***nec1*, a Gene Conferring a Necrogenic Phenotype, Is Conserved in Plant-Pathogenic *Streptomyces* spp. and Linked to a Transposase Pseudogene**

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We are investigating the genetic basis for, and evolution of, plant pathogenicity in *Streptomyces* spp. The plant-pathogenic species *S. scabies*, *S. acidiscabies*, and *S. turgidiscabies* cause the scab disease of potato and produce the phytotoxins, thaxtomins. Forty-three *Streptomyces* strains representing the three species were evaluated; all thaxtomin A-producing *Streptomyces* strains were pathogenic on potato tubers and all but one hybridized to *nec1* and ORF*tnp*, two genes previously cloned from *S. scabies* ATCC 41973. *nec1* confers a pathogenic phenotype on *S. lividans* TK24, a nonpathogen, and ORF*tnp* is a transposase pseudogene located 5' to *nec1*. The eight nonpathogenic strains tested neither produced thaxtomin A nor hybridized to *nec1*. ORF*tnp* and *nec1* occurred on a single *Pvu*II restriction fragment in all thaxtomin A-producing *Streptomyces* strains. The nucleotide sequences of the homologs of *nec1* and ORF*tnp* from two pathogenic strains each of *S. scabies*, *S. acidiscabies*, and *S. turgidiscabies* were identical; oligonucleotide primers specific to this gene amplified homologs from all strains that hybridized to *nec1*. We propose that *nec1* and ORF*tnp* have been horizontally mobilized from *S. scabies* to *S. acidiscabies* and *S. turgidiscabies*, and that *nec1* is involved in pathogenicity and physically linked to the thaxtomin A biosynthetic genes.

Additional keywords: horizontal gene transfer, pathogenicity determinants.

Streptomyces spp. are gram-positive, filamentous prokaryotes, many of which produce biologically active secondary metabolites. These organisms have DNA with a high proportion of guanine and cytosine and a single linear chromosome (Lin et al. 1993). Almost all *Streptomyces* spp. are soil-inhabiting saprophytes. However, a few species are pathogenic and cause diseases of underground structures of diverse plant species. *Streptomyces scabies* (Lambert and Loria 1989a), a cause of potato scab and similar diseases of tap

root crops, was the first described and is the best studied of the pathogenic species. *Streptomyces acidiscabies* (Lambert and Loria 1989b) produces symptoms like those of *S. scabies* (Manzer et al. 1977) and has a similar host range (Lambert and Loria 1990), although the two species are not closely related, based on DNA-DNA homology (Healy and Lambert 1991) and 16S rRNA sequences (Takeuchi et al. 1996). Another *Streptomyces* sp. that is pathogenic on potato has been described from the island of Hokkaido in Japan (Miyajima et al. 1998). This species, *S. turgidiscabies*, has a wide host range among root crops and produces symptoms like those of *S. scabies* on potato tubers, suggesting a common mechanism of pathogenicity. However, this recently described species is distinctly different from both *S. acidiscabies* and *S. scabies*, based on DNA-DNA homology and ribosomal DNA sequence comparisons, as well as morphological characteristics (Miyajima et al. 1998; Takeuchi et al. 1996).

Thaxtomins, a family of modified dipeptide phytotoxins, are produced by strains of *S. scabies* and *S. acidiscabies* and may play a role in the pathogenicity of these species on potato (King et al. 1991, 1989; Lawrence et al. 1990). Thaxtomins A and B were the first of the nine members of the family to be described. Thaxtomin A is the most abundant of these toxins in potato tuber tissue infected by *S. scabies*. When purified toxin is applied to plant tissue, it causes necrosis and cell hypertrophy, both of which are symptoms of tuber infection by *S. scabies* (Lawrence et al. 1990). Production of thaxtomins is perfectly correlated to pathogenicity in all strains tested to date including *S. scabies*, *S. acidiscabies*, and the sweet potato pathogen *S. ipomoeae* (King et al. 1994), suggesting that thaxtomins may be a common pathogenicity determinant among diverse *Streptomyces* spp. that cause plant disease.

