Evidence That Thaxtomin C Is a Pathogenicity Determinant of *Streptomyces ipomoeae*, the Causative Agent of Streptomyces Soil Rot Disease of Sweet Potato

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Streptomyces ipomoeae is the causal agent of Streptomyces soil rot of sweet potato, a disease marked by highly necrotic destruction of adventitious roots, including the development of necrotic lesions on the fleshy storage roots. *Streptomyces* potato scab pathogens produce a phytotoxin (thaxtomin A) that appears to facilitate their entrance into host plants. *S. ipomoeae* produces a less-modified thaxtomin derivative (thaxtomin C) whose role in pathogenicity has not been examined. Here, we cloned and sequenced the thaxtomin gene cluster (*txt*) of *S. ipomoeae*, and we then constructed targeted *txt* mutants that no longer produced thaxtomin C. The mutants were unable to penetrate intact adventitious roots but still caused necrosis on storage-root tissue. These results, taken in context with previous histopathological study of *S. ipomoeae* infection, suggest that thaxtomin C plays an essential role in inter- and intracellular penetration of adventitious sweet potato roots by *S. ipomoeae*. Once inside the plant host, the pathogen uses one or more yet-to-be-determined factors to necrotize root tissue, including that of any storage roots it encounters.

The mycelial spore-forming genus *Streptomyces* comprises predominantly saprophytic soil bacteria; nevertheless, a few species have acquired the ability to cause disease of underground plant structures such as roots and tubers. Species such as *Streptomyces scabies*, *Streptomyces acidiscabies*, and *Streptomyces turgidiscabies* are among the handful identified that cause potato scab, a disease that is characterized by raised, pitted, or superficial lesions forming on the tuber surface. Scab-causing *Streptomyces* spp. actually display no host or tissue specificity and, thus, cause scab-like lesions on actively growing tissue from a variety of crop and model plants (Loria et al. 2006).

Typical scab production is associated with the production of phytotoxins known as thaxtomins with the primary constituent being thaxtomin A, a cyclic dipeptide derived from L-phenylalanine and L-tryptophan, which includes a 4-nitroindole com-

ponent as well as N-methyl and hydroxyl group additions (Loria et al. 2008). Purified thaxtomin produced by *S. scabies* induces scab-like lesions on immature potato tubers (Lawrence et al. 1990). Recent evidence suggests thaxtomin A acts primarily to block cellulose synthesis while also inducing a host defense response (Bischoff et al. 2009; Scheible et al. 2003). The resulting depletion of cellulose in plant cell walls appears to facilitate penetration by streptomycete pathogens (Loria et al. 2008).

Genes required for thaxtomin A biosynthesis are present at a single locus (*txt*) on the chromosome of scab-producing species (Loria et al. 2008). Immediate precursors 4-nitrotryptophan and L-phenylalanine are methylated and cyclized via the action of two peptide synthetases encoded by the *txtA* and *txtB* genes, while post-cyclic hydroxylation is performed by the *txtC*-encoded P450 monooxygenase (Bignell et al. 2010; Johnson et al. 2009). Formation of 4-nitrotryptophan from tryptophan involves the product of the *nos/txtD* gene, a nitric oxide synthase, along with possibly a separate P450 monooxygenase product, whose gene (*txtE*) appears to be co-transcribed with *nos* (Bignell et al. 2010). Thaxtomin gene knockout mutants (e.g., *txtA*, *nos*) were found to be avirulent on potato tubers (Healy et al. 2000; Kers et al. 2004). Inducers of thaxtomin gene expression include the plant constituents suberin and cellobiose (Johnson et al. 2007; Lerat et al. 2010), the latter of which binds to the *txtR* gene product, which is a member of the AraC/XylS family of transcriptional regulators, in order to activate *txt* gene transcription (Joshi et al. 2007).

In contrast to scab-producing streptomycetes, the pathogen *Streptomyces ipomoeae* appears to naturally infect only family Convolvulaceae plant spp., including sweet potato (*Ipomoea batatas* [L.] Lam.) (Clark and Watson 1983), though infection of cultured potato minitubers has also been demonstrated (King et al. 1994). Infection of sweet potato leads to Streptomyces soil rot disease, which is distinguished from scab by its highly necrotic destruction of plant roots (Loria et al. 2003). Root development in sweet potato begins when adventitious roots originate from root primordia located on vines as well as the cut end of preexisting roots (Belehu et al. 2004; Togari 1950). Adventitious roots may become lignified or may develop into fleshy storage roots (Togari 1950; Wilson and Lowe 1973). While *S. ipomoeae* does produce thaxtomins on infected sweet potato roots and potato minituber tissue, the major constituent is a less-modified, nonhydroxylated form known as thaxtomin C (Fig. 1) (King et al. 1994). These differences in

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*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary figure and one supplementary table are published online.

host range, disease symptoms, and thaxtomin biosynthesis for *S. ipomoeae*, along with the notable absence of virulence factors known to be conserved in *Streptomyces* scab pathogens (e.g., Nec1, TomA) (Bignell et al. 2010), suggest that aspects of its pathogenicity may be novel.

