TtxH is a key component of the thaxtomin biosynthetic machinery in the potato common scab pathogen *Streptomyces scabies*

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**SUMMARY**

*Streptomyces scabies* causes potato common scab disease, which reduces the quality and market value of affected tubers. The predominant pathogenicity determinant produced by *S. scabies* is the thaxtomin A phytotoxin, which is essential for common scab disease development. Production of thaxtomin A involves the nonribosomal peptide synthetases (NRPSs) TtxA and TtxB, both of which contain an adenylation (A-) domain for selecting and activating the appropriate amino acid during thaxtomin biosynthesis. The genome of *S. scabies* B7.22 contains three small MbtH-like protein (MLP)-coding genes, one of which (txtH) is present in the thaxtomin biosynthesis gene cluster. MLP family members are typically required for the proper folding of NRPS A-domains and/or stimulating their activities. This study investigated the importance of TtxH during thaxtomin biosynthesis in *S. scabies*. Biochemical studies showed that TtxH is required for promoting the soluble expression of both the TtxA and TtxB A-domains in *Escherichia coli*, and amino acid residues essential for this activity were identified. Deletion of *txtH* in *S. scabies* significantly reduced thaxtomin A production, and deletion of one of the two additional MLP homologues in *S. scabies* completely abolished production. Engineered expression of all three *S. scabies* MLPs could restore thaxtomin A production in a triple MLP-deficient strain, while engineered expression of MLPs from other *Streptomyces* spp. could not. Furthermore, the constructed MLP mutants were reduced in virulence compared to wild-type *S. scabies*. The results of our study confirm that TtxH plays a key role in thaxtomin A biosynthesis and plant pathogenicity in *S. scabies*.

**Keywords:** adenylation domain, MbtH-like protein, nonribosomal peptide synthetases, potato common scab, *Streptomyces scabies*, thaxtomin A.

**INTRODUCTION**

Over 580 species of *Streptomyces* have been identified to date (Garrity et al., 2007), of which only a very small number have the ability to infect living plant tissue and cause plant diseases (Bignell et al., 2010a). One of the best studied plant-pathogenic species is *Streptomyces scabies* (syn. *S. scabiei*), which causes common scab disease of potato (Bignell et al., 2014; Loria et al., 2006). The main symptom associated with this disease is the formation of superficial, raised or deep-pitted lesions on the tuber surface, and these lesions reduce the market value of affected potatoes, leading to economic losses for potato growers (Dees and Wanner, 2012). As *S. scabies* is neither tissue- nor host-specific, it can cause scab disease symptoms on other economically important root crops such as radish, carrot, beet and turnip (Dees and Wanner, 2012). Also, the seedlings of model plants such as *Arabidopsis thaliana* and *Nicotiana tabacum* can be infected by *S. scabies*, resulting in root stunting, swelling, necrosis and seedling death (Loria et al., 2006).

The primary pathogenicity determinant produced by *S. scabies* consists of a family of specialized phytotoxins called the thaxtomins, which are cyclic dipeptides (King and Calhoun, 2009). Eleven different thaxtomins have been described, of which thaxtomin A is the predominant member produced by *S. scabies* and other scab-causing pathogens such as *Streptomyces turgidiscabies*, *Streptomyces acidiscabies*, *Streptomyces europaeiscabiei* and *Streptomyces stelliscabiei* (King and Calhoun, 2009; King et al., 1989). A positive correlation was established between the pathogenicity of *S. scabies* strains and their ability to produce thaxtomin A (King et al., 1991), and disruption of thaxtomin biosynthesis in *S. acidiscabies* abolished the ability of the pathogen to cause necrotic lesions on potato tubers (Healy et al., 2000). Thaxtomin A targets the plant cell wall by functioning as a cellulose synthesis inhibitor (Scheible et al., 2003), and its production is induced by cellulobiose and cellotriose, which are the smallest subunits of cellulose (Johnson et al., 2007). In *A. thaliana*, thaxtomin A has been shown to affect the expression of genes involved in cell wall synthesis, and it also reduces the number of cellulose synthase complexes in the plant cell plasma membrane (Bischoff et al., 2009). In addition, thaxtomin A elicits an early defence response in *Arabidopsis* by inducing the influx...
of Ca\(^{2+}\) and the efflux of H\(^+\) ions (Bischoff et al., 2009; Errakhi et al., 2008; Tegg et al., 2005).

The biosynthetic gene cluster responsible for the synthesis of thaxtomin A and related analogues is highly conserved in scab-causing Streptomyces spp. and consists of seven genes: txtA, txtB, txtC, txtD, txtE, txtH and txtR (Fig. 1). txtD encodes a nitric oxide synthase that generates nitric oxide (NO) from \(l\)-arginine, and txtE encodes a novel cytochrome P450 monooxygenase that nitrates \(l\)-tryptophan using the NO to produce the intermediate 4-nitro-\(l\)-tryptophan (4-NTrp) (Barry et al., 2012; Johnson et al., 2009). Two nonribosomal peptide synthetases (NRPSs) encoded by txtA and txtB have been proposed to synthesize thaxtomin D using \(l\)-phenylalanine and 4-NTrp as substrates, respectively (Healy et al., 2000; Johnson et al., 2009; Loria et al., 2008). NRPSs are a family of large proteins that produce nonribosomal peptide molecules with diverse structures and activities. NRPSs consist of multiple enzymatic domains, of which the adenylation domain (A-domain) is responsible for selecting and activating the amino acid substrate for incorporation into the peptide product (Süssmuth and Mainz, 2017). The txtC gene encodes a cytochrome P450 monooxygenase that introduces two hydroxyl groups onto the thaxtomin D backbone to generate the final thaxtomin A product (Fig. 1) (Healy et al., 2002), and txtR encodes a cluster-situated regulator that activates the expression of the thaxtomin biosynthetic genes (Joshi et al., 2007). Additionally, a small gene called txtH is located between txtB and txtC and encodes a protein belonging to the MbtH-like protein (MLP) family (Bignell et al., 2010a).

MLPs are small (~70 amino acids) proteins that are usually encoded within NRPS gene clusters (Baltz, 2011). Recent studies have shown that they play essential roles in promoting the proper folding and activity of the NRPS A-domains, though for reasons currently unknown, not all NRPS A-domains require an MLP for proper function (Boll et al., 2011; Felnagle et al., 2010; McMahon et al., 2012; Schomer and Thomas, 2017; Zhang et al., 2010). It has also been revealed that MLPs from different pathways can, in some instances, functionally complement each other with varying efficiencies (Boll et al., 2011; Lautru et al., 2007; Mori et al., 2018; Schomer and Thomas, 2017; Wolpert et al., 2007; Zhang et al., 2010).

In the current study, we used multiple approaches to investigate the requirement of TxtH and other MLPs during the biosynthesis of thaxtomin A in S. scabies.