Our goal is to identify plant pathogenicity determinants in *Streptomyces*. We cloned a gene, *nec1*, from *S. scabies* 84.34 (ATCC 41973) that allows the nonpathogen *Streptomyces lividans* to necrotize and colonize potato tuber disks (PTDs) and produce scablike symptoms on immature potato tubers (Bukhalid and Loria 1997). *S. lividans* expressing *nec1* does not produce thaxtomin A, but does produce an unidentified, extracellular, water-soluble compound that causes necrosis on potato tuber disks. No significant homologies to *nec1* were found in the nucleic acid and protein data bases. *nec1* has a low G+C content (54%), suggesting that it has moved horizontally from another genus. In *S. scabies*, *nec1* is adjacent to an open reading frame (ORF), designated ORF*tnp*, that has a

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high level of identity to members of the *Staphylococcus aureus* IS256 family of transposases (Collins and Stephens 1991; Komeda et al. 1996; Rouch et al. 1987). This family of transposases is known to mobilize antibiotic resistance genes among clinically important bacteria.

Southern analysis of 10 strains of *S. scabies* and *S. acidiscabies* and 11 nonpathogenic *Streptomyces* spp. strains suggested that *nec1* is associated with thaxtomin A production and linked to ORF*tnp* in *Streptomyces* strains that are pathogenic on potato (Bukhalid and Loria 1997). These data are consistent with the hypothesis that *nec1* is involved in pathogenicity and was mobilized into *S. scabies* and *S. acidiscabies*, perhaps through a transposition event mediated by ORF*tnp*. The objectives of this research were (i) to test the hypothesis that the presence of *nec1* is predictive of plant pathogenicity and thaxtomin A production in a large geographically diverse group of *Streptomyces* strains, including those of *S. scabies*, *S. acidiscabies*, and *S. turgidiscabies*; (ii) to determine if *nec1* is consistently linked to ORF*tnp* in these strains, as this would suggest simultaneous mobilization of both genes; (iii) to determine if *nec1* is functionally conserved in these strains by evaluating the phenotype of the *nec1* homologs from plant-pathogenic strains of other *Streptomyces* spp., when expressed in the nonpathogen *S. lividans*; and, (iv) to evaluate DNA sequence divergence among *nec1* and ORF*tnp* homologs from the three species, as a measure of the time over which horizontal gene transfer may have occurred.

RESULTS

Evaluation of *Streptomyces* strains for thaxtomin A production and pathogenicity.

Streptomyces strains were evaluated for production of the cyclic dipeptide phytotoxin, thaxtomin A, and their ability to produce disease on immature potato tubers. The morphological and physiological characteristics of phytopathogenic *Streptomyces* strains were consistent with species descriptions of either *S. scabies*, *S. acidiscabies*, or *S. turgidiscabies*; non-pathogenic species were not characterized. The pathogenicity of *Streptomyces* strains was assayed with a previously described potato tuber bioassay (Fig. 1). Thaxtomins were extracted from culture supernatants and fractionated by thin layer chromatography (TLC). The phytotoxicity of thaxtomin A-containing extracts was confirmed with a previously described PTD assay. All strains that were pathogenic also produced thaxtomin A (Table 1), as revealed by a yellow band comigrating with a thaxtomin A standard on TLC plates. These extracts were necrogenic on PTDs. None of the non-pathogenic strains produced thaxtomin A or were necrogenic on PTDs. All strains of *S. turgidiscabies*, a newly described phytopathogenic species from Japan (Miyajima et al. 1998), produced thaxtomin A (Table 1) and were pathogenic on potato tubers (Fig. 1).

Presence of *nec1* and ORF*tnp* homologs in *Streptomyces* strains.

Genomic DNA from all strains was restricted with the endonuclease *Bam*HI and subjected to Southern analysis with internal cloned fragments of *nec1* (305-bp *Pst*I-*Rsa*I fragment) and ORF*tnp* (490-bp *Sst*II-*Cla*I fragment) as probes (Bukhalid and Loria 1997). DNA from all 43 phytopathogenic strains

hybridized to a single fragment when *nec1* was used as a probe (Fig. 2), except for a single *S. scabies* strain from South Africa, CEK-018, that is pathogenic and produces thaxtomin A (Fig. 1). None of the nonpathogenic strains had *nec1* homologs. The size of the fragment hybridizing to *nec1* varied among the 23 *S. scabies* strains. Strains of *S. acidiscabies* and *S. turgidiscabies* did not vary significantly in their hybridization patterns.