To begin elucidating the mechanisms used by *S. ipomoeae* to cause disease, we have cloned and sequenced the genes (*txt*) involved in biosynthesis of thaxtomin C. *S. ipomoeae txt* deletion mutants were constructed and found to be avirulent when inoculated onto adventitious roots of sweet potato, yet were still able to elicit a necrotic response on exposed storage-root tissue. We propose a model of infection of sweet potato by *S. ipomoeae*, including a potential role for thaxtomin C in a process that appears to be distinct from other plant pathogenic streptomycetes.

RESULTS

Identification of a thaxtomin C biosynthetic gene cluster in *S. ipomoeae*.

Colony blot hybridization using a radiolabeled probe specific for the *S. turgidiscabies nos* gene and a previously described cosmid library of *S. ipomoeae* 91-03 (Zhang et al. 2003) identified three potential *nos*-containing cosmids. Physical mapping revealed that the inserts for two of the cosmids had undergone extensive deletions while, based on its size, the insert for the remaining cosmid (pSIP19) appeared to be intact (data not shown). Nucleotide sequencing of pSIP19 revealed an insert of 41,441 bp, which was subjected to Frameplot analysis to identify putative *Streptomyces* open reading frames (ORF) and manual annotation of each ORF using BLASTP analysis; the end result of these manipulations was the identification of the entire putative thaxtomin C gene cluster of *S. ipomoeae* 91-03, along with several potential mobile genetic elements flanking the cluster, as well as additional cloned genes of postulated or unknown function (Supplementary Table S1).

Homologs of *txtA*, *txtB*, *txtR*, *nos*, and *txtE* genes all showed strong similarity at the amino acid level to their counterparts in *Streptomyces* scab pathogens; moreover, the gene organization in *S. ipomoeae* was also similar with *txtB* immediately following *txtA*, and *txtR*, *txtE*, and *nos* all oriented in the opposite direction, with *nos* immediately following *txtE* (Fig. 2). Located between the divergently transcribed *txtA* and *txtR* genes was a small ORF (termed *txtH*) (Fig. 2) that was also found in the thaxtomin gene clusters of *Streptomyces* scab pathogens (Bignell et al. 2010) and which appears to encode a member of the MbtH-like protein superfamily. These proteins play an unknown but apparently essential role in the biosynthesis of secondary metabolites in other bacteria (Lautru et al. 2007; Wolpert et al. 2007).

Conspicuously absent in the thaxtomin C cluster present on pSIP19 was a *txtC* homolog, a finding consistent with the non-hydroxylated structure of thaxtomin C (Fig. 1). The *txtC* gene is located downstream of *txtB* in *Streptomyces* scab pathogens (Loria et al. 2008). As only 2.6-kb of *txtB* downstream se-

quence was included in the pSIP19 insert, additional colony blot hybridization using a relevant *txtB* gene probe was used to retrieve a cloned 7.2-kb *SacI* fragment that corresponded to sequence extending from the 3' end of *txtB* to a position nearly 7 kb downstream from this gene on the strain 91-03 chromosome. Nucleotide sequencing and subsequent analysis of ORF indicated that a *txtC* homolog was not encoded in this extended downstream region (data not shown). Moreover, as noted previously (Bignell et al. 2010), a search of the recently completed draft genome sequence of *S. ipomoeae* 91-03 (GenBank accession number AEJC000000000) showed no evidence of a *txtC* gene. Thus, the cloned thaxtomin C gene cluster on pSIP19 appeared to be intact. Southern blotting results of strain 91-03, along with three other randomly selected *S. ipomoeae* strains (78-61, 88-35, and 97-19), using an *S. ipomoeae nos* gene probe, showed evidence of a single conserved thaxtomin C gene cluster in each strain (data not shown).

Construction and assessment of pathogenicity of *S. ipomoeae txt* mutants.

To assess the role of thaxtomin C in pathogenicity, *txt* knockout mutants were constructed (details below) and were tested for their ability to cause disease. One mutant strain (DG1) was derived from a multigene deletion extending from the 5' portion of *txtB* to the 3' end of *txtE* (Fig. 2A), while the other mutant (DG5) contained an in-frame deletion of *txtA* (Fig. 2B). Southern blotting using a radiolabeled probe specific for the 3' portion of *txtB* and *SacI*-digested or *NcoI*-digested genomic DNA for DG1 (Fig. 2A) and *NotI*-*EcoRV*-digested or *XhoI*-*SbfI*-digested DNA for DG5 (Fig. 2B) confirmed the construction of these knockout mutants. Similar to the results for thaxtomin gene-knockout mutants of *Streptomyces* scab pathogens (Healy et al. 2000; Kers et al. 2004), no obvious growth or morphological defects were evident for strains DG1 and DG5 (data not shown).

Pathogenicity of *S. ipomoeae txt* deletion mutants was first assessed by exposing the adventitious roots of single vine cuttings of sweet potato plants to soil infested with either strain DG1 or DG5. Following five weeks of incubation in a greenhouse, roots were examined and were rated for necrosis, using the 0 to 5 scale of Moyer and associates (1984). Roots inoculated in such a manner with either DG1 or DG5 were devoid of necrosis and were indistinguishable from noninoculated control roots or adventitious roots exposed to the avirulent *S. ipomoeae* neotype strain B12321 (Fig. 3; Table 1), which was previously shown to be nonpathogenic on sweet potato (Clark et al. 1998; King et al. 1994). Meanwhile, roots inoculated with strain DG8, which is DG5 complemented with the wild-type *txtA* gene, showed evidence of necrosis that approximated the amount seen with the pathogenic parental strain 91-03 itself (Fig. 3; Table 1).

