Using the *TxtAB* Operon to Quantify Pathogenic *Streptomyces* in Potato Tubers and Soil

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ABSTRACT

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The phytotoxin thaxtomin, produced by plant pathogenic *Streptomyces* species, is the only known pathogenicity determinant for common scab diseases of potato and other root and tuber crops. Genes encoding thaxtomin synthetase (*txtAB*) are found on a pathogenicity island characteristic of genetically diverse plant pathogenic *Streptomyces* species. In this study, an SYBR Green quantitative real-time polymerase chain reaction (PCR) assay using primers designed to anneal to the *txtAB* operon of *Streptomyces* was developed to quantify pathogenic bacterial populations in potatoes and soil. The real-time PCR assay was specific for pathogenic *Streptomyces* strains. The detection limit of the assay was 10 fg of the

target DNA, or one genome equivalent. Cycle threshold (Ct) values were linearly correlated with the concentration of the target DNA (correlation coefficient $R^2 = 0.99$) and were not affected by the presence of plant DNA extracts, indicating the usefulness of the assay for quantitative analyses of the pathogenic bacteria in plant tissues. The amount of pathogenic *Streptomyces* DNA in total DNA extracts from 1 g asymptomatic and symptomatic tubers was quantified using the assay and ranged from 10^1 to 10^6 pg. A standard curve was established to quantify pathogenic *Streptomyces* in soil. Using the standard curve, numbers of pathogenic *Streptomyces* colony forming units were extrapolated to range from 10^3 to 10^6 per gram of soil from potato fields where common scab was found. This real-time PCR assay using primers designed from the *txtAB* operon allows rapid, accurate, and cost effective quantification of pathogenic *Streptomyces* strains in potato tubers and in soil.

Streptomyces is the most widely studied and well known genus of *Actinobacteria*, a group of gram-positive filamentous bacteria that are predominantly soil saprophytes. Of the more than 400 *Streptomyces* species described, only a few are known to be pathogenic and these species infect the underground portions of a range of host plants including potato, carrot, radish, beet, and other tap root crops (11,26). Scab lesions on the surfaces of root and tuber vegetables caused by pathogenic *Streptomyces* species reduce vegetable quality and marketability, resulting in significant economic loss (14). Among the plant diseases caused by *Streptomyces*, potato common scab is the most economically important (22,26).

Streptomyces is both seed- and soilborne. Streptomycetes can survive for many years in soil in the absence of potatoes due to their ability to live saprophytically and infect other plants (21). The bacteria also can be spread on infected seed tubers (32,37) and tubers harboring low levels of pathogenic *Streptomyces*, and without visible symptoms, can transmit common scab to their progeny (32). There have been reports that the inoculum density of pathogenic *Streptomyces* on tuber surfaces and in soil was positively related to scab disease severity and incidence (17,18, 33). Plate counting methods have been used to estimate quantities of pathogenic *Streptomyces* in soil and on tubers (6,17,18,31,32), but they lack specificity and are too tedious to accommodate large numbers of samples. To relate pathogen quantities to disease outcome in field situations, a fast, cheap, specific and sensitive

method is needed that can accurately quantify the pathogenic bacteria on tubers and in soil and can accommodate large sample numbers. Ideally, the method would identify all pathogenic strains of the several common scab-causing *Streptomyces* species.

Conventional polymerase chain reaction (PCR) assays using primers targeted to variable regions in 16S rRNA genes have been developed for species-specific detection of pathogenic *Streptomyces* on scabby tubers (24,34). However, species identification may give no indication of its pathogenicity, since both pathogenic and nonpathogenic strains have been classified within the same species (34). Molecular analyses, including 16S rDNA sequence comparisons (20,30), fatty acid methyl ester profiles (28), restriction fragment length polymorphisms (8), and DNA-DNA hybridization (12), have been carried out to identify markers specific for pathogenicity, but results showed genetic diversity among pathogenic strains.

Recently, pathogenicity genes, including genes for the biosynthesis of thaxtomin (*txtAB*, *txtC*), have been found to be clustered in a large chromosomal region that functions as a pathogenicity island (PAI) in *Streptomyces* (19,27). Thaxtomin plays a critical role in the pathogenicity of scab-producing strains and is a pathogenicity determinant on potato tubers (10,13,23,25). A 100% correlation between pathogenicity and the production of thaxtomin on oatmeal agar or the presence of thaxtomin biosynthetic *txtAB* genes has been reported (3,27,32–34). A second gene, *necl*, is conserved among most scab-causing *Streptomyces* species and is associated with a necrogenic phenotype on potato tubers (3–5,16). While a good correlation between the presence of the *necl* gene and pathogenicity has been reported, some nonpathogenic *necl*-positive strains (31) and numerous pathogenic *necl*-negative strains (2,3,20,33–35) have been described. Therefore, the *txtAB* operon was chosen as a marker for pathogenic *Streptomyces*.