The ORF*tnp* probe strongly hybridized to one restriction fragment in all of the *S. scabies*, *S. acidiscabies*, and *S. turgidiscabies* strains tested. In all the *S. scabies* strains, however, ORF*tnp* weakly hybridized to one additional restriction fragment. Unlike *nec1*, ORF*tnp* homologs were present in some of the nonpathogens; *Streptomyces* spp. strains DHL-1, 88.24, and TO-2, which were originally isolated from scab lesions on potato, had a single copy of ORF*tnp* (Table 1; Fig. 2). *S. scabies* strains were also diverse in their hybridization patterns to ORF*tnp*, while *S. acidiscabies* and *S. turgidiscabies* strains did not vary.

Southern analysis was consistent with linkage of *nec1* and ORF*tnp* in the phytopathogenic strains tested. The *Bam*HI digest demonstrated that ORF*tnp* and *nec1* are on the same restriction fragment in the *S. scabies* and the *S. turgidiscabies* strains (Fig. 2). However, there is a *Bam*HI restriction site in the *nec1*/ORF*tnp* intergenic region in *S. acidiscabies*. Southern blot analysis with the restriction endonuclease *Pvu*II, which does not have a site in the intergenic region, confirmed the linkage of ORF*tnp* and *nec1* in *S. acidiscabies* (data not shown).

The sequence of *nec1* in *S. scabies*, *S. acidiscabies*, and *S. turgidiscabies* strains is identical.

To evaluate sequence divergence among *nec1* homologs present in strains of the three pathogenic species, oligonucleotide primers specific to the sequences of *nec1* and ORF*tnp*

Fig. 1. Immature potato tubers infected with (a–c) *Streptomyces turgidiscabies* (Hi-C-20, Ko-g-6, Ko-g-2) and (d) *S. scabies* (SB-742) strains from Japan, (e–h) *S. scabies* (CEK-038A, CEK-038B, CEK-037B, CEK-018) from South Africa, and (i, j) *S. scabies* (84.34, 87.22) from the U.S. Nonpathogen *S. lividans* TK24 (k) included as a control.

were designed. The coding sequences of these two genes were polymerase chain reaction (PCR)-amplified from two *S. scabies* strains, ATCC 33282 and 87.22, two *S. acidiscabies* strains, ATCC 49003 and 84.104, and two *S. turgidiscabies* strains, Hi-C-13 and Car-8. The sequence of the primers for *nec1*, Nf and Nr, and for ORF*tmp*, Tf and Tr, was based on the

sequence from *S. scabies* strain 84.34 (ATCC 41973) (Bukhalid and Loria 1997) (Fig. 3). The predicted 0.72-kb and 0.55-kb products for the coding sequences of *nec1* and ORF*tmp*, respectively, were obtained from all six strains. A second set of primers, If and Ir, was used to amplify the intergenic region; the predicted 0.57-kb products were obtained

Table 1. *Streptomyces* strains and their country of origin, hosts, pathogenicity on immature potato tubers, thaxtomin A production, and hybridization to ORF*tmp* and *nec1*