To assess the ability of *txt* mutants to elicit a necrotic response on host tissue, the surface of storage root slices was inoculated with agar plugs containing spores of strains DG1 or DG5. After incubation at 30°C for several days, the *txt* mutants DG1 and DG5, like the parental strain 91-03, produced black, pitted, necrotic lesions accompanied by hyphal growth in and around the lesions (Fig. 4). Inoculation with either agar plugs alone or plugs containing spores of strain B12321 did not produce any lesions, though hyphal growth of the latter strain on the storage root surface was clearly evident (Fig. 4).

Analysis of in situ *txt* gene transcription and thaxtomin production of *S. ipomoeae txt* mutants.

In contrast to streptomycete scab pathogens, *S. ipomoeae* grown in media containing certain complex carbohydrates does not produce detectable amounts of thaxtomin; instead, thaxto-

Fig. 1. Structure of thaxtomin C produced by *Streptomyces ipomoeae*.

min C has been isolated successfully only in small amounts from infected storage-root tissue (King et al. 1994). Our *txt* mutants were, therefore, next characterized for *txt* gene transcription and thaxtomin C production in situ on storage roots. Using a sensitive reverse transcription-polymerase chain reaction (RT-PCR) approach, total plant and bacterial RNA was iso-

lated from storage root slices that had, before, been inoculated with spores of various *S. ipomoeae* strains and incubated for 2 days at 30°C. The RNA was then used as template in RT-PCR reactions specific for either *txtA* or an unrelated, previously described bacteriocin gene (*ipoA*) of *S. ipomoeae* (Zhang et al. 2003).

Predicted fragments of 393 bp (*txtA*) and 467 bp (*ipoA*) for storage roots inoculated with strain 91-03 (Fig. 5, lanes 8 and 9, respectively) but not for noninoculated storage roots (lanes 2 and 3) confirmed the specificity of the RT-PCR analysis for these *S. ipomoeae* genes. In comparison, a lack of *txtA*-specific product for storage roots inoculated with strain 91-03 but for which total RNA was isolated immediately upon inoculation (lane 23), suggested that *S. ipomoeae txt* gene transcription may be normally induced upon either spore germination, infection of sweet potato, or both.

As expected, no *txtA* transcription was observed for storage roots inoculated with either mutant strain DG1 (Fig. 5, lane 11) or DG5 (Fig. 5, lane 14), though tissue necrosis at the site of inoculation was evident again for both (data not shown). In situ *txtA* transcription was confirmed for the complemented mutant DG8 (Fig. 5, lane 17), while no evidence of *txtA* (or *ipoA*) expression was seen for storage roots inoculated with the neotype strain B12321 (Fig. 5, lanes 5 and 6). Finally, though thaxtomin C production by *S. ipomoeae* was not previously detected in tryptic soy broth (TSB) (King et al. 1994), *txtA* transcription in strain 91-03 TSB cultures was evident by this analysis (Fig. 5, lane 20).

Storage-root tissue infected with the various *S. ipomoeae* strains was next analyzed for the presence of thaxtomin C. In an effort to maximize potential phytotoxin production, slices of storage roots were briefly immersed in TSB cultures of each strain, and following incubation of the slices for typically 8 to 9 days at 30°C, thaxtomin C was extracted, based on the method described previously (King et al. 1994). As shown in Figure 6, strain 91-03 extracts showed a strong absorbance peak by high-performance liquid chromatography (HPLC), which had a retention time identical to that seen for authentic thaxtomin C standard (provided by R. King). The presence of thaxtomin C for strain 91-03 was consistent with previous results for this strain (King et al. 1994). This same peak was absent for the

txtA deletion strain DG5 but was restored in the complemented mutant DG8. As expected, this peak was also missing for the other deletion mutant DG1 as well as strain B12321 (Supplementary Fig. S1), with the latter result also confirming previous results for the neotype strain (King et al. 1994). Taken together, the in situ transcription and thaxtomin C results demonstrate that the *txt* deletion mutants DG1 and DG5 are specifically defective for production of thaxtomin C.

DISCUSSION

In a previous histopathological study of *S. ipomoeae* infection of sweet potato, the ability of the bacterium to penetrate adventitious roots both intra- and intercellularly was demonstrated, while it appeared to be unable to breach the intact periderm of storage roots (Clark and Matthews 1987). The latter observation was consistent with early field observations by Adams (1929), who noted that typical soil rot (pox) lesions on storage roots appear to be established through rootlet (i.e., adventitious root) infection. Here, we found evidence implicating thaxtomin C in root infection but not in the intrinsic process by which *S. ipo-*

Table 1. Adventitious root necrosis ratings^a

Strain ^b	Vine cutting				
	1	2	3	4	Ave. ^c
VGM only	0	0	0	0	0.00
B12321	0	0	0	0	0.00
DG1	0	0	0	0	0.00
DG5	0	0	0	0	0.00
DG8	3	3	2	3	2.75
91-03	3	3	4	2	3.00

^a Necrosis ratings for four independent Jewel cultivar vine cuttings inoculated with the indicated *Streptomyces ipomoeae* strains using a 0 to 5 scale in which 0 = no necrosis and 5 = 100% necrosis.