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Since real-time PCR can be applied to quantify pathogenic *Streptomyces* and the *txtAB* operon is the best marker for pathogenic strains, the aims of the present study were to (i) develop a quantitative real-time PCR assay specific for pathogenic *Streptomyces* strains using the *txtAB* operon as the target gene, (ii) use the assay to quantify pathogenic *Streptomyces* strains in potato tubers, and (iii) use the assay to quantify pathogenic *Streptomyces* strains in naturally infested field soils.

MATERIALS AND METHODS

Streptomyces strains. The *Streptomyces* strains analyzed in this study are listed in Table 1. Type strains of ATCC 49173 (*S. scabies*, syn. *S. scabiei*), ATCC 33282 (*S. europaeiscabiei*), ATCC 700248 (*S. turgidiscabiei*), ATCC 700526 (*S. aureofaciens*), and ATCC 49003 (*S. acidiscabiei*) were obtained from American Type Culture Collection (ATCC, Manassas, VA). The other *Streptomyces* strains and species were isolated from scab lesions on potato tubers according to the method of Wanner (33), and tested for pathogenicity on radishes and/or potatoes (33,35; L. A. Wanner, unpublished data). Species determinations were made by cloning and sequencing the 16S ribosomal RNA gene and comparing the *rrn* sequences obtained to sequences deposited in GenBank (34; D. K. Lakshman and L. A. Wanner, unpublished data).

Potato tuber samples. Twenty-four symptomatic (common scab or powdery scab lesions) and asymptomatic potato tuber samples were used in this study (Table 2). Tubers from Pennsylvania were collected from research plots at the Russell Larson Agriculture Research Center at Rock Springs, used in disease resistance screening. The tubers from other states were sent by potato growers or researchers for identification of scab diseases. Pathogen-free mini-tubers cv. Russet Burbank (Summit Plant Laboratories, Inc., Fort Collins, CO) were used as negative controls. Tubers were washed gently under running tap water to remove soil particles from the surface. For symptomatic tubers, 1 g of scab lesion tissue was used for DNA extraction. For asymptomatic tubers, 1 g of peel tissue was used for DNA extraction.

Soil samples. Thirty-eight soil samples were collected from potato fields in six states where common scab is found (Table 3). In Pennsylvania, eight soil samples were randomly collected from one field. In other states, one sample was collected for each field. Two soil samples were collected from a field with powdery scab but no common scab history in Alamosa, Colorado, and used as negative controls (Table 3). Potato varieties, susceptibility, and incidence of common scab disease were recorded (Table 3). The disease incidence was calculated as a percentage of common scab tubers out of the total number of tubers sampled from the field. Soil samples (500 g) were collected at harvest. Samples were well-mixed before triplicate 1-g aliquots were taken for DNA extraction. *Streptomyces*-spiked soils were prepared and used to determine PCR sensitivity and to generate a standard curve for PCR quantification. Bacterial spores were harvested using a sterile glass rod to gently scrape 2- to 3-week old *Streptomyces* cultures on yeast-malt extract (YME) agar plates (29) flooded with 5 ml of sterile water. Spore numbers were determined by counting on a hemacytometer slide, and the number of colony forming units (CFU) verified by agar plate counts. Playground sand (purchased from Home Depot) was mixed 1:1 (vol/vol) with ProMix Plus. The soil mixture was sterilized by autoclaving 6 liter volumes three times, with 2 to 3 days between autoclavings. Autoclaved sand/soil was spiked with 10^1 , 10^2 , 10^3 , 10^4 , 10^5 , 10^6 , 10^7 , 10^8 , and 10^9 CFU of pathogenic *Streptomyces* strain ME01-11h per gram by adding a dilution series of bacterial spores in a volume of 100 μ l to 1 g of sterile soil. Triplicate samples were prepared for each spore dilution and used for DNA extractions.

DNA extraction. *Streptomyces* strains were grown at 28°C for 3 days in YME, and cells were pelleted to obtain approximately 100-mg aliquots that were stored at -20°C until use. DNA was isolated from frozen cells using a Bio101 FAST DNA kit (Q-Biogene, now MP Biomedicals, Solon, OH). DNA was extracted from potato tuber tissue using the CTAB (hexadecylmethylammonium bromide) method (9). Soil DNA was extracted using a Bio101 Soil DNA kit (Q-Biogene, now MP Biomedicals) and a FAST-prep machine according to manufacturer protocols, with

