synthetic standards CFA-L-Ile and CFA-L-Val, were used for qualitative identification of the COR-like metabolites. The ChemStation software (version B.04.03; Agilent Technologies Canada Inc.) was used for acquiring and analyzing all of the data.

LC-MS analysis of the *S. scabiei* culture extracts was performed at the Centre for Chemical Analysis, Research and Training (Department of Chemistry, Memorial University) 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 conditions identical to those for analytical HPLC, and detection was by UV radiation (230 nm) and ESI-MS in negative ion mode.

Purification of the COR-like metabolites.

Culture extracts were first analyzed using high-performance thin-layer chromatography (TLC) kieselgel 60 F₂₅₄ plates (Merck Canada Inc., Kirkland, PQ, Canada) in order to identify the COR-like metabolites. Extracts were spotted alongside the COR and CFA-L-Val standards and were separated using a mobile phase composed of ethyl acetate, isopropanol, acetic acid, and H₂O in a ratio of 195:4:0.5:0.5. Sample components were then detected by quenching of fluorescence under short-wave UV light.

Fig. 8. Bioactivity of CFA-L-Ile purified from *Streptomyces scabiei* Δ txtA/51-1 soy flour mannitol broth culture supernatants. **A**, Potato tuber slice bioassay showing the induction of tissue hypertrophy by equimolar amounts (16 nmol) of COR (dissolved in MeOH) and CFA-L-Ile (dissolved in dimethyl sulfoxide [DMSO]). Treatment with the metabolite solvents served as negative controls. **B**, Radish seedling bioassay showing the effect of different amounts (0.9, 9, and 90 nmol) of pure COR (dissolved in MeOH) and CFA-L-Ile (dissolved in DMSO) on seedling root and shoot length. The mean root and shoot length for 10 radish seedlings per treatment is shown, and the error bars represent the standard deviation from the mean. Treatments that produced a statistically significant result compared with the relevant solvent control treatments (MeOH and DMSO) are indicated by * ($P \leq 0.001$).

Large-scale concentrated extracts were separated by preparative TLC using silica gel GF plates (1,000 μm) with a preabsorbant zone (Analtech, Newark, DE, U.S.A.). Sample separation and visualization were as described above, and the COR-like metabolites were recovered from the gel by elution with 100% methanol. The methanol extract was concentrated approximately 20 $\times$, filtered through a 0.2- μm syringe filter, then preparatively chromatographed using the Agilent 1260 Infinity LC system described above. Samples were repetitively loaded onto a Zorbax SB-C18, semipreparative column (9.4 by 250 mm, 5-micron; Agilent Technologies Inc.) set at 50°C and using a constant flow rate of 4 ml/min. The initial mobile phase (A/B, 30:70%) was maintained for 7.5 min, after which the ratio was increased linearly to A/B 50:50% over a period of 12.5 min. This ratio was held for 5 min and was then returned to the initial solvent concentration using a linear gradient over 7.5 min. Fractions corresponding to single peaks were collected, pooled in preweighed vials, dried, and reweighed in order to obtain the amount of each compound collected. The residual material was dissolved in 100% (vol/vol) methanol or DMSO and was stored at -20°C.

HR-ESI-MS.

Samples of the purified metabolites (100 μg) were transferred to clean vials and were dried prior to shipment to the McMaster Regional Centre for Mass Spectrometry facility (Department of Chemistry, McMaster University) for HR-ESI-MS analysis.

Amino acid feeding studies.

SFMB cultures (5 ml) of the $\Delta\text{txtA}/51$ -1 strain were grown for 3 days, after which autoclaved aqueous solutions or suspensions of L-Val, D-Val, or L-norvaline (Sigma Aldrich Canada) were added to triplicate cultures to a final concentration of 10 mM. The cultures were incubated for an additional 4 days prior to harvesting and extraction of the culture supernatants.

Targeted deletion of the *S. scabies* *cfl* gene.

Deletion of the *cfl* gene from the chromosome of *S. scabies* $\Delta\text{txtA}/51$ -1 was achieved using the Redirect PCR targeting system (Gust et al. 2003a,b). An [*hyg+oriT*] cassette was PCR amplified from pIJ10700 using primers DRB651 and DRB652, after which it was gel purified and introduced into arabinose-induced *E. coli* BW25113 containing pIJ790 and Cosmid 1770 (Table 1) by electroporation. Mutant cosmids in which *cfl* was replaced with the [*hyg+oriT*] cassette were subsequently isolated, and gene replacement was verified by PCR analysis using the primer pairs DRB662-RED-Down and DRB663°RED-Up. A single clone of the mutant cosmid (1770/ Δcfl) was then introduced into *E. coli* ET12567 harboring pUZ8002 (Table 1) prior to transfer into *S. scabies* $\Delta\text{txtA}/51$ -1 by intergeneric conjugation (Kieser et al. 2000). Hygromycin-B-resistant exconjugants that arose were screened for kanamycin sensitivity to confirm that a double-crossover event had taken place. The resulting mutant isolates were verified by PCR using the same primer pairs used for verification of the 1770/ Δcfl cosmid.

Genetic complementation of the *S. scabies* Δcfl strain.

A complementation plasmid harboring the *S. scabies* *cfl* gene was constructed as follows. First, a 1,365-bp fragment containing the *neo* gene and promoter region was PCR amplified from Cosmid 1770 using the primers MSA11 and MSA12 and was cloned into pGEM-T Easy. The cloned PCR product was then released from the plasmid by digestion with *EcoRV*, after which it was gel purified and ligated into the *EcoRV* site of pIJ10257 to yield pMSAK13 (Table 1). Next, the *cfl* gene was PCR amplified from Cosmid 1770 using the primers MSA5 and MSA13 and was cloned into pGEM-T Easy. After sequencing to confirm that no mutations were present, the cloned insert was

released by digestion with *NdeI* and *XhoI* and was gel purified and ligated into similarly digested pMSAK13, yielding pMSAK13/*cfl*. The plasmids pMSAK13 and pMSAK13/*cfl* were then introduced into *E. coli* ET12567 harboring pUZ8002 prior to transfer into the *S. scabies* Δcfl strain by intergeneric conjugation.

ACKNOWLEDGMENTS

We thank C. Bender for providing the CFA, CFA-L-Ile, and CFA-L-Val standards; and K. Tahlan for critical reading of the manuscript. This work was supported by a Natural Sciences and Engineering Research Council of Canada Discovery Grant (386696-2010) and by a Newfoundland and Labrador Research and Development Corporation Ignite R&D grant (5404.1207.102) to D. R. D. Bignell. J. K. Fyans was supported by a Government of Canada Postdoctoral Research Fellowship, and M. S. Altowairish was supported by a King Abdullah Scholarship.

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