^b *S. ipomoeae* strains. VGM = vermiculite growth medium

^c Average rating per *S. ipomoeae* strain treatment.

Fig. 3. Sweet potato adventitious root pathogenicity assay. Plants of the susceptible cultivar Jewel were grown for 37 days in soil amended with sterile vermiculite growth medium (VGM) or with VGM cultures of the individual *Streptomyces ipomoeae* strains noted. Four replicate plants were used per strain, and roots were scored for necrosis following growth.

Fig. 4. Sweet potato storage-root slice pathogenicity assay. A peeled storage-root slice of the cultivar Beauregard was inoculated with agar plugs consisting of *Streptomyces ipomoeae* growth agar (SIGA) or SIGA plugs on which the indicated *S. ipomoeae* strains had sporulated. Slices were examined for necrosis and were photographed 5 days after inoculation. At least four independent assays were performed for each strain with similar results occurring in each case.

moeae causes necrosis. The latter result is consistent with previous data in which addition of purified thaxtomin C to potato minitubers resulted in scab-like symptoms (King et al. 1994). Thaxtomin A has been shown to be a potent inhibitor of cellulose biosynthesis (Bischoff et al. 2009; Scheible et al. 2003), which presumably allows streptomycete scab pathogens to penetrate host tissue in regions of active cell growth and tissue expansion. It seems reasonable to postulate that thaxtomin C may play a very similar, if not identical, role for *S. ipomoeae*.

Taking into account these past and present observations, we propose a model for infection of sweet potato by *S. ipomoeae*. Initial infection of adventitious roots would be facilitated by thaxtomin C, which promotes both intra- and interepidermal penetration, potentially by inhibiting cellulose synthesis. Penetration would lead to necrotic destruction of root tissue by one or more additional uncharacterized virulence factors. Extensive hyphal colonization of infected root tissue, which occurs both intra- and intercellularly (Clark and Matthews 1987), would continue. Such colonization and necrosis of adventitious roots may interfere with storage-root initiation (Villordon et al. 2011). Additionally, infection of preexisting storage roots adjoining the site of infection may also occur. In this case, this may result either through continued lateral colonization by bacterial hyphae or as infected sections of adventitious roots come in contact with the developing storage roots or both (A. Villordon, *personal communication*). Necrotic destruction of storage-root tissue would ensue and would potentially involve one or more of the same virulence factors that led to adventitious root necrosis.

Production of thaxtomin A by *Streptomyces* scab pathogens is stimulated in laboratory media containing certain complex carbohydrates (Wach et al. 2007). Recently, cello-oligosaccharides were identified as inducers of thaxtomin A synthesis (Johnson et al. 2007) and one of these, cellobiose, the smallest subunit of cellulose, was shown to be a ligand of the *S. scabies* TxtR activator protein (Joshi et al. 2007). Cello-oligosaccharides have, thus, been proposed to function as signaling molecules that alert scab pathogens to regions of actively expanding plant tissue and stimulate thaxtomin A production. The use of cellulose subunits to signal sites of potential infection is consistent with the broad host range of *Streptomyces* scab pathogens (Loria et al. 2006).

In contrast, thaxtomin C production by *S. ipomoeae* is not detectable in the same plant-based media used for thaxtomin A production (King et al. 1994; *our unpublished results*). These

results raise the possibility that cello-oligosaccharides are not inducers of thaxtomin C synthesis in *S. ipomoeae*. The exact ligand or ligands that interact with *S. ipomoeae* TxtR to induce thaxtomin gene transcription (as potentially evidenced by RT-PCR analysis here) thus remains to be determined. That such a molecule or molecules may somehow uniquely represent family *Convolvulaceae* plant species and, therefore, reflect the narrower host range seen for this pathogen is an intriguing possibility that remains to be explored.

Sequence analysis of pSIP19 here also revealed the presence of numerous putative transposase genes and insertion sequences (IS) flanking the thaxtomin C gene cluster. Interestingly, the thaxtomin A locus of *S. turgidiscabies* strain Car8 is also associated with transposase genes and IS and is part of a large mobilizable pathogenicity island; meanwhile, this same locus in *S. scabies* also appears to be associated with a genomic island (Huguet-Tapia et al. 2011). It is possible, then, that the thax-