TABLE 1. Description of *Streptomyces* strains used in this study

Strain ^a	Closest genus and species ^b (GenBank accession number)	Origin	Pathogenicity ^c	<i>txtAB</i> ^d	<i>Nec1</i> ^e
ME01-11h	<i>S. scabies</i>	Typical common scab lesion on potato tuber, Presque Isle, ME	+	+	+
ME02-6991.2B	<i>S. glauciniger</i>	lesion atypical for common scab, potato tuber; Presque Isle, ME	-	-	-
ME02-6985.2A	<i>Ochrobactrum anthropii</i>	Netted surface potato tuber; Presque Isle, ME	-	-	-
ME03-5709D	<i>S. coelestis</i> (EU080957)	Typical common scab lesion on potato tuber, Presque Isle, ME	-	-	-
ND04-1A	<i>S. clavifer</i> ; <i>S. griseus</i>	Typical common scab lesion on potato tuber, Williston, ND	-	-	-
WI03-5B	<i>S. griseus</i> ; <i>S. setoni</i> (EU080963)	Typical common scab lesion on potato tuber, Hancock, WI	-	-	-
ID01-15B	<i>S. badius</i> ; <i>S. sindensis</i> (EU080940)	Typical common scab lesion on potato tuber, American Falls, ID	-	-	-
NY02-3A	<i>S. stelliscabiei</i> (DQ861640)	Typical common scab lesion on potato tuber, Ithaca, NY	+	+	+
ID01-12c	<i>Streptomyces</i> sp. IdX (DQ341443)	Typical common scab lesion on potato tuber, location not noted, ID	+	+	-
OH01-5D	<i>S. scabies</i> (DQ861637)	Typical common scab lesion on potato tuber, Urbana, OH	+	+	+
ATCC 49173	<i>S. scabies</i> (type strain) (gi971124)	Potato tuber New York	+	+	+
ID01-16c	<i>S. europaeiscabiei</i> (DQ861638)	Typical common scab lesion on potato tuber; American Falls, ID	+	+	+
ATCC 33282	<i>S. scabiei</i> (<i>S. europaeiscabiei</i> REF) (gi5263068)	Soil	+	+	+
ATCC 700248	<i>S. turgidiscabiei</i> (type strain) (AB026221)	Potato tuber, Hokkaido, Japan, 1993	+	+	+
ATCC 700526	<i>S. aureofaciens</i> (type strain) (gi3550677)	Scab lesion on potato tuber, Loimaa, Finland, 1995	-	-	-
ATCC 49003	<i>S. acidiscabiei</i> (type strain) (AB026220)	Potato tuber, ME	+	+	+

^a Strains with names beginning with the two-letter abbreviation for U.S. state from which the isolate was made are from the Wanner collection of *Streptomyces* isolates at USDA-ARS in Beltsville, MD; the digits following letters indicate the year of isolation and a unique identifier. ATCC strains came from the American Type Culture Collection in Manassas, VA.

^b Based on sequence of the 16S rRNA gene. GenBank accession numbers for 16S rRNA gene sequence are given in parentheses. If no GenBank accession number is given, the species assignment is based on unpublished 16S rDNA sequence data (L. A. Wanner, unpublished data).

^c Pathogenicity tested on radishes and/or potatoes (33,35).

^d Presence (+) or absence (-) of the thaxtomin biosynthesis operon *txtAB* as determined by real-time polymerase chain reaction (PCR) using primer pair StrepF/R.

^e Presence (+) or absence (-) of the *nec1* gene as determined by real-time PCR using primer pair NecTqF1/R1.

the minor modification of an additional ethanol:salt wash before eluting the DNA in 200 µl of TE buffer or sterile distilled water.

Primer design and SYBR Green real-time PCR amplification. Sequences deposited in GenBank for the *txtAB* gene from *S. acidiscabies* (AF255732) and from *S. turgidiscabies* (AY707081) were aligned using the Clustal W alignment tool in DNASTar software (DNASTar Inc., Madison, WI). A real-time PCR forward primer, StrepF, and a reverse primer, StrepR (Table 4), were designed from a region of 100% similarity using Primer Express version 2.0 software (Applied Biosystems, Foster City, CA). The specificity of the primer pair was checked by comparing the NCBI nonredundant nucleotide sequence database for similarity to other organisms using BLASTn, and by real-time PCR amplifications of DNA from potato tissues and potato pathogens *Alternaria solani*, *Colletotrichum coccodes*, *Fusarium sambucinum*, *Helminthosporium solani*, *Spongopora subterranea*, and *Phytophthora infestans*. A real-time PCR primer pair NecTqF1/R1 (Table 4) designed from the *nec1* gene region by Cullen and Lees (7) was used in this study to examine the relationship between the presence of *nec1* gene and pathogenicity. A third real-time PCR primer pair CoxF/R (Table 4) designed to amplify the potato cytochrome oxidase gene by Weller et al. (36) was used as a control to detect potato DNA in *Streptomyces*-negative samples.