RESULTS AND DISCUSSION

Bioinformatics analysis of S. scabies TxtH

The TxtH amino acid sequence was aligned with that of other MLP homologues from the database, including some that have

![Fig. 1](image-url)
been previously characterized (Fig. 2A; Table S1). Pairwise comparisons revealed that TxtH shares the greatest degree of amino acid identity (100%) with the corresponding homologues from the thaxtomin biosynthetic gene clusters in the potato scab pathogens *S. acidiscabies* and *S. europaeiscabiei* (Table S2). In contrast, the TxtH homologue from the thaxtomin biosynthetic gene cluster of another scab pathogen, *S. turgidiscabies*, shares only 80% amino acid identity with the *S. scabies* TxtH. This is consistent with a previous phylogenetic analysis that suggested that the thaxtomin biosynthetic gene clusters from *S. scabies* and *S. acidiscabies* are more closely related to each other than to the *S. turgidiscabies* gene cluster (Huguet-Tapia et al., 2016). As expected, the TxtH homologues from the pathogenic *Streptomyces* spp. formed a well-supported clade in the constructed phylogenetic tree (Fig. 2B). Interestingly, an MLP (ACMO1_RS10820) from the nonpathogenic species *Streptomyces viridochromogenes* also clustered together with theTxtH homologues from the pathogenic species and showed a very high degree of amino acid identity (97%) with TxtH from *S. scabies*, *S. europaeiscabiei* and *S. acidiscabies* (Fig. 2B and Table S2). An analysis of the *S. viridochromogenes* genome sequence (accession number PRJNA238534) revealed that the MLP is encoded in the vicinity...
of four other genes that show strong similarity to thaxtomin biosynthetic genes, though three of the genes appear to be pseudo-genes. Two other MLPs encoded in the S. scabies 87.22 genome, SCAB_3331 (herein referred to as MLP\(_{lipo}\)) and SCAB_85461 (herein referred to as MLP\(_{scab}\)), both share only 52.3\% amino acid identity with TxtH (Table S1) and cluster together in a separate clade as compared to the one containingTxtH (Fig. 2B). The MLP\(_{lipo}\)-encoding gene is located within an NRPS gene cluster that is responsible for the biosynthesis of a putative lipopeptide metabolite (Yaxley, 2009), whereas the MLP\(_{scab}\)-encoding gene is localized within the NRPS gene cluster that synthesizes the siderophore scabichelin (Kodani et al., 2013). An orphan MLP (MXAN_3118) from Myxococcus xanthus DK 1622 showed the least amino acid identity (33.8\%) with TxtH in the pairwise comparison (Table S2). A recent study showed that MXAN_3118, which is not located within or near any NRPS biosynthetic gene clusters, can interact in vivo and in vitro with several different NRPSs in M. xanthus (Esquilin-Lebron et al., 2018).

Baltz (2011) previously proposed a signature sequence \([\text{NxExQxSxWP(x)}5\text{PxGW(x)}12\text{L(x)}6\text{WTDxRP}]\) consisting of multiple amino acid residues that are invariant in most MLPs, all of which are also conserved inTxtH and in other MLP homologues analysed here (Fig. 2A). Structural analysis of the PA2412 MLP from Pseudomonas aeruginosa PAO1 revealed that several of these residues, including the three highly conserved tryptophan residues, lie on one face of the protein, which is thought to interact with conserved components of the cognate NRPS (Drake et al., 2007). The structure of SIgN1, a 3-methylaspartate-adenylating enzyme with an MLP domain at its N-terminus, revealed that two of the conserved tryptophan residues (W25 and W35) from the MLP domain are located at the interface between the MLP and the A-domain and are important for this interaction (Herbst et al., 2013). Analysis of mutants defective in equivalent residues (W22A/W32A) in another MLP, PacJ, showed that they contribute to PacJ's ability to form a complex with the cognate PacL NRPS to stimulate the adenylation activity of the synthetase (Zhang et al., 2010). Based on these studies, we predict that the conserved amino acid residues inTxtH play an important role in its interaction with the thaxtomin NRPS in S. scabies.

**TxtH is required for promoting the solubility of the TtxT A- and TtxB A-domains**

Previously it was shown that YbdZ, an MLP encoded in the enterobactin biosynthetic gene cluster of *Escherichia coli*, can interact with adenylating enzymes from different NRPS biosynthesis pathways (Felnagle et al., 2010). Therefore, the TtxT A- and TtxB A-domains were expressed as N-terminal 6× histidine (His\(_6\))-tagged proteins in an *E. coli* ybdZ mutant (BL21(DE3)ybdZ:aac(3)IV) to avoid any potential interference caused by YbdZ during co-expression studies using TxtH (Table 1). Each A-domain (herein referred to as His\(_6\)-TtxT-A) of TtxT (Table 1). Each A-domain (herein referred to as His\(_6\)-TtxT-A) and His\(_6\)-TtxB-A) was expressed in the presence or absence of TxtH, which itself either contained or lacked an N-terminal His\(_6\)-tag, to rule out any influence that the tag might have on the function of TxtH. The ability of TxtH to promote the solubility of each A-domain was then determined by western blot

### Table 1  Bacterial strains used in this study.

| Strain | Description | Resistance† | Reference or source |
|--------|-------------|-------------|---------------------|
| **Escherichia coli strains** | | | |
| DH5\(\alpha\) | General cloning host | n/a | Gibco-BRL |
| NEB5\(\alpha\) | DHS\(\alpha\) derivative, high efficiency competent cells | n/a | New England Biolabs |
| BL21(DE3)ybdZ:aac(3)IV | BL21(DE3) derivative, ybdZ replaced with an apramycin resistance cassette (aac(3)IV) | Apra\(^a\) | Herbst et al., 2013 |
| ET12567/pU28002 | dam\(^{-}\), dcm\(^{-}\), hsdS\(^{-}\); non-methylating conjugation host | Kan\(^{R}\), Cm\(^{R}\) | MacNeil et al., 1992 |
| **Streptomyces scabies strains** | | | |
| 87.22 | Wild-type strain | n/a | Loria et al., 1995 |
| 87.22/\(\Delta\)mlp\(_{lipo}\)/\(\Delta\)txtH | Strain 87.22 containing plasmid pIJ12738/\(\Delta\)mlp\(_{lipo}\)/\(\Delta\)txtH inserted into the chromosome | n/a | This study |
| \(\Delta\)mlp\(_{lipo}\) | mlp\(_{lipo}\) deletion mutant derivative of strain 87.22 | n/a | This study |
| \(\Delta\)txtH | txtH deletion mutant derivative of strain 87.22 | n/a | This study |
| \(\Delta\)mlp\(_{lipo}\)/\(\Delta\)txtH | txtH deletion mutant derivative of strain \(\Delta\)mlp\(_{lipo}\) | Apra\(^{a}\) | This study |
| \(\Delta\)txtH/\(\Delta\)mlp\(_{scab}\) | mlp\(_{scab}\) deletion mutant derivative of strain \(\Delta\)txtH | Apra\(^{a}\), Hyg\(^{a}\) | This study |
| \(\Delta\)mlp\(_{lipo}\)/\(\Delta\)txtH/\(\Delta\)mlp\(_{scab}\) | mlp\(_{scab}\) deletion mutant derivative of strain \(\Delta\)mlp\(_{lipo}\)/\(\Delta\)txtH | Apra\(^{a}\), Hyg\(^{a}\) | This study |

\(^{a}\)Apra\(^{R}\), Kan\(^{R}\), Cm\(^{R}\) and Hyg\(^{R}\) = apramycin, kanamycin, chloramphenicol and hygromycin resistance, respectively.
analysis of isolated soluble protein fractions using an anti-His\textsubscript{6} antibody.

As shown in Fig. 3A, only trace levels of soluble His\textsubscript{6}-TxtA-A and His\textsubscript{6}-TxtB-A protein were detected in E. coli when expressed in the absence of TxtH, whereas both proteins were readily detectable in soluble form when co-expressed with the MLP. The solubility promoting activity of TxtH was observed regardless of whether or not the protein contained an N-terminal His\textsubscript{6} tag (Fig. 3A), indicating that the tag did not interfere with the activity of the protein. Our results therefore suggest that TxtH likely functions as a chaperone that is essential for the proper folding of both A-domains in the thaxtomin NRPSs, a role that is consistent with that proposed for other MLPs (Imker et al., 2010; Zhang et al., 2010; Zolova and Garneau-Tsodikova, 2012).