Table 2. Primers used in this study

Primer	Sequence
AmR_5	5' ATCAGCGGTGGAGTGAATGTCGT
AmR_SalI3	5' AAAAAGTCGACTCATGAGTCAGCC
ipoA-5'	5' AAAAAGGATCCACAAGGTCGGCTCCC AGTGAG
ipoA-3'	5' AAAAAGATCTCTGTCGCTCAGACGCGC AGG
JK017	5' CGCTGCATCGGCCGCCTTTACTGG
JK018	5' TGCCGGGACTCCTCGTGGTGGTTG
KDC081	5' GCTGTATCGGCCGCCTGTA
KDC082	5' GCAGAGTTCGCCAGGTCGTG
SP6-downstream	5' CGGAGATGTCCACCGCCAC
SP6-downstream-2	5' AGCGGTGCGACTTCGATATGC
SP6 universal	5' ATTTAGGTGACACTATAG
T7-downstream	5' CCGCAGGTGGGTGATCGAAA
T7-downstream-2	5' CCCAGTCCCAGATGACCGCA
T7 universal	5' TAATACGACTCACTATAGGG
txtA1	5' CGGCGTGAGGGTGTCTGCCAG
txtA2	5' GGAGCACGCTCCGAGGCAAC
txtA3EcoRI52	5' AGATGAATTCGGTTCGGAGTGAGCAG
txtA3SacI3	5' AACCGAGCTCACTCTGACATCTCGT
txtA5SacI5	5' AGGAGAGCTCCATTGCGCATTTTCT
txtA5XbaI32	5' GGCTCTAGATCTTCATCATGGAGA
txtA-RT-R	5' CGCAAGAGCGGAGCAAGGGT
txtB1	5' AGGGAGCGGTAGCGGAAAC
txtB2	5' GGCGATCAGGGAAGTGGCTC
txtB3	5' GCCCGAGTAGACGCCGATG
txtB4	5' AGCTCCTCGCCCGACCCGT

Fig. 5. In situ transcriptional analysis of the *txtA* gene in various *Streptomyces ipomoeae* strains inoculated onto sweet potato storage-root slices. Total plant and bacterial RNA was isolated from infected storage roots 2 days after inoculation, and expression of the *txtA* gene or the previously described *ipoA* gene of *S. ipomoeae* (Zhang et al. 2003) was probed by reverse transcription-polymerase chain reaction (RT-PCR). Signs above lanes indicate where *txtA* or *ipoA* expression was probed (+) or not probed (-); similarly, reactions containing reverse transcriptase (+) or not containing the enzyme (-) are indicated. Results were analyzed by gel electrophoresis and ethidium bromide staining of bands. Lanes 1, 2, and 3, RNA was from noninoculated storage roots; lanes 4, 5, and 6, RNA was from storage roots inoculated with strain B12321; lanes 7, 8, and 9, RNA was from storage roots inoculated with strain 91-03; lanes 10, 11, and 12, RNA was from storage roots inoculated with strain DG1; lanes 13, 14, and 15, RNA was from storage roots inoculated with strain DG5; lanes 16, 17, and 18, RNA was from storage roots inoculated with strain DG8; lanes 19, 20, and 21, RNA was from exponentially growing tryptic soy broth cultures of strain 91-03; lanes 22, 23, and 24, RNA was isolated immediately following inoculation of storage roots with strain 91-03. M = 1 kb Plus DNA Ladder (Invitrogen). All RT-PCR analyses were performed at least twice with independent RNA preparations, and similar results were obtained in each case.

tomin gene cluster of *S. ipomoeae* was acquired horizontally, perhaps through transposition. If such transmission included a *txtC* gene homolog, then this gene must have been lost subsequently through some type of recombination event. Whether the thaxtomin gene cluster of *S. ipomoeae*, like its counterparts in *Streptomyces* scab pathogens, is part of a larger genomic island region remains an interesting question to be examined.

It is well-documented that the *S. ipomoeae* neotype strain B12321 is no longer pathogenic on sweet potato (Clark et al. 1998; King et al. 1994), and previous evidence suggested that the strain was no longer able to produce thaxtomin on infected sweet potato or potato minituber tissue (King et al. 1994). Here, we found no evidence of either *txtA* or *ipoA* gene transcription in strain B12321, raising the possibility that their expression has been altered through mutation. In the case of *txtA*, additional Southern blot analysis indicated that the entire thaxtomin C gene cluster has been deleted from this strain (our unpublished results). Given that thaxtomin mutant strains DG1

and DG5 were still necrotic on storage-root tissue while B12321 was not (Fig. 4), these data argue that B12321 may be deleted for additional loci that are important for eliciting a necrotic response on susceptible host tissue. Such additional deletion of DNA may be the basis for previous findings that demonstrated lower DNA relatedness as well as differences in *AseI* digestion and rep-PCR profiles for strain B12321 in relation to other *S. ipomoeae* strains (Clark et al. 1998).

MATERIALS AND METHODS

Bacterial strains, plasmids, and general DNA manipulations.

Streptomyces ipomoeae strains B12321 (ATCC-25462), 91-03, 78-61, 88-35, and 97-19 (81-44) have been described previously (Clark et al. 1998), while *S. turgidiscabies* 94-20 (SS-12) is an isolate from Japan. The construction of the 91-03 derivatives DG1, DG5, and DG8 are described in a subsequent section. The *Escherichia coli* host for cloning was BRL2288 (Pettis et al. 2001), while *E. coli* S17.1 (Simon et al. 1983) was used as the donor for intergeneric conjugation. The apramycin-resistant (*Am^r*) plasmids pOJ260 and pSET152 (Biernan et al. 1992) and the ampicillin-resistant plasmid pUC18 (Yanisch-Perron et al. 1985) have been described previously. Plasmid pSP72 is an ampicillin-resistant *E. coli* cloning vector from Promega Corp. (Madison, WI, U.S.A.).