The primer pair StrepF/R was initially tested in an SYBR Green real-time PCR assay. The parameters tested included the concentrations of MgCl₂ (2.5 to 5 mM), StrepF/R primers (0.2 to 1 µM), and DNA templates. Based on the results of these tests, optimized real-time PCR amplifications were performed in 25-µl reaction mixtures containing 1× QuantiTect SYBR Green PCR Master Mix (QIAGEN, Valencia, CA) and 0.3 µM of each primer. Ten nanograms of *Streptomyces* DNA, or 0.5 µl of tuber DNA extract, or 10 µl of soil DNA extract was used as DNA template per reaction. Real-time PCR amplifications were carried out on a Smart Cycler (Cepheid, Sunnyvale, CA) with an initial activation step at 95°C for 15 min, followed by 45 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Melting curve analysis was

performed for each reaction to check the presence of primer dimers or nonspecific products. In the initial tests, the PCR products were separated by electrophoresis on 1.5% agarose gels to verify the size of the amplified products and to check for the presence of primer dimers or nonspecific bands.

The sensitivity limit and quantification range of the SYBR Green real-time PCR assay was evaluated by using serial dilution of genomic DNA ranging from 10 ng to 1 fg from pathogenic *Streptomyces* strain OH01-5D. A standard curve was constructed by plotting Ct values versus the log of the initial concentration of *Streptomyces* DNA. To determine whether the presence of plant DNA and co-extracted compounds affect the amplification of target bacterial DNA, the SYBR Green real-time PCR assay was performed using the serial dilution of pathogenic *Streptomyces* DNA plus 1 µl of DNA extract from mini-tuber tissues per reaction.

To test whether the DNA extracts from field soil samples were PCR-inhibitor-free, 10 ng of potato DNA from mini-tuber was added to the reaction mix containing 10 µl of field soil DNA extracts and amplified by real-time PCR using potato primer pair CoxF/R. As a control, 10 µl of sterilized distilled water was used to replace the same volume of DNA extracts from soil, with only 10 ng of potato DNA in the reaction mix. *Cox* Ct values were obtained from each soil sample and the control. ΔCt was calculated as follows: ΔCt = (Ct of *Cox* in sample tested) – (Ct of *Cox* in control).

Sequencing of real-time PCR products. To ensure that the real-time PCR products were amplified from pathogenic *Streptomyces* DNA, the PCR products of four samples, two tuber samples, T1 and T12 (Table 2), and two soil samples, PA1 and ID1 (Table 3), were sequenced. Following real-time PCR, the products were separated on a 1% low-melting agarose gel, purified with the QIAquick PCR Gel Purification Kit (QIAGEN, Valencia, CA), and sequenced using primers StrepF and StrepR with the ABI automated sequencer at the Nucleic Acid Facility, Pennsylvania State University.

TABLE 2. The results of real-time polymerase chain reaction (PCR) quantification of pathogenic *Streptomyces* on potato tuber samples

Sample	Symptom	Variety	Origin	Ct value ^a	Amount of pathogenic <i>Streptomyces</i> DNA (pg) ^b
T1	Common scab	Snowden	NY	20.41 ± 0.16	6.49 × 10 ⁶
T2	Common scab	Snowden	NY	21.54 ± 0.03	2.93 × 10 ⁶
T3	Common scab	Snowden	NY	19.40 ± 0.17	9.60 × 10 ⁶
T4	Common scab	Unknown	MN	24.12 ± 0.48	5.05 × 10 ⁵
T5	Common scab	Unknown	MN	22.14 ± 0.39	1.98 × 10 ⁶
T6	Common scab	Unknown	ME	21.38 ± 0.30	3.32 × 10 ⁶
T7	Common scab	Klondike Goldust	ID	26.14 ± 0.19	1.19 × 10 ⁵
T8	Common scab	Shepody	PA	26.06 ± 0.23	1.27 × 10 ⁵
T9	Scab ^c	Shepody	PA	38.16 ± 0.39	2.81 × 10 ¹
T10	Scab	Shepody	PA	36.69 ± 0.78	8.33 × 10 ¹
T11	Scab	Shepody	PA	27.70 ± 0.31	4.06 × 10 ⁴
T12	Scab	Shepody	PA	31.99 ± 0.27	2.04 × 10 ³
T13	Scab	Bake King	NY	28.98 ± 0.21	1.66 × 10 ⁴
T14	Scab	Green Mountain	NY	28.71 ± 0.23	2.00 × 10 ⁴
T15	Scab	Chippewa	NY	26.04 ± 0.36	1.25 × 10 ⁵
T16	Asymptomatic	Dark Red Norland	PA	39.49 ± 1.76	1.83 × 10 ¹
T17	Asymptomatic	Dark Red Norland	PA	39.62 ± 1.61	1.53 × 10 ¹
T18	Asymptomatic	Dark Red Norland	PA	38.66 ± 0.62	2.05 × 10 ¹
T19	Asymptomatic	Dark Red Norland	PA	ND ^d	ND
T20	Asymptomatic	Dark Red Norland	PA	ND	ND
T21	Asymptomatic	Dark Red Norland	PA	ND	ND
T22	Powdery scab	Unknown	WY	ND	ND
T23	Powdery scab	Unknown	WY	ND	ND
T24	Asymptomatic	Pathogen-free mini-tuber cv. Russet Burbank	CO	ND	ND

^a Data are means ± standard deviation from three replicate quantitative real-time PCR amplifications.