To further explore the role of the highly conserved amino acid residues in the MLP signature sequence of TxtH, we constructed several His\textsubscript{6}-TxtH point mutants (N17A, Q21A, S23A, S23Y, L24A, W25A, W35A, W55A, T56A and D57A) and then co-expressed each mutant protein with His\textsubscript{6}-TxtA-A and His\textsubscript{6}-TxtB-A. As shown in Fig. 3B, the solubility of both the His\textsubscript{6}-TxtA-A and His\textsubscript{6}-TxtB-A proteins was reduced or abolished when co-expressed with all of the TxtH point mutants. Of particular note is the S23Y mutation, which resulted in complete loss of soluble protein for both A-domains. Herbst and colleagues showed that the same mutation in the MLP domain of the SIgN1 hybrid adenylase resulted in a 5-fold reduction in adenylation activity of the enzyme, most likely due to impairment of the interaction between the MLP and adenylation domains by the bulky tyrosyl residue (Herbst et al., 2013). In contrast, the S23A mutation in TxtH caused a drastic reduction of soluble His\textsubscript{6}-TxtB-A protein but did not lead to a complete loss of soluble protein, and it only slightly reduced the solubility of the His\textsubscript{6}-TxtA-A protein (Fig. 3B). This is possibly due to the fact that an alanine side chain is less bulky than a tyrosine side chain and may therefore cause less steric interference during the interaction of the MLP with the A-domains.

All three highly conserved tryptophan residues in TxtH (W25, W35, W55) (Fig. 2A) were found to be essential for promoting the solubility of His\textsubscript{6}-TxtB-A, whereas only W35 and W55 are essential for promoting His\textsubscript{6}-TxtA-A solubility (Fig. 3B). The W55 residue is part of the highly conserved WTDxRP motif, which in the P. aeruginosa PA2412 occurs between two alpha helices and was proposed to play a role in the proper orientation of the C-terminal helix (Drake et al., 2007), whereas in MbtH from M. tuberculosis the motif lies within a disordered region (Buchko et al., 2010). Our results show that in addition to W55, two other residues within this motif (T56, D57) are critical for the ability of TxtH to promote the solubility of His\textsubscript{6}-TxtB-A, whereas neither residue is essential for obtaining soluble His\textsubscript{6}-TxtA-A, though His\textsubscript{6}-TxtA-A solubility was clearly affected in the presence of these point mutants. Other TxtH residues that were found to be essential for promoting the solubility of His\textsubscript{6}-TxtB-A are N17 and L24. Overall, our results show that all of the highly conserved amino acid residues found in the MLP signature sequence are important for the solubility-promoting activity of TxtH. We anticipate that structural studies examining the interaction of TxtH with each
A-domain will provide further insight into the specific function of these residues during such interactions.

Loss of MLPs abolishes thaxtomin A production in S. scabies

To examine the in vivo role of txtH in the thaxtomin A biosynthetic pathway, we deleted txtH from the S. scabies chromosome (Fig. S1). Four mutant isolates were examined for thaxtomin A production, and all were found to produce significantly less thaxtomin A as compared to the wild-type strain (Fig. 4A, B). Production in the ΔtxtH1 mutant isolate was partially restored when txtH was expressed from an integrative plasmid using the strong, constitutive ermEp* promoter (Fig. 4C). Notably, two other metabolites with retention times of 3.82 and 4.64 min were found to accumulate at very low levels in the ΔtxtH1 mutant isolates but not in wild-type S. scabies (Fig. 4B, peaks ▼ and ▽). Liquid chromatography-high resolution electrospray ionization mass spectrometry (LC-HRESIMS) analysis of the ΔtxtH1 mutant culture extract in negative ion mode revealed a pseudomolecular [M-H]− ion at m/z 421.1524 for peak ▼ and a pseudomolecular [M-H]− ion at m/z 405.1577 for peak ▽, which is consistent with the accumulation of thaxtomin B and D, respectively (King and Calhoun, 2009). Thaxtomin D was previously reported to accumulate in a ΔtxtC mutant of S. acidiscabies (Healy et al., 2002), suggesting that there may be some polar effects on the
expression of txtC caused by the deletion of txtH, even though the orientation of the inserted apramycin resistance cassette was the same as the original txtH gene (Figs S1 and S2). Indeed, semi-quantitative RT-PCR analysis showed that the txtC transcription level was reduced in the ΔtxtH mutant compared to the wild-type strain, though expression of txtC could still be detected in the mutant, especially at higher PCR cycle numbers (Fig. 5; data not shown).

It has been reported that MLPs from different pathways can functionally complement each other (Boll et al., 2011; Lautru et al., 2007; Mori et al., 2018; Schomer and Thomas, 2017; Wolpert et al., 2007; Zhang et al., 2010). In organisms where multiple MLPs are encoded in a single genome, the deletion of a single MLP often does not abolish the production of the cognate metabolite, but instead metabolite production is eliminated only when all copies of MLP-encoding genes are removed from the host genome (Lautru et al., 2007; Wolpert et al., 2007). As the S. scabies genome harbours two additional MLP-encoding genes, mlp_lipo and mlp_scab, it is possible that either or both MLPs might be able to partially compensate for the loss of txtH in the ΔtxtH mutant. When we deleted mlp_lipo from the wild-type S. scabies chromosome (Fig. S3), thaxtomin A production was similar in the Δmlp_lipo mutant as compared to the wild-type strain (data not shown). Deletion of both txtH and mlp_lipo resulted in thaxtomin A production levels that are similar or slightly reduced as compared to the ΔtxtH single mutant, whereas deletion of txtH and mlp_scab abolished thaxtomin A production completely, and similar results were observed when all three MLP genes were deleted (Fig. 4B). Both the mlp_lipo and mlp_scab genes were shown to be expressed in wild-type S. scabies and in the ΔtxtH mutant under thaxtomin-inducing conditions (Fig. 5), suggesting that the lack of thaxtomin A production in the ΔtxtH/Δmlp_scab mutant was not due to a lack of transcription of the mlp_lipo gene. Interestingly, both MLP_lipo and MLP_scab were able to promote the soluble expression of theTxtA and TxtB A-domains in E. coli, though the solubility-promoting activity of MLP_scab was less efficient for the His6-TxtB-A protein (Fig. 3C). This suggests that despite the inability of the ΔtxtH/Δmlp_scab mutant to produce detectable levels of thaxtomin A, both MLP_lipo and MLP_scab have the ability to functionally replace TxtH in its interaction with the thaxtomin NRPS A-domains. Further investigations will be required to determine the reason for the lack of detectable thaxtomin A production in the ΔtxtH/Δmlp_scab mutant.

**Engineered expression of MLPs in wild-type S. scabies and in the MLP triple mutant**

To further explore the ability of MLPs from different biosynthetic pathways to promote thaxtomin A production in the absence of txtH, we constructed several plasmids that over-express different MLP-encoding genes using the ermEp* promoter and then introduced them into the Δmlp_lipo/ΔtxtH/Δmlp_scab mutant. As shown in Fig. 4D, overexpression of mlp_lipo and mlp_scab from S. scabies restored thaxtomin A production in the triple mutant to levels similar to that observed when txtH was overexpressed, confirming that both MLPs can functionally replace txtH in the thaxtomin biosynthetic pathway. We note that overexpression of txtH, mlp_lipo and mlp_scab also led to accumulation of the thaxtomin B and D biosynthetic intermediates (Fig. S5), confirming that there are some polar effects of the ΔtxtH mutation on expression of the downstream txtC gene. In contrast, overexpression of the MLP-encoding genes cdaX from Streptomyces coelicolor and clav_p1293 from Streptomyces clavuligerus did not restore thaxtomin metabolite production in the S. scabies triple MLP mutant (Fig. 4D), suggesting that neither MLP can exhibit functional cross-talk with TxtH. Both CdaX and Clav_p1293 localize in different phylogenetic clades from TxtH (Fig. 2B), though CdaX is predicted to be closely related to MLP_lipo and MLP_scab both of which can exhibit cross-talk with TxtH (Fig. 4D). Interestingly, a recent study by Schomer and Thomas (2017) also showed that while some non-cognate MLPs are able to functionally replace the YbdZ MLP in the E. coli enterobactin biosynthetic pathway, others cannot, and no apparent correlation between MLP functionality and sequence similarity could be identified (Schomer and Thomas, 2017).