Isolation of plasmid DNA from *E. coli* was done using a QIAprep Spin miniprep kit (Qiagen Inc., Valencia, CA, U.S.A.), following the manufacturer's protocol. Isolation of *S. ipomoeae* genomic DNA was performed as previously described (Kieser et al. 2000). Restriction and modification enzymes were obtained from New England Biolabs (Ipswich, MA, U.S.A.) and were used according to the supplier's specifications. Primers used in this study are listed in Table 2. PCR analyses performed in order to generate DNA fragments for cloning were carried out with Cloned Pfu DNA polymerase (Stratagene, La Jolla, CA, U.S.A.), while all other PCR analyses were performed with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, U.S.A.). Each 25- μ l PCR reaction mixture consisted of 2.5 μ l of 10 $\times$ buffer, 2.5 μ l of 25 mM MgCl₂, if required, 2.5 μ l of dimethyl sulfoxide, 4 μ l of 1.25 mM dNTPs, 0.5 μ l of each 20 μ M primer, $\leq$ 150 ng of DNA template, distilled H₂O, and 0.3 μ l of 5 U of AmpliTaq polymerase or 0.5 μ l of 2.5 U of Pfu polymerase per microliter. Except where noted otherwise, PCR analyses were subjected to an initial temperature of 96°C for 4 min, followed by 30 cycles consisting of 94°C for 45 s, 61.6°C for 45 s, and 72°C for 1 min, with a final extension at 72°C for 7 min.

Media, culture conditions, and bacteriological methods.

E. coli strains were grown at 37°C in Luria Bertani (LB) broth (Difco, Detroit) or on LB agar, both of which were supplemented, as appropriate, with Am to 100 μ g ml⁻¹ or ampicillin to 50 μ g ml⁻¹. Mycelia for *S. ipomoeae* strains were preserved at -80°C in 20% glycerol and were revived at 30°C in TSB (Difco) that was supplemented, as appropriate, with Am at 5 μ g ml⁻¹ and nalidixic acid at 25 μ g ml⁻¹. Except when noted otherwise, *S. ipomoeae* spores were prepared on *S. ipomoeae* growth agar (SIGA) (Clark et al. 1998). Patching of transconjugant colonies was performed as previously described (Pettis et al. 2001). Inocula for adventitious root pathogenicity assays were produced on vermiculite growth medium (VGM) as described (Clark et al. 1998). Intergeneric conjugation from *E. coli* to *S. ipomoeae* was performed as described (Guan and Pettis 2009), using International Streptomyces Project Medium 4 (ISP4; Difco) as the sole medium for preparation of *S. ipomoeae* spores and for conjugation.

Fig. 6. High-performance liquid chromatography (HPLC) of extracts of sweet potato storage-root slices infected with various *Streptomyces ipomoeae* strains. Peeled storage-root slices of the cultivar Beauregard were exposed to individual *S. ipomoeae* strain cultures and, following incubation for, typically, 8 to 9 days, thaxtomin C was extracted and analyzed by HPLC, using an Econosil C18 10- μ m column (4.6 $\times$ 250 mm). An isocratic eluant of 75% H₂O and 25% MeCN was employed, and a flow rate of 1 ml min⁻¹. Detection was at 380 nm. A chromatogram of authentic thaxtomin C is also presented. Each extract was analyzed twice by HPLC, and multiple extracts were prepared and analyzed for strains DG5 and DG8. Similar results were obtained in every case for a given strain.

Colony blot and Southern blot hybridizations and nucleotide sequencing.

Colony blotting was performed as previously described (Zhang et al. 2003) with the following modifications. Colonies were transferred to Hybond XL (GE Healthcare, Piscataway, NJ, U.S.A.) membranes and, following the amplification step, lysis, denaturation, and neutralization steps were performed according to the manufacturer. Nucleic acids were then cross-linked to the membranes by using UV light. Hybridization conditions were as described previously (Brasch et al. 1993). Radiolabeled probes were generated using an Invitrogen random priming kit (Carlsbad, CA, U.S.A.) and a 436-bp portion of the *S. turgidiscabies* *nos* gene as template. The latter fragment was generated by PCR using primers KDC081 and KDC082 along with *S. turgidiscabies* 94-20 genomic DNA. Finished blots were exposed to Hyperfilm (GE Healthcare) at -80°C for visualization of spots.

Cosmid sequencing was performed by SeqWright, Inc. (Houston, TX, U.S.A.). Briefly, cosmid pSIP19 was fragmented into 1- to 3-kb fragments, which were subcloned into pUC18. A total of 384 randomly selected clones were subjected to dye-terminator cycle sequencing using M13 universal forward and reverse primers, and reactions were analyzed on an ABI model 3730xl DNA sequencer. The 768 sequencing reads generated were then assembled into a single contig without any gaps. Following manual analysis of the sequencing reads in order to resolve a few uncalled base positions, a final sequence of 49,332 bp was generated, which included an insert of 41,441 bp. The insert region was then analyzed for ORF using the FRAME algorithm (Bibb et al. 1984) as provided by the Frameplot 2.3.2 website. Database similarity searches using the BLASTP algorithm were enabled by the National Center for Biotechnology Information website.