^b In total, DNA extracts were from 1 g of tuber tissue. Pathogenic *Streptomyces* DNA was calculated by comparing mean cycle threshold (Ct) value to standard curve and then multiplying by dilution factor for each sample.

^c The visible lesion was not a typical symptom of common scab; could be common scab or powdery scab.

^d ND = pathogenic *Streptomyces* was not detected.

RESULTS

Specificity of the real-time PCR assay. The sequences of primers StrepF and StrepR were compared with sequences in GenBank using a BLAST search and no similarity to other organisms was found. An SYBR Green real-time PCR assay using primer pair StrepF/R was optimized with 10 ng of DNA per reaction. The DNA was isolated from a pathogenic *Streptomyces* strain, OH01-5D. Under optimal conditions (described above), the primer pair amplified a predicted 71-bp fragment with a Ct value of 20.96, and no nonspecific products or primer dimers were generated after 45 cycles of amplification as confirmed by melting curve analyses and agarose gel observations (data not shown). No fluorescence was detected in real-time PCR using DNA from potato tissues or from common potato tuber pathogens *Alternaria solani*, *Colletotrichum coccodes*, *Fusarium sambucinum*, *Helminthosporium solani*, *Spongopora subterranean*, and *Phytophthora infestans* (data not shown). The capacity of the PCR assay to discriminate between pathogenic and nonpathogenic strains was tested against DNA from nine known pathogenic *Streptomyces* strains representing seven species and from seven nonpathogenic

species. Fluorescence signal was detected for each of the nine pathogenic strains and no signal was detected for any nonpathogenic species (Table 1). The sequences of real-time PCR products from two tuber samples T1 and T12 and two soil samples PA1 and ID1 were identical to the sequences of the pathogenic *Streptomyces txtAB* gene. These results showed that the PCR assay was specific for pathogenic *Streptomyces* species.

For comparison, a primer pair NecTqF1/R1 designed from *necI* gene was also used in the SYBR Green real-time PCR assay against DNA from the nine pathogenic *Streptomyces* strains and seven nonpathogenic species. No fluorescence signal was generated from seven nonpathogenic *Streptomyces* species and one pathogenic strain (strain ID01-12C), while signal was detected for eight other pathogenic *Streptomyces* strains (Table 1).

Sensitivity and quantification range of the real-time PCR assay. The detection limit and quantification range of the SYBR Green real-time PCR assay using primer pair StrepF/R was determined by using serial dilution of pathogenic *Streptomyces* DNA. The real-time PCR amplification profiles are shown in Figure 1A. The reliable lower limit of detection was 10 fg of genomic *Streptomyces* DNA. Ct values were variable for 1 fg DNA and,

TABLE 3. The results of real-time polymerase chain reaction (PCR) quantification of pathogenic *Streptomyces* in potato field soil samples