Strain ^a	Origin ^b	Host	Pathogenicity on PMT ^{c,d}	Thaxtomin A production ^d	Hybridization	
					<i>nec1</i>	ORF <i>tmp</i>
<i>S. scabies</i>						
84-34 (ATCC 41973)T	United States	Potato	+	+	+	+
84-102	United States	Potato	+	+	+	+
87-22	United States	Potato	+	+	+	+
87	United States	Potato	+	+	+	+
89	United States	Potato	+	+	+	+
Pon-P	United States	Potato	+	+	+	+
ATCC 33282	Hungary	Potato	+	+	+	+
SB-742	Japan	Potato	+	+	+	+
S-7	Japan	Potato	+	+	+	+
CEK-010	South Africa	Potato	+	+	+	+
CEK-018	South Africa	Potato	+	+	—	—
CEK-037A	South Africa	Potato	+	+	+	+
CEK-037B	South Africa	Potato	+	+	+	+
CEK-038A	South Africa	Potato	+	+	+	+
CEK-038B	South Africa	Potato	+	+	+	+
DNK-043	South Africa	Potato	+	+	+	+
DNK-034	South Africa	Potato	+	+	+	+
DNK-025A	South Africa	Potato	+	+	+	+
DNK-005	South Africa	Potato	+	+	+	+
DNK-020	South Africa	Potato	+	+	+	+
DNK-G01	South Africa	Peanuts	+	+	+	+
DNK-007	South Africa	Potato	+	+	+	+
VNT-048	South Africa	Potato	+	+	+	+
<i>S. acidiscabies</i>						
ATCC 49003T	United States	Potato	+	+	+	+
84-104	United States	Potato	+	+	+	+
85-06	United States	Potato	+	+	+	+
84-182	United States	Potato	+	+	+	+
84-45	United States	Potato	+	+	+	+
<i>S. turgidiscabies</i>						
S-2	Japan	Potato	+	+	+	+
SS-9	Japan	Sugar beet	+	+	+	+
SS-13	Japan	Sugar beet	+	+	+	+
SS-12	Japan	Sugar beet	+	+	+	+
Hi-C-13	Japan	Carrot	+	+	+	+
Hi-C-16	Japan	Carrot	+	+	+	+
Hi-C-19	Japan	Carrot	+	+	+	+
Hi-C-20	Japan	Carrot	+	+	+	+
Hi-C-36	Japan	Carrot	+	+	+	+
Ko-g-2	Japan	Great burdock	+	+	+	+
Ko-g-6	Japan	Great burdock	+	+	+	+
Car-3	Japan	Carrot	+	+	+	+
Car-8	Japan	Carrot	+	+	+	+
Hi-R-11	Japan	Radish	+	+	+	+
Hi-R-15	Japan	Radish	+	+	+	+
<i>S. griseus</i> ATCC10246	NA	NA	—	—	—	—
<i>S. coelicolor</i> M146	NA	NA	—	—	—	—
<i>Streptomyces</i> spp.						
To-2	Japan	Potato	—	—	—	+
88.24	United States	Potato	—	—	—	—
88.25	United States	Potato	—	—	—	—
83.40	United States	Potato	—	—	—	—
DHL-1	United States	Potato	—	—	—	+
DHL-2	United States	Potato	—	—	—	—

^a T, Type strain of the species.

^b NA, not known or applicable.

^c PMT, Potato minitubers.

^d +, thaxtomin A production, pathogenicity on potato tubers, and DNA hybridization; –, no activity or hybridization.

from both *S. scabies* and *S. turgidiscabies* strains, while a 2.0-kb product was obtained from the *S. acidiscabies* strains.

Alignment of sequences of the PCR-amplified *nec1* and ORFmp from the six strains with the sequence of their homologs from *S. scabies* 84.34 (Bukhalid and Loria 1997) demonstrated that both of these genes had identical sequences in all seven strains. The sequences of the intergenic region of the four *S. scabies* and *S. turgidiscabies* strains were also identical to that of strain 84.34. Interestingly, in the *S. acidiscabies* strains, the intergenic sequence was identical to that of strain 84.34, except that it also contained a 1.5-kb IS element (Fig. 4) recently identified in pathogenic *Streptomyces* spp. (F. Healy, unpublished).

The *nec1* homologs from *S. turgidiscabies* are functionally conserved.

DNA fragments encoding the *nec1* homologs from *S. turgidiscabies* Hi-C-13 and Car-8 and *S. scabies* 87.22 were amplified with restriction site-modified primers. An oligonucleotide primer pair was designed so as to amplify the coding sequence in addition to 168 bp upstream of the translational start codon of *nec1*. *Bam*HI and *Eco*RI sites were introduced 5' of the forward (5'-TCCCCGACGCCGAGCAAACCC-3')

and reverse (Nr) primers (Fig. 3), respectively. The PCR products were cloned in the *Streptomyces/Escherichia coli* shuttle plasmid vector, pUWL-KS (Wehmeier 1995), where the expression of *nec1* is driven by its native promoter. *S. lividans* TK24 protoplasts were transformed with the *nec1* constructs from the *S. scabies* and *S. turgidiscabies* strains. Transformants carrying plasmids encoding the *nec1* homologs were necrogenic on PTDs (Fig. 5); nontransformed *S. lividans* and this strain transformed with the plasmid alone were not necrogenic.

nec1-specific oligonucleotide primers amplify the 0.7-kb gene from all the *nec1*-containing strains.