Previously, it was reported that the overexpression of cognate and non-cognate MLPs in vivo increases vancomycin production in the high-producing strain Amycolatopsis orientalis KFCC10990P (Lee et al., 2016). We investigated whether overexpression of txtH, mlp_lipo, mlp_scab, cdaX and clav_p1293 in S. scabies.
scabies 87.22 enhances thaxtomin A production in this strain; however, none of the overexpression strains produced significantly higher levels of thaxtomin A compared to the control strain (data not shown). Other studies have shown that an A-domain requires a 1:1 molar ratio with its MLP partner for the maximum enzyme activity, and increasing the amount of MLPs beyond this optimal ratio did not stimulate the adenylating activity beyond a point (Boll et al., 2011; Zhang et al., 2010). Our results suggest that a similar situation may exist with TxtH and its cognate NRPS, though further investigations into this are needed.

Plant pathogenic phenotype of the S. scabies MLP mutants

We conducted a potato tuber slice assay in order to compare the virulence phenotype of the different S. scabies MLP mutant strains. As expected, S. scabies 87.22 readily colonized the surface of the potato tuber tissue and caused significant necrosis of the tissue after 10 days post-inoculation (Fig. 6). The ΔtxtH and Δmlp_{lip}/ΔtxtH mutants also colonized the tissue and induced tissue necrosis, though both strains were less efficient at doing so than the wild-type strain. In contrast, there was very little visible growth of the Δmlp_{scab}/ΔtxtH and Δmlp_{lip}/ΔtxtHΔmlp_{scab} mutant strains on the tuber tissue, and both strains caused very little necrosis of the tissue (Fig. 6). Given that a positive correlation has been noted between the production of thaxtomin A and the virulence of scab-causing Streptomyces spp. (Healy et al., 2000; King et al., 1991), the observed virulence phenotype of the different MLP mutant strains is consistent with the corresponding thaxtomin A production profiles observed in liquid culture (Fig. 4B). It remains to be determined whether production of the putative lipopeptide metabolite and the scabichelin siderophore are also affected in the MLP mutant strains and whether these metabolites also contribute to the pathogenicity of S. scabies. As siderophore production is known to contribute to the virulence phenotype of plant-pathogenic bacteria (Franza et al., 2005; Taguchi et al., 2010), it will be interesting to further investigate the role of scabichelin in S. scabies plant pathogenicity.

Concluding remarks

This study demonstrated the importance of TxtH in the biosynthesis of thaxtomin A in S. scabies. In particular, TxtH is required for promoting the soluble expression of both A-domains from the thaxtomin NRPS in E. coli, suggesting that it performs a chaperone-like role to enable the proper folding of the NRPS in S. scabies. Amino acid residues that contribute to the solubility-promoting activity of TxtH have been revealed in this study, and future structural investigations will provide important insights into the role of these residues in mediating interactions between TxtH and the thaxtomin NRPSs. We also showed that MLP_{lip} from the putative lipopeptide biosynthetic pathway and MLP_{scab} from the scabichelin biosynthetic pathway can functionally replace TxtH in the thaxtomin biosynthetic pathway, whereas two MLPs from other Streptomyces species cannot. Further investigations are required to better understand the mechanisms behind MLP cross-talk and why certain MLPs from different pathways can functionally complement each other while others are unable to do so. Finally, our study confirmed that TxtH is important for the plant pathogenic phenotype of S. scabies.

EXPERIMENTAL PROCEDURES

Bacterial strains, cultivation and maintenance

The bacterial strains used in this study are listed in Table 1. Escherichia coli strains were cultivated at 37 °C unless otherwise stated. Liquid cultures were grown with shaking (200 – 250 rpm)
in Luria-Bertani (LB) Lennox broth (Fisher Scientific, Ottawa, ON, Canada), low salt LB broth (1% w/v tryptone; 0.5% w/v yeast extract; 0.25% w/v NaCl), super optimal broth (SOB) or super optimal broth with catabolite repression (SOC) medium (New England Biolabs, Whitby, ON, Canada), while solid cultures were grown on LB Lennox (or low salt LB) medium containing 1.5% w/v agar. When necessary, the growth medium was supplemented with 50 μg/mL apramycin (Sigma Aldrich, Oakville, ON, Canada), 50 μg/mL kanamycin or hygromycin B (Millipore Sigma, Oakville, ON, Canada), or 25 μg/mL chloramphenicol (Acros Organics, Geel, Belgium) (final concentration). Escherichia coli strains were maintained at 4 °C for short-term storage or at −80 °C as glycerol stocks for long-term storage.

*Streptomyces scabies* strains were cultured at 28 °C unless otherwise indicated. Liquid cultures were typically grown with shaking (200 rpm) in trypticase soy broth (TSB; BD Biosciences, Mississauga, ON, Canada) medium with stainless-steel springs. Plate cultures were routinely grown on potato mash agar (PMA; Fyans et al., 1987) that had been modified by replacing the malt extract with Bacto Yeast Extract (Becton Dickinson, Mississauga, Canada) as per the manufacturer’s instructions, except that 5% v/v DMSO was included in the reactions. All oligonucleotide primers used for cloning, PCR, site-directed mutagenesis and sequencing were purchased from Integrated DNA Technologies (Coralville, IA, USA) and are listed in Table S3. DNA sequencing was performed by The Centre for Applied Genomics (Toronto, Canada). *Streptomyces* genomic DNA was isolated from mycelia harvested from 1–2 day-old TSB cultures using the QIAamp® DNA mini kit as per the manufacturer’s protocol (QIAGEN Inc, Toronto, ON, Canada).

**Construction of protein expression plasmids**

Plasmids were constructed for overexpression of TtxH in *E. coli* with and without an N-terminal His$_6$ tag as well as for overexpression of N-terminal His$_6$-tagged MLP$_{lipo}$ and MLP$_{scab}$. The ttxH gene was PCR-amplified using cosmid 1989 as template and using primers PL150 and PL36 for construction of the untagged TtxH expression plasmid, and PL35 and PL36 for construction of the His$_6$-TtxH expression plasmid. The resulting PCR products were directly cloned into the expression vector pET28b via the *NdeI/EcoRI* and *Ncol/EcoRI* restriction sites to give pET28b/His$_6$-ttxH and pET28b/ttxH, respectively. MLP$_{lipo}$ was PCR-amplified from genomic DNA using primers PL163 and PL164, and MLP$_{scab}$ was PCR-amplified from cosmid 57 using primers PL165 and PL166. The PCR products were directly cloned into the *NdeI/EcoRI* restriction sites of pET28b to give pET28b/His$_6$-MLP$_{lipo}$ and pET28b/His$_6$-MLP$_{scab}$.

Plasmids were also constructed for overexpression of the TtxA and TtxB A-domains as N-terminal His$_6$-tagged proteins. The DNA sequences encoding TtxA-A and TtxB-A were PCR amplified using the primer pairs PL37/PL38 and PL40/PL41, respectively, and using cosmid 1989 as template. The products were cloned into the pGEM-T vector as per the manufacturer’s instructions, after which the inserts were released by digestion with *EcoRI* and *HindIII* and were cloned into similarly digested pACYCDuet-1 to give pACYCDuet-1/His$_6$-ttxA-A and pACYCDuet-1/His$_6$-ttxB-A. The cloned inserts in all constructed expression vectors were verified by DNA sequencing.

**Site-directed mutagenesis of TtxH**

Site-directed mutagenesis of TtxH was performed using the QuikChange II Site-directed Mutagenesis Kit (Agilent Technologies Canada, Inc., Mississauga, ON, Canada) as per the manufacturer’s instructions. Mutagenic primers for the desired mutation was designed online with QuikChange® Primer Design Program (https://www.genomics.agilent.com/primerDesignProgram.jsp). The desired mutations were verified by DNA sequencing.