Field location	Potato variety or description	Susceptibility of potato variety ^a	Common scab incidence ^b	Soil sample	Ct value ^c	Amount of pathogenic <i>Streptomyces</i> (CFU per gram of soil) ^d
Danielsville, PA ^e	Yukon Gold	S	84%	PA1	36.92 ± 0.31	1.50 × 10 ³
				PA2	35.53 ± 0.54	3.97 × 10 ³
				PA3	36.19 ± 1.11	2.99 × 10 ³
				PA4	32.82 ± 0.63	2.59 × 10 ⁴
				PA5	35.80 ± 0.82	3.56 × 10 ³
				PA6	36.22 ± 0.55	2.52 × 10 ³
				PA7	37.06 ± 0.98	1.54 × 10 ³
				PA8	37.03 ± 1.03	1.64 × 10 ³
Palmer, AK ^e	Sweetheart	Unknown	NR ^f	AK1	33.97 ± 0.23	1.11 × 10 ⁴
	All Blue	Unknown	NR	AK2	33.16 ± 0.61	2.00 × 10 ⁴
Presque Isle, ME ^e	White	S	100	ME1	34.81 ± 0.51	6.45 × 10 ³
	Lightly Russet	S	100	ME2	32.86 ± 0.16	2.37 × 10 ⁴
	Small Russet	S	100	ME3	35.96 ± 0.04	2.87 × 10 ³
Aberdeen, ID ^e	VC0967-2R/Y	I	73	ID1	35.57 ± 0.47	3.83 × 10 ³
	W1836-1rus	R	62	ID2	35.10 ± 2.44	0.91 × 10 ⁴
	AF2211-9	S	95	ID3	36.21 ± 0.59	2.52 × 10 ³
	AF2211-9	S	89	ID4	33.79 ± 0.67	1.31 × 10 ⁴
	B1952-2	S	89	ID5	35.67 ± 1.78	4.88 × 10 ³
	Freedom Russet	R	26	ID6	33.80 ± 1.52	1.59 × 10 ⁴
	B1952-2	S	92	ID7	33.83 ± 0.14	1.22 × 10 ⁴
	Atlantic	I/S	64	ID8	35.36 ± 0.84	4.68 × 10 ³
	AF2291-10	I/S	86	ID9	35.34 ± 2.00	6.52 × 10 ³
	NR	Unknown	NR	MN1	30.03 ± 0.32	1.63 × 10 ⁵
E. Grand Forks, MN ^e	NR	Unknown	NR	MN2	36.40 ± 1.63	2.82 × 10 ³
	NR	Unknown	NR	MN3	29.48 ± 0.06	2.35 × 10 ⁵
	NR	Unknown	NR	MN4	32.04 ± 1.09	4.69 × 10 ⁴
	NR	Unknown	NR	MN5	31.41 ± 0.25	6.38 × 10 ⁴
	NR	Unknown	NR	MN6	32.34 ± 0.84	3.64 × 10 ⁴
	NR	Unknown	NR	MN7	34.21 ± 1.44	1.18 × 10 ⁴
	NR	Unknown	NR	MN8	32.09 ± 2.25	6.53 × 10 ⁴
	NR	Unknown	NR	MN9	36.87 ± 0.48	1.58 × 10 ³
	NR	Unknown	NR	MN10	28.29 ± 0.65	5.52 × 10 ⁵
	NR	Unknown	NR	MN11	29.70 ± 0.35	2.05 × 10 ⁵
	NR	Unknown	NR	MN12	29.80 ± 0.71	1.99 × 10 ⁵
	Russet	Unknown	NR	WI1	26.94 ± 1.72	1.80 × 10 ⁶
Portage Co., WI ^e	Washburn Co., WI ^e	MN99460-21	Unknown	WI2	32.17 ± 0.63	3.94 × 10 ⁴
Marquette Co., WI ^e	Norland	S	NR	WI3	38.57 ± 0.25	0.49 × 10 ³
Waushara Co., WI ^e	Norland	S	NR	WI4	37.41 ± 1.23	1.26 × 10 ³
Alamosa, CO ^g	Unknown	Unknown	No disease	CO1	ND ^h	ND
				CO2	ND	ND

^a S = susceptible; I = intermediate; R = resistant.

^b The disease incidence was calculated as a percentage of scabby tubers out of total number of tubers from the field.

^c Data are means ± standard deviation from three replicate DNA extractions and quantitative real-time PCR amplifications.

^d Calculated by comparing mean cycle threshold (Ct) value to standard curve.

^e Location with a history of common scab.

^f NR = not recorded.

^g Location with a history of powdery scab but no common scab.

^h ND = pathogenic *Streptomyces* was not detected.

therefore, not included in the analysis. Based on a PCR assay of three replicate serial dilutions of *Streptomyces* DNA, a standard curve was constructed (Fig. 1B). The standard curve showed a linear response with a high correlation coefficient ($R^2 = 0.9946$). The efficiency of the real-time PCR amplification was 100%, determined using the formula, $(10^{[-1/\text{slope}]} - 1) \times 100$, indicating that the assay could be used for the accurate quantification of the bacterial DNA.

When 1 µl of DNA extract from mini-tuber tissues was added to serial dilutions of *Streptomyces* DNA ranging from 10 ng to 10 fg, results of real-time PCR assays based on three replicate experiments showed the mean Ct values for each dilution were similar to those obtained from serial dilution of *Streptomyces* DNA without added plant DNA extracts (data not shown), indicating that the real-time PCR can be used to quantify *Streptomyces* in infected plant tissues.

Quantification of pathogenic *Streptomyces* DNA in plant tissues. Twenty-four tuber samples were assessed by the SYBR green real-time PCR assay. Each tuber was evaluated three times by PCR and the amount of *Streptomyces* DNA was calculated by referring Ct values to the standard curve. The Ct values and the amount of *Streptomyces* DNA are shown in Table 2. Eight tubers with typical common scab symptoms were all positive for pathogenic *Streptomyces*, and 1.19×10^5 to 6.49×10^6 pg of *Streptomyces* DNA was detected in total DNA extracts from 1 g of tuber lesion tissue. Seven scabby tubers which showed atypical common scab symptoms similar to powdery scab were also all positive for pathogenic *Streptomyces*, although 10- to 10,000-fold less *Streptomyces* DNA (2.81×10^1 to 1.25×10^5 pg) was detected in total DNA extracts from 1 g of tuber lesion tissue. Low levels of pathogenic *Streptomyces* were also detected in three out of six asymptomatic tubers: 1.53×10^1 to 2.05×10^1 pg of *Streptomyces* DNA was detected in total DNA extracts from 1 g of tuber tissue. Pathogenic *Streptomyces* DNA was not detected in two typical powdery scab tubers or a pathogen-free mini-tuber.