The sequence identity of *nec1* homologs in seven pathogenic strains of *Streptomyces* spp. suggested that the sequence

Fig. 3. Map of *nec1*, ORFmp, and ORF2, and their respective oligonucleotide primers (half arrows).

Fig. 2. Southern analysis of a *Bam*HI digest of representative *Streptomyces* strains with internal fragments of (A) *nec1* (305 bp) and (B) ORFmp (490 bp). Plant-pathogenic strains included (a–i) *S. scabies* strains 84.34, 87.22, Pon-P, ATCC33282, S-7, SB-742, CEK-038A, DNK-007, VNT-048; (j–n) *S. turgidiscabies* strains S-2, SS-9, Hi-R-11, Ko-g-2, Hi-C-13, and (o–r) *S. acidiscabies* strains ATCC 49003, 84.104, 84.45, 84.182. Nonpathogenic strains included (s) *S. coelicolor* M146; (x) *S. griseus* ATCC10246, and (t–w, y) *Streptomyces* spp. strains DHL-1, DHL-2, 88.24, 88.25, To-2. Pathogenicity (Path) and thaxtomin A (ThaxA) production by strains are indicated. Size markers (kb) are shown to the left.

of this gene is conserved in all strains that hybridize to *nec1*. The oligonucleotide primers Nf and Nr were used to amplify the coding sequence of *nec1* from the *S. scabies*, *S. turgidiscabies*, and *S. acidiscabies* strains listed in Table 1. The predicted 0.7-kb product was amplified from all of the pathogenic *Streptomyces* strains that hybridized to *nec1*, demonstrating that these primers could be useful in the detection of pathogenic *Streptomyces* strains.

DISCUSSION

Our data support the hypothesis that thaxtomin A production is a pathogenicity determinant in plant-pathogenic *Streptomyces* spp. All strains that produced thaxtomin A were pathogenic on potato tubers and all strains that were pathogenic on potato tubers produced thaxtomin A. We included representative strains of three *Streptomyces* spp. known to be pathogenic on plants from three geographic locations. This is the most comprehensive analysis of the relationship between thaxtomin A production and pathogenicity yet reported, and the first report of thaxtomin A production by *S. turgidiscabies*. The data support the hypothesis that thaxtomins are host-nonspecific pathogenicity determinants in plant-pathogenic *Streptomyces* spp. Phytotoxins produced by most plant prokaryotes are host-nonspecific virulence, rather than pathogenicity, determinants (Gross 1991). In contrast, many fungal plant pathogens produce host-selective phytotoxins that are pathogenicity determinants (Walton 1996).

nec1 was structurally conserved among all but one of the pathogenic strains examined, based on Southern analysis and PCR amplification with *nec1*-specific primers. Further, *nec1* was functionally conserved among at least four strains representing *S. scabies* and *S. turgidiscabies*. This is not surprising, as our data demonstrated that the sequence of *nec1* in these four strains and two additional strains of *S. acidiscabies* is identical. The genetic diversity of *S. scabies*, *S. acidiscabies*, and *S. turgidiscabies* is well established. Therefore, *nec1* sequence identity among strains of these three species indicates that horizontal transfer of *nec1* among these strains has occurred relatively recently. Further, the low G+C content of *nec1* indicates that this gene did not evolve in the genus *Streptomyces*, but was horizontally transferred into *Streptomyces* spp. from an organism with a G+C content of approximately 55%.