**Co-expression of His$_6$-TtxA-A and His$_6$-TtxB-A with MLPs**

The BL21(DE3) ybdZ::aac(3)IV bacterial strain was used for co-expression of His$_6$-TtxA-A or His$_6$-TtxB-A with tagged or untagged MLP proteins. Strains containing either pACYCDuet-1/His$_6$-ttxA-A or pACYCDuet-1/His$_6$-ttxB-A with and without pET28b/ttxH, pET28b/His$_6$-ttxH (wild-type or point mutants), pET28b/His$_6$-MLP$_{lipo}$ or pET28b/His$_6$-MLP$_{scab}$ were grown overnight in 3 mL of...
### Table 2  Plasmids and cosmids used in this study.

| Plasmid or cosmid | Description | Resistance† | Reference or source |
|------------------|-------------|-------------|---------------------|
| pGEM-T           | General cloning vector | Amp<sup>R</sup> | Promega Corporation   |
| pGEM-T EASY      | General cloning vector | Amp<sup>R</sup> | Promega Corporation   |
| pGEM-T EASY/Δmlp<sub>ipo</sub> | pGEM-T EASY derivative containing a 3725 bp insert with a deletion of the mlp<sub>ipo</sub> gene | Amp<sup>R</sup> | This study |
| pET28b           | N- or C-terminal 6 × histidine fusion tag protein expression vector with T7 promoter and lac operator | Kan<sup>R</sup> | Novagen            |
| pET28b/His<sub>6</sub>-txtH | pET28b derivative containing a DNA fragment for expression of the His<sub>6</sub>-TxtH protein | Kan<sup>R</sup> | This study |
| pET28b/His<sub>6</sub>-mlp<sub>ipo</sub> | pET28b derivative containing a DNA fragment for expression of the His<sub>6</sub>-MLP<sub>ipo</sub> protein | Kan<sup>R</sup> | This study |
| pET28b/His<sub>6</sub>-mlp<sub>scab</sub> | pET28b derivative containing a DNA fragment for expression of the His<sub>6</sub>-MLP<sub>scab</sub> protein | Kan<sup>R</sup> | This study |
| pET28b/txTH      | pET28b derivative containing a DNA fragment for expression of the untagged TxTH protein | Kan<sup>R</sup> | This study |
| pACYCDuet-1      | N- terminal 6 × histidine fusion tag expression vector with T7 promoter and lac operator | Cml<sup>R</sup> | Novagen            |
| pACYCDuet-1/His<sub>6</sub>-txtA-A | pACYCDuet-1 derivative containing a DNA fragment for expression of the His<sub>6</sub>-TxtA-A protein | Cml<sup>R</sup> | This study |
| pACYCDuet-1/His<sub>6</sub>-txB-A | pACYCDuet-1 derivative containing a DNA fragment for expression of the His<sub>6</sub>-TxB-A protein | Cml<sup>R</sup> | This study |
| pIJ12738         | Conjugative plasmid, non-replicative in Streptomyces, containing MCS and I-Scel site | Apra<sup>R</sup> | Fernández-Martínez and Bibb, 2014 |
| pIJ12738/Δmlp<sub>ipo</sub> | pIJ12738 derivative containing two flanking regions of mlp<sub>ipo</sub> | Apra<sup>R</sup> | This study |
| pIJ12742         | Conjugative plasmid containing the temperature-sensitive replication origin and the codon optimized I-Scel gene under the control of the strong constitutive ermEp<sup>*</sup> promoter | Thio<sup>R</sup> | Fernández-Martínez and Bibb, 2014 |
| pIJ773           | Template for PCR amplification of the aac(3)IV-oriT cassette used for PCR targeting | Apra<sup>R</sup> | Gust et al., 2003a |
| pIJ10700         | Template for PCR amplification of the hyg-oriT cassette used for PCR targeting | Hyg<sup>R</sup> | Gust et al., 2003b |
| Cosmid 1989      | SuperCos1 derivative containing the S. scabies thaxtomin A biosynthetic gene cluster | Amp<sup>R</sup>, Kan<sup>R</sup> | Zhang et al., 2016 |
| Cosmid 57        | SuperCos1 derivative containing the S. scabies mlp<sub>scab</sub> gene | Amp<sup>R</sup>, Kan<sup>R</sup> | This study |
| Cosmid 1989/ΔtxtH | Cosmid 1989 derivative containing the aac(3)IV-oriT cassette in place of the txtH gene | Amp<sup>R</sup>, Apra<sup>R</sup> | This study |
| Cosmid 57/Δmlp<sub>scab</sub> | Cosmid 57 derivative containing the hyg-oriT cassette in place of the mlp<sub>scab</sub> gene | Amp<sup>R</sup>, Kan<sup>R</sup>, Hyg<sup>R</sup> | This study |
| pRLDB50-1a       | Overexpression plasmid containing the strong constitutive ermEp<sup>*</sup> promoter | Apra<sup>R</sup>, Thio<sup>R</sup> | Bignell et al., 2010b |
| pRLDB50-1a/txTH  | pRLDB50-1a derivative containing the S. scabies txtH gene | Apra<sup>R</sup>, Thio<sup>R</sup> | This study |
| pRLDB50-1a/mlp<sub>ipo</sub> | pRLDB50-1a derivative containing the S. scabies mlp<sub>ipo</sub> gene | Apra<sup>R</sup>, Thio<sup>R</sup> | This study |
| pRLDB50-1a/mlp<sub>scab</sub> | pRLDB50-1a derivative containing the S. scabies mlp<sub>scab</sub> gene | Apra<sup>R</sup>, Thio<sup>R</sup> | This study |
| pRLDB50-1a/clav_p1293 | pRLDB50-1a derivative containing the S. clavuligerus clav_p1293 gene | Apra<sup>R</sup>, Thio<sup>R</sup> | This study |
| pRLDB50-1a/cdaX  | pRLDB50-1a derivative containing the S. coelicolor cdaX gene | Apra<sup>R</sup>, Thio<sup>R</sup> | This study |

†Amp<sup>R</sup>, Apra<sup>R</sup>, Kan<sup>R</sup>, Cml<sup>R</sup>, Thio<sup>R</sup> and Hyg<sup>R</sup> = ampicillin, apramycin, kanamycin, chloramphenicol, thiostrepton and hygromycin resistance, respectively.
LB medium supplemented with 1% glucose, apramycin and chloramphenicol. Kanamycin was additionally included for strains containing the MLP expression plasmids. The overnight cultures were subcultured (1% v/v) into 50 mL of fresh LB containing appropriate antibiotics, and the cultures were incubated at 37 °C and 200 rpm until the OD₆₀₀ was 0.4–0.6. Then, the cells were induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and were further incubated at 16 °C and 200 rpm for 48 h. Cells from 1 mL of culture were harvested by centrifugation and were resuspended in 200 μL of 50 mM Tris-HCl (pH 8.0) containing 1 × Complete EDTA-free protease inhibitor (Roche Diagnostics, Laval, QC, Canada). The cells were then lysed by sonication for 25 s (10 s pulses alternating with 10 s pauses, 40% Amp) and the cell debris was removed by centrifugation (1 min at 16 000 rpm). The supernatants containing soluble proteins were collected and the protein concentration was quantified using a Bradford protein assay kit (Fisher Scientific).

**Western blot analysis**

Soluble protein extracts (10 μg) were subjected to standard sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) before being transferred to Amersham™ Hybond™ ECL membrane (GE Healthcare Canada Inc., Mississauga, ON, Canada) as per the manufacturer’s instructions. Membranes were blocked overnight in TBS-T buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl and 0.05% v/v Tween 20) containing 5% w/v skim milk, and were then incubated with 6 × His Epitope Tag Antibody (Fisher Scientific) at a 1:2000 dilution. The membranes were washed several times with TBS-T buffer and were then incubated with the secondary antibody (Fisher Scientific) at a 1:2000 dilution. The membranes were processed using ECL™ western blotting detection reagent (GE Healthcare) and were visualized an ImageQuant LAS4000 Biomolecular Imager (GE Healthcare).