For samples negative for pathogenic *Streptomyces*, a second SYBR Green real-time PCR assay using potato primer pair CoxF/R was performed to confirm whether the DNA extracts contained PCR inhibitors that would prevent PCR amplifications and result in false negatives. Fluorescence signal was detected in all of these samples (data not shown), indicating that these samples were truly *Streptomyces* negative, or the amount of *Streptomyces* DNA in the samples was below the detection limit, and that PCR inhibitors were not likely a problem.

Quantification of pathogenic *Streptomyces* in soils. To determine the detection limit and the relationship between the Ct values and the concentrations of pathogenic *Streptomyces* in soils, SYBR Green real-time PCR with the primer pair StrepF/R, was carried out using DNA extracts from ten *Streptomyces*-spiked soil samples containing from 10^9 to 0 CFU of pathogenic *Streptomyces* per gram of soil. The reliable detection limit in soil was 10^3 CFU per gram of soil. *Streptomyces* was not detected in samples containing 0 or 10^1 CFU per gram of soil. Ct values were variable for samples containing 10^2 CFU. The data for the 10^1 and 10^2 CFU levels were therefore omitted from the standard curve. The real-time PCR amplification profiles are shown in Figure 2A. A standard curve was calculated based on three replicate experiments (Fig. 2B). A linear regression was obtained with an R^2

value of 0.9931. The efficiency of the real-time PCR amplification was 97%, indicating that the assay could be used for the quantification of the bacteria in soil.

Thirty-eight soil samples which were collected from potato fields where common scab was found, were assessed by the real-time PCR assay. Pathogenic *Streptomyces* was detected in all of these samples. By comparing Ct values to the standard curve, the concentrations of pathogenic *Streptomyces* were calculated, and the mean concentration values from three experiments are shown in Table 3. Pathogenic *Streptomyces* were not detected in two soil samples collected from a field with powdery scab but no history of common scab (Table 3).

In tests to determine whether the DNA extracts from field soils contained PCR inhibitors, potato DNA was added to the field soil DNA extracts. In SYBR Green PCR assays, the *Cox* Ct value of the potato DNA control was 24.16, and *Cox* Ct values of potato DNA added to DNA extracts from field soils ranged from 24.18 to 25.14 (Table 5). Δ Ct ranged from 0.02 to 0.98 (Table 5), indicating that *Cox* was amplified at a similar efficiency between field soils and control and among field soils. These results demonstrated that field soil DNA extracts showed no PCR inhibition.

DISCUSSION

In this investigation we developed a quantitative real-time PCR assay specific to plant pathogenic *Streptomyces*. The SYBR Green

Fig. 1. A, Real-time polymerase chain reaction (PCR) amplification profile for a representative dilution series of DNA from pathogenic *Streptomyces scabies* strain OH01-5D. **B,** Standard curve obtained by plotting threshold cycle (Ct) values against log serial dilutions of DNA (ng) from pathogenic *Streptomyces* strain OH01-5D. The standard curve is the result of three separate PCR analyses.

TABLE 4. Real-time polymerase chain reaction primers used in this study

Target organism	Primer	Sequences (5' – 3')	Target gene	Product size (bp)	Reference
Pathogenic <i>Streptomyces</i>	StrepF	GCAGGACGCTCACCAGGTA	<i>txtAB</i>	71	This work
	StrepR	ACTTCGACACCGTTGTCCTCA			
Pathogenic <i>Streptomyces</i>	NecTqF1	TCGCACTCTTGGAGATCTCATG	<i>nec1</i>	83	Cullen and Lees (7)
	NecTqR1	TCGTAAGAACGCGACGCTTT			
Potato	CoxF	CGTCGCATTCCAGATTATCCA	<i>cox</i>	79	Weller et al. (36)
	CoxR	CAACTACGGATATATAAGAGCCAAA			

assay developed uses primer pair StrepF/R designed to the sequence of the *Streptomyces txtAB* operon, which encodes a non-ribosomal peptide synthetase that is found in all pathogenic *Streptomyces* (13,19,27,33,34). When tested against DNA from 16 *Streptomyces* strains, potato tissues, and selected other soilborne potato pathogens, fluorescent signal was detected from nine strains previously determined to be pathogenic that represent all species of plant-pathogenic *Streptomyces* so far reported to occur in Europe and North America (2,5,20,34,35). No fluorescent signal was obtained from seven nonpathogenic *Streptomyces* strains, from other common potato tuber pathogens, or from tissue-culture disease-free tubers, and no primer dimer was observed.