The function of *nec1* is not presently known. The necrogenic phenotype of *nec1* suggests that this gene plays a role in plant pathogenicity in streptomycetes. A previous study with fewer *Streptomyces* strains had found a perfect correlation between the presence of *nec1* and thaxtomin A production

(Bukhalid and Loria 1997). In this study, we identified one thaxtomin A-producing strain, CEK-018, out of 43 that did not hybridize to *nec1*. We believe that the absence of a *nec1* homolog in CEK-018 indicates that *nec1* is not directly involved in thaxtomin A biosynthesis but is, perhaps, a virulence factor in plant-pathogenic streptomycetes. However, the high correlation between the presence of *nec1* and thaxtomin A production in this diverse group of *Streptomyces* strains does suggest physical linkage between *nec1* and the thaxtomin A biosynthetic genes. Linkage would facilitate the simultaneous transfer of these genes on a mobile genetic element, such as a plasmid or a conjugative transposon.

The physical linkage of *nec1* and ORF*tnp* in a large number of genetically and geographically diverse, plant-pathogenic *Streptomyces* strains suggests that ORF*tnp* may have been involved in the horizontal transfer of *nec1* into *Streptomyces*

Fig. 5. Necrogenic phenotype on potato tuber disks (PTDs) of *Streptomyces lividans* TK24 expressing *nec1* homologs from two strains of *S. turgidiscabies*, (A) Hi-C-13 and (B) Car-8, along with the disks inoculated with the wild-type strains (C) Hi-C-13 and (D) Car-8. E, *S. lividans* TK24 harboring the vector alone is included as a control.

Fig. 4. Arrangement of *nec1*, ORF*tnp*, ORF2, and the IS element in *Streptomyces scabies*, *S. acidiscabies*, and *S. turgidiscabies*. Sequences of *nec1* and ORF*tnp* are identical in strains representing all three species. Two *PvuII* restriction sites shown are conserved in at least four strains of each species, based on Southern analysis (data not presented).

spp. ORF tnp is a transposase pseudogene with a high level of amino acid identity to the putative transposases of the IS1164 from *Rhodococcus rhodochrous* (68.2%) and the IS1407 from *Mycobacterium celatum* (68.4%), as well as other transposases of the *Staphylococcus aureus* IS256 family of transposons. Members of this family of transposons are implicated in horizontal movement of genes conferring a fitness advantage, such as antibiotic resistance genes, among clinically important bacteria (Rouch et al. 1987; Tauch et al. 1995), or are associated with genes involved in the biodegradation of toxic nitriles (Komeda et al. 1996). ORF tnp is an interrupted ORF; a frameshift mutation at nucleotide 245 introduced a stop codon in the N terminus of ORF tnp , reducing it to a 168 amino acid pseudogene. The frameshift-corrected sequence would encode a putative 413 amino acid polypeptide (data not presented) (Bukhalid and Loria 1997). The mutation in the coding sequence of ORF tnp might have played a major role in the stabilization of *nec1* in the *S. scabiei* genome. These data are consistent with the hypothesis that *nec1* was mobilized into *S. scabiei*, through a transposition event mediated by ORF tnp , and subsequently mobilized within a larger DNA fragment, containing ORF tnp , into other *Streptomyces* spp. through a different mechanism. Southern analysis also showed that a *PvuII* site in the sequence approximately 3.0 kb downstream of *nec1* is also highly conserved (Fig. 4), indicating that a much larger DNA fragment may have been mobilized among pathogenic *Streptomyces* spp.

Hybridization patterns of *nec1* and ORF tnp support other data indicating that *S. scabiei* is a much more genetically diverse, and therefore more ancient, species than are *S. acidiscabies* and *S. turgidiscabies*. Our knowledge about the emergence of *S. acidiscabies* and *S. turgidiscabies* as economically important pathogens is also consistent with a relatively recent origin for these species. Strains that were eventually named *S. acidiscabies* were isolated in the 1940s from potato scab lesions on potatoes grown in acidic soils (Manzer et al. 1977). Low soil pH had been used by potato growers to suppress *S. scabiei* (Manzer et al. 1977) and may have facilitated transfer of genetic determinants for pathogenicity from *S. scabiei* to the acid-tolerant species *S. acidiscabies*. *S. turgidiscabies* was recently described from the island of Hokkaido in Japan. *S. scabiei*, which is also well established there, was likely introduced to Hokkaido with potato seed tubers from the northeastern United States, approximately 50 years ago. We expect that transfer of pathogenicity-associated genes from *S. scabiei* to *S. turgidiscabies* occurred during the last 50 years on Hokkaido.