For cloning of the 7.2-kb *SacI* fragment that extends downstream of *txtB*, strain 91-03 *SacI*-digested genomic DNA in the range of 6 to 8 kb in size was ligated into pSP72, and this mixture was used to transform *E. coli* DH10B by electroporation. Transformants were screened by colony blot hybridization as described above, except that the probe was generated by random priming as described previously (Grau et al. 2008), using a 697-bp *txtB* fragment as template. This fragment was synthesized by PCR, using primers txtB3 and txtB4 along with pSIP19 DNA. One positive clone (pSIP43) was found by restriction mapping to contain an insert of approximately 7.2 kb, which was then sequenced by the method described previously (Wang and Pettis 2010), using universal primers SP6 and T7 as well as primers SP6-downstream, T7-downstream, SP6-downstream-2, and T7-downstream-2. The finished sequence was subjected to BLASTN analysis.

Southern blotting was performed as described previously (Brasch et al. 1993; Grau et al. 2008). Genomic DNA for strains 91-03, 78-61, 88-35, and 97-19 was digested individually with *EcoRI*, *PstI*, or *SacI* and, following gel electrophoresis and blotting, fragments were probed with a *S. ipomoeae* *nos*-specific radiolabeled probe that was produced by random priming (Brasch et al. 1993; Grau et al. 2008) using a 0.7-kb fragment as template. The latter fragment had been generated by PCR using primers JK017 and JK018 and one of the *nos*-containing cosmids isolated in this study. All four strains showed evidence of a single *nos* gene contained within a physical map that included *SacI* fragments of 6.2, 1.5, and 0.4 kb in size.

Construction of *txt* null mutants and complementation analysis.

For construction of strain DG1, the 11.6-kb *BglII* fragment extending from the 5' portion of *txtB* to the 3' end of *nos* was cloned into pOJ260 at the *BamHI* site. This construct, pSIP45, was digested with *BamHI*, and the ends of the resulting 6.9-kb

fragment were ligated together in order to create plasmid pSIP50. Following conjugation of pSIP50 from *E. coli* S17.1 to *S. ipomoeae* 91-03, transconjugants that resulted from single crossover homologous recombination between the incoming plasmid and resident chromosomal sequences were selected by overlaying with Am (25 $\mu\text{g ml}^{-1}$) and nalidixic acid (25 $\mu\text{g ml}^{-1}$); these transconjugants were then verified by PCR using their genomic DNA along with primers AmR_5, and AmR_SalI3. *S. ipomoeae* strains that underwent an additional subsequent homologous recombination event were identified by first patching Am^r transconjugants nonselectively on SIGA for two to three consecutive life cycles. Spores were then harvested, diluted, and plated on ISP4 without selection, to generate 50 to 300 CFU per plate, and colonies were replica-plated to ISP4 with and without Am (25 $\mu\text{g ml}^{-1}$) in order to identify Am^s recombinants. Using this method, 25 Am^s isolates were identified out of more than 4,000 colonies screened. Twelve of the 25 Am^s isolates were tentatively identified by PCR as the putative *txt* multigene deletion mutant (as opposed to wild-type revertants), by using their genomic DNA and primers txtA1 and txtA2. Mutant strains were then verified by Southern blotting, which was performed using the general protocol described earlier, along with *SacI*-digested and *NcoI*-digested DNA of each clone and a radiolabeled probe produced by random priming of a 726-bp portion of *txtB*. This fragment had been generated by PCR using primers txtB1 and txtB2 along with pSIP19 as template. One verified mutant was designated strain DG1.

For DG5 construction, 2.0-kb regions upstream and downstream of *txtA* were amplified by PCR from pSIP19 with the primer pairs txtA5SacI5 and txtA5XbaI32 and txtA3EcoRI52 and txtA3SacI3, respectively. PCR analyses were performed as described earlier, except that the annealing temperature was 57°C and the elongation time was 5 min for each cycle. The upstream fragment was digested with *SacI* and *XbaI* and was cloned into plasmid pSP72 at these same restriction sites to generate pSP72-txtA5-2. The downstream fragment was then digested with *EcoRI* and *SacI* and was cloned into these same sites on pSP72-txtA5-2 to create pSP72-txtA5-2-txtA3-2, which, thus, contained an in-frame deletion of almost the entire *txtA* gene. Following sequencing to ensure its correctness, the 4.0-kb insert of this plasmid was removed by digesting with *PstI* and *EcoRI*, and the fragment was ligated into pOJ260 at these same sites, resulting in pSIP52. Intergeneric conjugation of pSIP52 to *S. ipomoeae* 91-03, selection of Am^r single-crossover transconjugants, and isolation and subsequent screening of Am^s recombinants was performed as described for DG1 construction. After screening more than 11,000 CFU, 20 Am^s isolates were identified, of which only 1 appeared to have the *txtA* deletion, by the PCR method used to identify strain DG1. Southern blotting involving *NotI*-*EcoRV*-digested and *XhoI*-*SbfI*-digested genomic DNA along with the same probe used for DG1 analysis confirmed the correctness of this isolate, which was designated DG5.