**Construction of an MLP-deficient strain of S. scabies**

A markerless deletion mutant of the mlplipo gene was generated using the meganuclease I-SceI system (Fernández-Martínez and Bibb, 2014). A 1766 bp region upstream of mlplipo (5′ mlplipo) was amplified using S. scabies 87.22 genomic DNA as template and using primers PL3 and PL4 to generate a DNA fragment with terminal XbaI and BamHI sites. A 1959 bp region downstream of the gene (3′ mlplipo) was separately amplified using the same template and primers PL5 and PL6 to generate a DNA fragment with terminal BamHI and EcoRI sites. These two flanking fragments were each cloned into pGEM-T EASY (Table 2) generating pGEM-T EASY'S mlplipo and pGEM-T EASY'3′ mlplipo, the inserts of which were confirmed by DNA sequencing. The 3′ mlplipo insert was then released following digestion with EcoRI and BamHI and was cloned into similarly digested pGEM-T EASY'S mlplipo to generate pGEM-T EASY'Δmlplipo, which contained a 3725 bp insert with a deletion of the mlplipo gene. Next, the 3725 bp insert was released by digestion with XbaI and EcoRI and was cloned into similarly digested pJJ12738 to give pJJ12738/Δmlplipo, which was then introduced into S. scabies 87.22 by intergeneric conjugation with E. coli as described before (Kieser et al., 2000). Apramycin-resistant exconjugants (assigned as 87.22/Δmlplipo_int) were selected and verified by PCR using primers PL62 and PL63. Then, the delivery vector pJJ12742 containing the codon optimized I-SceI gene under the control of the ermE’ promoter was introduced into verified S. scabies 87.22/Δmlplipo_int by conjugation with E. coli. The exconjugants were cultured on PMA at 37 °C in order to promote the loss of pJJ12742, which was confirmed by screening for sensitivity to thistrepton. Spores of thistrepton-sensitive exconjugants were then serially diluted in sterile water and were plated onto PMA plates to obtain single colonies, which were then screened for sensitivity to apramycin. Successful deletion of mlplipo was confirmed by PCR (Fig. S3).

The Redirect PCR targeting system (Gust et al., 2003a, 2003b) was used to construct the ΔtxtH, Δmlplipo/ΔtxtH, ΔtxtH/ΔlipoΔmlplipo/ΔtxtHΔlipo ΔlipoΔmlplipo/ΔtxtHΔlipo mutant strains. The txtH gene on cosmid 1989 was replaced with an extended apramycin resistance cassette [aac(3)IV-oriT] that was PCR-amplified using pJJ1773 as template and using primers DRB627 and DRB628. The mlplipo gene on cosmid 57 was replaced with an extended hygromycin resistance cassette [hyg-oriT] that was PCR-amplified using pJJ10700 as template and using primers PL153 and PL154. The ΔtxtH and ΔlipoΔmlplipo, mutant cosmids were verified by PCR (Fig. S2; data not shown) and were then introduced into S. scabies by intergeneric conjugation with E. coli. The resulting mutant strains were analysed by PCR to confirm replacement of the target genes (Figs S1 and S4).

**Construction of MLP overexpression plasmids**

The txtH, mlplipo, and mlplipo genes from S. scabies, together with the clav_p1293 and cdaX MLP-encoding genes from S. clavuligerus and S. coelicolor, respectively, were PCR-amplified using cosmid 1989 (for txtH) or genomic DNA (for mlplipo, mlplipo, clav_p1293 and cdaX) as template and using gene-specific primers with BamHI and XbaI restriction sites added (Table S3). The resulting products were digested with BamHI and XbaI restriction sites added (Table S3). The resulting products were digested with BamHI and XbaI and were ligated into similarly digested pRLDB50-1a (Bignell et al., 2010b) to generate pRLDB50-1a/txtH, pRLDB50-1a/ mlplipo, pRLDB50-1a/mlplipo, and pRLDB50-1a/clav_p1293 and pRLDB50-1a/cdaX (Table 2). The expression plasmids along with the control plasmid pRLDB50-1a were then introduced into S. scabies 87.22 and the Δmlplipo/ΔtxtHΔlipoΔmlplipo mutant by intergeneric conjugation with E. coli.

**Quantification of thaxtomin A production**

Thaxtomin A was extracted from S. scabies OBBC cultures as described by Fyans et al. (2016). Quantification of thaxtomin A in the culture extracts was by reverse-phase HPLC using a standard curve.
that was constructed from known amounts of a pure thaxtomin A standard (Sigma Aldrich). The thaxtomin A production levels were normalized using dry cell weights (DCWs) as described before (Fyans et al., 2016) and were reported as ng thaxtomin A/mg DCW. Statistical analysis of the results was conducted in Minitab 18 using one-way ANOVAs with a posteriori multiple comparisons of least squared means performed using the Tukey test. P values ≤ 0.05 were considered statistically significant in all analyses.

**LC-HRESIMS analysis of S. scabies culture extracts**

LC-HRESIMS analysis of S. scabies culture extracts was performed at the Memorial University Centre for Chemical Analysis, Research and Training using an Agilent 1260 Infinity HPLC system interfaced to an Agilent 6230 orthogonal time-of-flight mass analyser. Separation was achieved using a ZORBAX SB-C18 analytical column (4.6 × 150 mm, 5 μm particle size) held at a constant temperature of 40 °C and an isotropic mobile phase consisting of 30% acetonitrile and 70% water at a constant flow rate of 1.0 mL/min. Metabolites were monitored by absorbance at 380 nm and by electrospray ionization MS in negative ion mode.

**Potato tuber slice bioassay**

The virulence phenotype of S. scabies strains was assessed using a potato tuber slice bioassay as described before (Loria et al., 1995). Streptomyces scabies strains were cultured on modified YMS agar for 14 days until well sporulated. Agar plugs were then prepared from the plates and inverted onto the tuber slices. The tuber slices were incubated at room temperature (−22−25 °C) in the dark in a moist chamber and were photographed after 10 days. The assay was performed twice in total.

**Total RNA isolation**

*Streptomyces scabies* mycelia (100–200 mg) from 42 h OBAC plates were placed into sterile 1.7 mL microcentrifuge tubes and were flash frozen in a dry ice/ethanol bath and then stored at −80 °C. Total RNA was isolated using a innuPREP RNA Mini Kit 2.0 and a SpeedMill PLUS tissue homogenizer (Analytik Jena AG, Jena, Germany) as per the manufacturer’s instructions. The resulting RNA samples were treated with DNase I (New England Biolabs) as directed by the manufacturer to remove trace amounts of genomic DNA, after which the DNase-treated RNA samples were quantified using a NanoDrop™ 1000 Spectrophotometer (Fisher Scientific). The integrity of the RNA was confirmed by agarose gel electrophoresis using a 1.2% w/v RNAse-free agarose gel in 1 × TBE (Tris-borate-EDTA) buffer. The RNA samples were stored at −80 °C.

**Reverse transcription PCR**

Reverse transcription (RT) was performed using SuperScript IV reverse transcriptase (Fisher Scientific) with 2 μg of DNase-treated total RNA and random hexamer primers as per the manufacturer’s instructions. A negative control reaction lacking the reverse transcriptase enzyme was included to verify the absence of genomic DNA in the RNA samples. RNA was removed from the synthesized cDNA by adding 1 μL of RNase H and incubating at 37 °C for 20 min. PCR was performed using 2 μL of the cDNA template. Amplification was conducted using Taq DNA polymerase (New England Biolabs) with 1 × Standard Taq Reaction Buffer, 250 μM dNTPs, 0.5 μM of gene-specific primers (Table S1) and 5% v/v DMSO. The PCRs were initiated by denaturing at 95 °C for 2 min followed by 22 (txtA, txtB, txtC, txtH), 25 (gyrA) or 27 (mlp<sub>in</sub>, mlp<sub>out</sub>) cycles of 95 °C for 15 s, 60 °C for 30 s and 68 °C for 15 s. After the amplification, 10 μL of each PCR product was analysed on a 1% agarose gel by electrophoresis.