The apparent specificity of *nec1* primers for three pathogenic *Streptomyces* spp. has practical applications. Plant disease diagnosticians now rely on potato tuber pathogenicity tests, a time-consuming and inconvenient assay, for determining the etiology of scablike diseases on tap root and tuber crops (Schaad 1988). If *nec1* primers are specific for pathogenic strains of *S. scabiei*, *S. acidiscabies*, and *S. turgidiscabies*, amplification of *nec1* might substitute for pathogenicity testing. This tool, in combination with relatively simple morphological evaluations, could potentially be very useful in identifying species responsible for scab diseases. In addition, it may be possible to use *nec1* primers for detection of these three species in soil, for studies on the ecology of plant-pathogenic *Streptomyces* spp., and for the development

of disease prediction models based on pathogen population densities.

MATERIALS AND METHODS

Culture media and conditions.

Luria Bertani medium (LB; Sigma, St. Louis, MO) and the International *Streptomyces* Project Medium 2 (ISP2; Difco, Detroit, MI) were used for the routine cultivation of *E. coli* and *Streptomyces* strains, respectively. CRM medium (Pigac and Schrempf 1995) was used to culture *Streptomyces* strains for DNA extractions. Oatmeal broth (OMB) and oatmeal agar (OMA) were used to assess thaxtomin A production and to produce inoculum for pathogenicity tests (Loria et al. 1995). Spore suspensions of all the *Streptomyces* strains were prepared as previously described (Hopwood et al. 1985). Antibiotics were added to media at the following final concentrations: thiostrepton (Th), 25 to 50 mg/liter; ampicillin (Ap), 100 mg/liter.

Streptomyces strains and their characterization.

Strains of *S. scabiei*, *S. acidiscabies*, *S. turgidiscabies*, and a number of nonpathogenic *Streptomyces* spp. were included in this study (Table 1). Strains were originally isolated from South Africa (14), Japan (18), Hungary (1), and the United States (16). Morphological and physiological characterization was based on methods of the International *Streptomyces* Project as described by Shirling and Gottlieb (1966). *S. lividans* TK24 was used for the expression of *nec1* homologs from *Streptomyces* spp.

Plant bioassays.

Immature potato tubers (Loria and Kempter 1986) were used to evaluate pathogenic activity, as described in Loria et al. (1995), with the following modifications. *Streptomyces* strains were grown in OMB amended with trace elements (Hopwood et al. 1985). Mycelium was washed in sterile, distilled water by centrifugation to remove the preformed thaxtomins in the OMB media, and then resuspended before it was used as inoculum. Six immature potato tubers were immersed for 5 min in mycelial suspensions of the test strains, followed by incubation at 100% relative humidity for 3 to 4 days at 22 to 24°C in the dark. Potato tubers were scored for scab lesions. A previously described (Loria et al. 1995) PTD assay was used to screen *S. lividans* transformants and chloroform extracts of culture supernatants for necrotizing ability.

Phytotoxin assay.

Methods for evaluation of thaxtomin A production by *Streptomyces* strains in culture media were similar to those used previously (Loria et al. 1995). Thaxtomins were extracted from the supernatant of the OMB cultures used in the potato tuber bioassay and fractionated by TLC. Necrogenic activity of the chloroform extracts was also tested in the PTD assay. Bands of interest were removed from the TLC plates, eluted in methanol, concentrated, and tested for necrogenic activity on PTD.

Recombinant DNA techniques.

Recombinant DNA techniques were carried out according to the methods of Hopwood et al. (1985) and Rao et al. (1987)

for *Streptomyces* and Sambrook et al. (1989) for *E. coli*, except for the preparation of competent *E. coli* DH5 α MCR cells (Hanahan 1985). For subcloning and preparation of DNA for probes, individual DNA restriction fragments were purified by separation on SeaKem GTG agarose gels (FMC BioProducts, Rockland, ME), followed by elution of DNA with GenElute columns (Supelco, Bellefonte, PA), according to the manufacturers' procedures. Restriction enzymes and DNA modification enzymes were purchased from GIBCO BRL (Grand Island, NY) and New England Biolabs (NEB; Beverly, MA).