To complement DG5, a 6.1-kb *SphI*-*EcoRV* fragment containing the entire *txtA* gene from pSIP45 was cloned into pSP72 at these same sites. The insert was then removed by digesting with *XhoI* and *EcoRV*, and the *XhoI* end was filled using Klenow fragment. The blunt-ended fragment was then cloned into the *EcoRV* site of plasmid pSET152, which carries the *Streptomyces* phage Φ C31 integrase gene and attachment site for site-specific integration into *Streptomyces* genomes (Bierman et al. 1992). The resulting construct pSIP60 was conjugated from *E. coli* S17.1 to *S. ipomoeae* DG5, and Am^r transconjugants were selected by overlaying with Am (25 $\mu\text{g ml}^{-1}$) and nalidixic acid (25 $\mu\text{g ml}^{-1}$). Transconjugants were verified by PCR for the presence of both the Am^r and *txtA* genes in

their genomic DNA by using primers AmR_5 and AmR_SalI3, and txtA1 and txtA2, respectively. One such transconjugant was designated DG8.

Pathogenicity assays.

Pathogenicity to adventitious roots was determined in a greenhouse, as described previously (Clark et al. 1998), using single vine cuttings of the susceptible cultivar Jewel. Necrosis was rated on a 0 to 5 scale as described (Moyer et al. 1984). Four replicate vine cuttings were used for each *S. ipomoeae* strain.

Storage-root slice pathogenicity assays were performed as previously described (Clark et al. 1998), except that inocula consisted of 7-mm (in diameter), 6- to -10-day-old circular plugs from sporulating cultures of each *S. ipomoeae* strain grown on SIGA. The plugs were excised from plates and were placed inverted (i.e., with spores facing down) onto the surface of the storage-root slices. The cultivar Beauregard was used here, and at least four tests were performed for each *S. ipomoeae* strain.

RNA extraction and RT-PCR.

For isolation of total RNA, agar plugs containing spores of individual *S. ipomoeae* strains were inoculated onto storage roots as described in the previous section. After 2 days, root tissue that was directly exposed to the inocula (this tissue showed evidence of necrosis for strains 91-03, DG1, and DG5), as well as some adjoining noninoculated tissue was excised with a scalpel, and total RNA was extracted using an RNeasy plant mini kit (Qiagen) according to the manufacturer's protocol. Strain 91-03 total RNA was also isolated from TSB cultures using TRI REAGENT (Molecular Research Center Inc., Cincinnati, OH, U.S.A.), as described previously (Schully and Pettis 2003). The integrity of the total RNA was determined by agarose gel electrophoresis. DNase (Promega Corp.) treatment was carried out as suggested by the manufacturer.

For two-step cDNA generation, first-strand synthesis was performed as previously described (Grau et al. 2008). Control reactions without reverse transcriptase were performed in parallel. Second-strand amplification PCR analyses were carried out as described previously (Grau et al. 2008), except that for the primers specific for the *ipoA* gene (i.e., *ipoA*-5' and *ipoA*-3') the annealing temperature was 64.6°C and the elongation time was 45 s for each cycle, while for primers specific for the *txtA* gene (i.e., *txtA*2 and *txtA*-RT-R) the annealing temperature was 59.2°C and the elongation time was 45 s for each cycle. Additionally, all PCR reactions were performed for 36 cycles. All RT-PCR analyses were performed at least twice with independent RNA preparations.

Thaxtomin C isolation and analysis.

The procedure is based on that described previously (King et al. 1994) with some modifications. Late-exponential or early-stationary phase *S. ipomoeae* TSB cultures (200 ml) were harvested, the cells were washed, and the resulting pellets were each resuspended in 20 ml of fresh TSB. Sweet potato storage roots of the Beauregard variety were peeled, the ends were cut off, and the roots were then surface-disinfested in 0.525% sodium hypochlorite for 10 min at room temperature. Slices (approximately 0.5 cm in thickness) were briefly immersed in a given *S. ipomoeae* concentrated culture and were then placed in a sterile mason jar containing moist paper towels. Following incubation at 30°C for, typically, 8 to 9 days, storage-root slices (approximately 50 g) were homogenized in 100 ml of acetone in a blender. The resulting slurry was filtered, and the filter cake was washed with acetone (2 × 25 ml). The filtrate was concentrated under reduced pressure,

without heating, until only aqueous orange solution (20 to 25 ml) remained, with an insoluble orange film. This material was transferred to a separatory funnel with more water (25 ml). The mixture was extracted with chloroform (2 × 75 ml) and was stored at room temperature overnight. The chloroform was evaporated, without heating, in the hour immediately prior to HPLC analysis. The orange residue was dissolved in methanol (150 µl), and 25 µl was injected. Chromatography details are given in Figure 6. Each extract was analyzed twice by HPLC, and multiple extracts were prepared and analyzed for strains DG5, DG8, and DG1. Similar results were obtained in every case for a given strain.

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AUTHOR RECOMMENDED INTERNET RESOURCES

Frameplot 2.3.2 website: www.nih.go.jp/~jun/cgi-bin/frameplot.pl
 National Center for Biotechnology Information website:
www.ncbi.nlm.nih.gov