**Bioinformatics analysis**

Identification of the adenylation domain within the TxtA and TxtB amino acid sequences was performed using the Pfam database (http://pfam.xfam.org/) (Finn et al., 2016). TxtH homologues were identified using the NCBI Protein Basic Local Alignment Search Tool (BLASTP) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Amino acid sequence alignment of TxtH and other MLPs was performed using ClustalW within the Geneious v. 6.1.2 software (Biomatters Inc., Newark, NJ, USA). The accession numbers for the protein sequences used in the alignment are listed in Table S1. The MLP phylogenetic tree was constructed by maximum likelihood with the MEGA 7 software (Kumar et al., 2016) using the Whelan and Goldman plus gamma (WAG + G) substitution model (Whelan and Goldman, 2001). Bootstrap analyses were performed with 1000 replicates.

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**REFERENCES**

Baltz, R.H. (2011) Function of MbtH homologs in nonribosomal peptide biosynthesis and applications in secondary metabolite discovery. J. Ind. Microbiol. Biotechnol. 38, 1747–1760.

Barry, S.M., Kers, J.A., Johnson, E.G., Song, L.J., Aston, P.R., Patel, B., Krasnoff, S.B., Crane, B.R., Gibson, D.M., Loria, R. and Challis, G.L. (2012) Cytochrome P450-catalyzed L-tryptophan nitration in thaxtomin phytotoxin biosynthesis. Nat. Chem. Biol. 8, 814–816.
Bignell, D.R.D., Huguet-Tapia, J.C., Joshi, M.V., Pettis, G.S. and Loria, R. (2010a) What does it take to be a plant pathogen: genomic insights from Streptomyces species. Antonie van Leeuwenhoek, 98, 179–194.

Bignell, D.R.D., Seipe, R.F., Huguet-Tapia, J.C., Chambers, A.H., Parry, R.J. and Loria, R. (2010b) Streptomyces scabies 87–22 contains a coronafacic acid-like biosynthetic cluster that contributes to plant-microbe interaction. Mol. Plant-Microbe Interact. 23, 161–175.

Bignell, D.R.D., Fyans, J.K. and Cheng, Z. (2014) Phytotoxins produced by plant pathogenic Streptomyces species. J. Appl. Microbiol. 116, 223–235.

Bischoff, V., Cookson, S.J., Wu, S. and Scheibe, W.R. (2009) Thaxtomin A affects CESA-complex density, expression of wall cell genes, wall cell composition, and causes ectopic lignification in Arabidopsis thaliana seedlings. J. Exp. Bot. 60, 955–965.

Boll, B., Taubitz, T. and Heide, L. (2011) The role of MbtH-like proteins in the adenylation of tyrosine during aminocoumarin and vancomycin biosynthesis. J. Biol. Chem. 286, 36281–36290.

Buchko, G.W., Kim, C.Y., Terwilliger, T.C. and Myler, P.J. (2016) Isolation and characterization of phytotoxins associated with Streptomyces scabies. Physiol. Mol. Plant Pathol. 44, 7100.

Dees, M.W. and Wanner, L.A. (2012) In search of better management of plant pathogens. Nat. Rev. Microbiol. 10, 1379–1393.

Drees, M.W. and Wanner, L.A. (2012) In search of better management of plant pathogens. Nat. Rev. Microbiol. 10, 1379–1393.

Esquilín-Lebrón, K.J., Boynton, T.O., Shimkets, L.J. and Thomas, M.G. (2018) An orphan MbtH-like protein interacts with multiple nonribosomal peptide synthetase modules. J. Mol. Biol. 342, 1736–1751.

Felnagle, E.A., Barkei, J.J., Park, H., Podevels, A.M., McMahon, M.D., Johnson, E.G., Krasnoff, S.B., Chung, W.C., Tao, T., Parry, R.J., Loria, R. and Gibson, D.M. (2009) 4-Nitrotryptophan is a substrate for the non-ribosomal peptide synthetase TxtB in the thaxtomin A biosynthetic pathway. J. Biol. Chem. 284, 18019–18029.

Franza, T., Mahe, B. and Expert, D. (1989) Correlation of phytotoxin production with pathogenicity of Streptomyces scabies isolates from scab-causing Streptomyces species. Physiol. Mol. Pathol. 75, 849–850.

Fyans, J.K., Aulton-Warish, M.S., Li, Y.T. and Bignell, D.R.D. (2015) Characterization of the coronatine-like phytotoxins produced by the common scab pathogen Streptomyces scabies. Mol. Plant-Microbe Interact. 28, 443–454.

Fyans, J.K., Bown, L. and Bignell, D.R.D. (2016) Isolation and characterization of plant-pathogenic Streptomyces species associated with common scab-infected potato tubers in Newfoundland. Phytopathology, 106, 123–131.

Garrity, G.M., Lilburn, T.G., Cole, J.R., Harrison, S.H., Euzéby, J. and Tindall, B.J. (2007) Part 10: The Bacteria. Phylum ‘Actinobacteria’: Class Actinobacteria, 399–541. In Taxonomic Outline of the Bacteria and Archaea, Release 7.7. Michigan State University Board of Trustees, East Lansing, MI.

Gust, B., Challis, G.L., Fowler, K., Kieser, T. and Chater, K.F. (2003a) PCR-targeted Streptomyces gene replacement identifies a protein domain needed for biosynthesis of the sesquisparte soil odor geosmin. Proc. Natl. Acad. Sci. USA. 100, 1541–1546.

Gust, B., O’Rourke, S., Bird, N., Kieser, T. and Chater, K.F. (2003b) Recombining in Streptomyces coelicolor. Norwich, UK: The John Innes Foundation.

Healy, F.G., Wach, M., Krasnoff, S.B., Gibson, D.M. and Loria, R. (2000) The txtAB genes of the plant pathogen Streptomyces acidiscabies encode a peptide synthetase required for phytotoxin thaxtomin A production and pathogenicity. Mol. Microbiol. 38, 794–804.

Healy, F.G., Krasnoff, S.B., Wach, M., Gibson, D.M. and Loria, R. (2002) Involvement of a cytochrome P450 monoxygenase in thaxtomin a biosynthesis by Streptomyces acidiscabies. J. Bacteriol. 184, 2019–2029.

Herbst, D.A., Boll, B., Zocher, G., Stehle, T. and Heide, L. (2013) Structural basis of the interaction of MbtH-like proteins, putative regulators of nonribosomal peptide biosynthesis, with adenylylating enzymes. J. Biol. Chem. 288, 1991–2003.

Huguet-Tapia, J.C., Lefebure, T., Badger, J.H., Guan, D., Pettis, G.S., Stanhope, M.J. and Loria, R. (2016) Genome content and phylogenomics reveal both ancestral and lateral evolutionary pathways in plantpathogenic Streptomyces species. Appl. Environ. Microbiol. 82, 2146–2155.

Ikeda, H., Kotaki, H. and Obara, S. (1987) Genetic studies of avermectin biosynthesis in Streptomyces avermitilis. J. Bacteriol. 169, 5615–5621.

Imker, H.J., Krahn, D., Clerc, J., Kaiser, M. and Walsh, C.T. (2010) N-acylation during glidobactin biosynthesis by the tridomain nonribosomal peptide synthetase module GlbF. Chem. Biol. 17, 1077–1083.

Johnson, E.G., Joshi, M.V., Gibson, D.M. and Loria, R. (2007) Cello-oligosaccharides released from host plants induce pathogenicity in scab-causing Streptomyces species. Physiol. Mol. Plant Pathol. 71, 18–25.