Southern blot analysis.

Total genomic DNA was extracted by a miniprep modification of the procedure of Rao et al. (1987). DNA was digested with *Bam*HI, separated by agarose gel electrophoresis, UV-nicked, transferred to Hybond-N⁺ nylon membranes (Amersham, Little Chalfont, England) by the manufacturer's alkaline transfer method, and hybridized to random-primed [α -³²P]dCTP-labeled DNA probes made with the Prime-It II labeling kit from Stratagene (La Jolla, CA). Prehybridization and hybridization were done at 65°C overnight in the following buffer: 2 $\times$ SSPE (1 $\times$ SSPE is 0.18 M NaCl, 10 mM NaPO₄, and 1 M EDTA [pH 7.7]), 5 $\times$ Denhardt's, 1% bovine serum albumin (BSA), and 5% sodium dodecyl sulfate (SDS; Amersham). The most stringent wash was 0.1 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.5% SDS for 15 min at 65°C. Autoradiography was performed with X-OMAT AR X-ray film (Eastman Kodak) at -80°C.

PCR amplification.

Streptomyces cultures were grown in 5 ml of CRM in test tubes for 24 to 48 h at 30°C. DNA from 2 ml of culture was extracted by washing mycelium in 1 ml of sterile, distilled water, followed by boiling mycelium in 200 μ l of water, and DNA in the supernatant was recovered by centrifugation. The primers for *nec1* and ORFmp were Nf (5'-ATGAGCGCGAACGGAAGCCCCGGA-3') and Nr (5'-GCAGGTCGTCACGAA GGATCG-3') and Tf (5'-ATGACCGCATCCGACA GTCTGCCC-3') and Tr (5'-TTGAT GATCCGCGCCCTCGC-3') (Fig. 3), respectively. The primers for the intergenic region were If (5'-GTTGTCTTCGGCGAGGGCGTGCAGG-3') and Ir (5'-AGCGGAAGGATT TGCGACCACAACG-3'). Amplification was carried out with *Taq* DNA polymerase (0.5 U; Perkin Elmer, Foster City, CA) or the high fidelity *pfu* DNA polymerase (2.5 U; Stratagene) under the following conditions: DNA denaturing at 95°C for 1 min, annealing at 60°C for 1 min, and elongation at 72°C for 2 min for 30 cycles. The amplified DNA was then run on 1% agarose gels. The PCR amplification experiments were performed twice with independent cultures for each strain.

Heterologous expression of *nec1*.

PCR-amplified DNA was digested with the *Bam*HI/*Eco*RI restriction enzymes and purified by agarose gel-electrophoresis. The bands were cut out and the DNA recovered with GenElute spin columns (Supelco). DNA fragments were ligated to the *Streptomyces/E. coli* shuttle plasmid vector, pUWL-KS (Wehmeier 1995), and used to transform *E. coli* DH5 α MCR. DNA from each of four recombinant plasmids containing the expected inserts was then used to transform *S.*

lividans TK24 protoplasts. Transformations were carried out in the presence of polyethylene glycol following the procedure of Rao et al. (1987). Four independent transformants were picked for each recombinant plasmid, cultured on oatmeal agar, and tested for necrogenic activity on PTD.

Nucleotide sequencing and analysis.

Cloned or pooled PCR products of *nec1* homologs were sequenced by the dideoxy-chain termination method with an automated sequencer (DNA Services, Biotechnology, Cornell University, Ithaca, NY). The *nec1* homologs from each strain were sequenced at least twice, from PCR products obtained from different DNA preparations, so as to eliminate the possibility of cross contamination of DNA templates. The T3 and T7 primers were used for sequencing both strands of the PCR products cloned in pUWL-KS, whereas the amplification reverse and forward primers were used for sequencing the pooled PCR products. PCR products were purified with the QIAquick columns from Qiagen (Chatsworth, CA). MEGALIGN and SEQMAN (DNASTAR, Madison, WI) were used for the multiple sequence alignments with the Clustal method and for sequence assembly and contig management, respectively.

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