Johnson, E.G., Krasnoff, S.B., Bignell, D.R.D., Chung, W.C., Tao, T., Parry, R.J., Loria, R. and Gibson, D.M. (2009) 4-Nitrotryptophan is a substrate for the non-ribosomal peptide synthetase TxtB in the thaxtomin A biosynthetic pathway. Mol. Microbiol. 73, 409–418.

Johnson, R.R., Lawrence, C.H., Clark, M.C. and Calhoun, L.A. (1991) Correlation of phytotoxin production with pathogenicity of Streptomyces scabies isolates from scab-infected potato-tubers. Am. Potato J. 68, 675–680.

Kodani, S., Bicz, J., Song, L.J., Deeth, R.J., Ohnishi-Kameyama, M., Yoshida, M., Ochi, K. and Challis, G.L. (2013) Structure and biosynthesis of scabichelin, a novel tris-hydroxamate siderophore produced by the plant pathogen Streptomyces scabies 87.12. Org. Biomol. Chem. 11, 4686–4694.

Kumar, S., Stecher, G. and Tamura, K. (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.

Lautru, S., Oves-Costales, D., Perndon, J.L. and Challis, G.L. (2007) MbtH-like protein-mediated cross-talk between non-ribosomal peptide antibiotic and siderophore biosynthetic pathways in Streptomyces coelicolor M145. Microbiology, 153, 1405–1412.
Lee, K.S., Lee, B.M., Ryu, J.H., Kim, D.H., Kim, Y.H. and Lim, S.K. (2016) Increased vancomycin production by overexpression of MbtH-like protein in *Amycolatopsis orientalis* KFC10990P. *Lett. Appl. Microbiol.* 63, 222–228.

Loria, R., Bukhalid, R.A., Creath, R.A., Leiner, R.H., Olivier, M. and Steffens, J.C. (1995) Differential production of thaxtomin by pathogenic *Streptomyces* species in vitro. *Phytopathology*, 85, 537–541.

Loria, R., Kers, J. and Joshi, M. (2006) Evolution of plant pathogenicity in *Streptomyces*. *Annu. Rev. Phytopathol.* 44, 469–487.

Loria, R., Bignell, D.R., Moll, S., Huguet-Tapia, J.C., Joshi, M.V., Johnson, E.G., Seipke, R.F. and Gibson, D.M. (2008) Thaxtinom biosynthesis: the path to plant pathogenicity in the genus *Streptomyces*. *Antonie van Leeuwenhoek*, 93, 4–10.

MacNeil, D.J., Gewain, K.M., Ruby, C.L., Dezeny, G., Gibbons, P.H. and MacNeil, T. (1992) Analysis of *Streptomyces* avermitilis genes required for avermectin biosynthesis utilizing a novel integrative vector. *Gene*, 111, 61–68.

McMahon, M.D., Rush, J.S. and Thomas, M.G. (2012) Analyses of MbtB, MbtE, and MbtF suggest revisions to the mycobactin biosynthesis pathway in *Mycobacterium tuberculosis*. *J. Bacteriol.*, 194, 2809–2818.

Mori, S., Green, K.D., Choi, R., Buchko, G.W., Fried, M.G. and Garneau-Tsodikova, S. (2018) Using MbtH-like proteins to alter the substrate profile of a nonribosomal peptide adenylation enzyme. *ChemBioChem* 19, 1–10.

Sambrook, J. and Russell, D.W. (2001) Molecular cloning: a laboratory manual (3-volume set). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Scheible, W.R., Fry, B., Kochevenko, A., Schindelasch, D., Zimmerli, L., Somerville, S., Loria, R. and Somerville, C.R. (2003) An Arabidopsis mutant resistant to thaxtomin A, a cellulose synthesis inhibitor from *Streptomyces* species. *Plant Cell*, 15, 1781–1794.

Schermer, R.A. and Thomas, M.G. (2017) Characterization of the functional variance in MbtH-like protein interactions with a nonribosomal peptide synthetase. *Biochemistry*, 56, 5380–5390.

Süssmuth, R.D. and Mainz, A. (2017) Nonribosomal peptide synthesis – principles and prospects. *Angew. Chem. Int. Ed.* 56, 3370–3821.

Taguchi, F., Suzuki, T., Inagaki, Y., Toyoda, K., Shiraishi, T. and Ichinose, Y. (2010) The siderophore pyoverdine of *Pseudomonas syringae* pv. *tabaci* 6605 is an intrinsic virulence factor in host tobacco infection. *J. Bacteriol.* 192, 117–126.

Tegg, R.S., Melian, L., Wilson, C.R. and Shabala, S. (2005) Plant cell growth and ion flux responses to the streptomycete phytotoxin thaxtomin A: calcium and hydrogen flux patterns revealed by the non-invasive MIFE technique. *Plant Cell Physiol.* 46, 638–648.

Whelan, S. and Goldman, N. (2001) A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. *Mol. Biol. Evol.* 18, 691–699.

Wolpert, M., Gust, B., Kammerer, B. and Heide, L. (2007) Effects of deletions of mbtH-like genes on chlorobiocin biosynthesis in *Streptomyces coelicolor*. *Microbiology*, 153, 1413–1423.

Yaxley, A.M. (2009) Study of the complete genome sequence of *Streptomyces scabies* (or *scabiei*) 87.22. (Doctoral dissertation, University of Warwick).

Zhang, W.J., Heemstra, J.R., Walsh, C.T. and Imker, H.J. (2010) Activation of the pacidamycin PacL adenylation domain by MbtH-like proteins. *Biochemistry*, 49, 9946–9947.

Zolova, O.E. and Garneau-Tsodikova, S. (2012) Importance of the MbtH-like protein TioT for production and activation of the thioacoraline adenylation domain of TioT. *MedChemComm*. 3, 950–955.

**Supporting Information**

Additional supporting information may be found in the online version of this article at the publisher’s web site.
mutant isolates (lanes 3–4). A negative control reaction was conducted for each primer set using water (lane 2) in place of template DNA, and a positive control was included for the PL71/PL72 primer set using Cosmid 57/Δmlp

scab as template (lane 5). The size (kb) of each product was estimated by comparison with the 1 kb ladder for the PL71/PL72 primer set (lane 1) and with the 100 bp ladder for the PL155/PL156 primer set (lane 1). (C) Agarose gel electrophoresis of the PCR products generated using genomic DNA from S. scabies 87.22 (lane 8, left image; lane 7, right image) and from the Δmlp

lipo/ΔtxtH/Δmlp

scab mutant isolates (lanes 3–6). A negative control reaction was conducted for each primer set using water in place of template DNA (lane 2), and a positive control was included for the PL71/PL72 primer set using cosmid 57/Δmlp

scab as template (lane 7, left image). The size (kb) of each product was estimated by comparison with the 1 kb ladder for the PL71/PL72 primer set (lane 1) and with the 100 bp ladder for the PL155/PL156 primer set (lane 1).

Fig. S5. Complementation of the Streptomyces scabies MLP triple mutant. HPLC chromatograms of culture extracts from wild-type S. scabies 87.22 (i), the triple MLP mutant (Δmlp

lipo/ΔtxtH/Δmlp

scab) (ii), the triple MLP mutant containing plasmid pRLD850-1a (iii), the triple MLP mutant containing the txtH expression plasmid (iv), the triple MLP mutant containing the mlp

lipo expression plasmid (v) and the triple MLP mutant containing the mlp

scab expression plasmid (vi). The peak corresponding to thaxtomin A in each chromatogram is indicated with the red asterisks, and the peaks corresponding to the thaxtomin B and thaxtomin D intermediates are indicated with ▼ and ▽, respectively.

Table S1. Accession numbers of MLP protein sequences used for constructing the amino acid alignment and phylogenetic tree.

Table S2. Pairwise comparison of amino acid identity (lower tier) and similarity (upper tier) for the MLPs included in this study.

Table S3. Oligonucleotide primers used in this study.