We obtained a detection limit of 10 fg of *Streptomyces* DNA, corresponding to approximately 1 bacterial cell according to the genome size of a *Streptomyces* species (1). Considering that *txtAB* is a single copy gene, this assay is highly sensitive. Using primers developed on multi-copy genes such as 16S rRNA may improve sensitivity, but it is difficult to design primers specific for all pathogenic strains because of the genetic similarity of 16S rRNA gene sequences in pathogenic and nonpathogenic *Streptomyces* strains, and because both pathogenic and nonpathogenic strains are found within a single species. Furthermore, quantification using single copy genes is not affected by variable numbers of tandem repeats found in multi-copy genes, and there is a potential to directly correlate Ct values with the pathogen biomass and/or with the number of propagules. Using serial dilutions of target DNA, high PCR amplification efficiency and high correlation coefficients between the amount of DNA and cycle thresholds were achieved. In addition, Ct values and correlation coefficients were not affected by the presence of plant extracts. These results demonstrate that the assay is appropriate for both qualitative and quantitative analyses of pathogen biomass in infected plant tissues.

For the PCR detection and quantification of pathogenic *Streptomyces* in soil, it is essential to have a method that extracts DNA free from PCR inhibitors. With DNA extracted using a Bio101 Soil DNA kit, we obtained a real-time PCR amplification efficiency of 97%, even when 10 µl of DNA extract (or 40% of

the PCR volume) was used for amplification. No PCR inhibition was seen in field soil DNA extracts using a *Cox* real-time PCR assay, demonstrating that the DNA extraction method used adequately removes PCR inhibitors commonly co-extracted with DNA from soil. We conclude the assay is reliable, specific, and sensitive for the detection and quantification of pathogenic *Streptomyces* strains on tubers or in soil.

The assay was used to quantify the amount of pathogen in potato tuber and field soil samples. Approximately 10⁵ to 10⁶ pg of DNA of pathogenic *Streptomyces*, corresponding to approximately 10⁷ to 10⁸ bacterial cells, was detected in total DNA extracts from 1 g of infected tubers with typical common scab symptoms. These numbers are similar to those of Wang and Lazarovits (31), who estimated 2 to 3 × 10⁸ CFU/g of scabby tuber tissue. Populations in lesions from growers' potato storage after winter were estimated to range from 2 × 10⁵ to 2 × 10⁸ CFU/g of lesion tissue (31).

Actinobacteria have recently been estimated to make up an average of 13% of all soil bacteria, about a quarter of which can

TABLE 5. Real-time polymerase chain reaction (PCR) assays to test for PCR inhibitors in DNA extracts from field soil samples^a

Soil sample	<i>Cox</i> Ct value	ΔCt ^b
PA1	24.28	0.12
PA2	24.31	0.15
PA3	24.84	0.68
PA4	24.86	0.70
PA5	24.62	0.46
PA6	24.18	0.02
PA7	24.70	0.54
PA8	24.23	0.07
AK1	24.42	0.26
AK2	24.51	0.35
ME1	24.80	0.64
ME2	24.70	0.54
ME3	25.12	0.96
ID1	24.95	0.79
ID2	24.36	0.20
ID3	24.48	0.32
ID4	24.23	0.07
ID5	24.83	0.67
ID6	24.39	0.23
ID7	24.33	0.17
ID8	24.83	0.67
ID9	24.18	0.02
MN1	24.39	0.23
MN2	24.79	0.63
MN3	24.35	0.19
MN4	24.65	0.49
MN5	24.99	0.83
MN6	24.84	0.68
MN7	25.12	0.96
MN8	24.72	0.56
MN9	24.64	0.48
MN10	25.14	0.98
MN11	25.14	0.98
MN12	24.77	0.61
WI1	24.91	0.75
WI2	24.93	0.77
WI3	24.30	0.14
WI4	24.86	0.70
CO1	24.97	0.81
CO2	24.58	0.42
Control ^c	24.16	

^a Ten microliters of DNA extracts from each soil sample plus 10 ng of potato DNA from mini-tuber was used for real-time PCR amplification using potato primer pair CoxF/R.

^b ΔCt = (Ct of *Cox* in sample tested) – (Ct of *Cox* in control). ΔCt ranged from 0.02 to 0.98, indicating that *Cox* was amplified at a similar efficiency between field soils and control and among field soils.

^c Ten microliters of sterilized distilled water was used to replace the same volume of DNA extracts from soil, with only 10 ng of potato DNA in the reaction mix.

Fig. 2. A, Real-time polymerase chain reaction (PCR) amplification profile for DNA from soil spiked with 10³, 10⁴, 10⁵, 10⁶, 10⁷, 10⁸, and 10⁹ colony forming units (CFU) of pathogenic *Streptomyces* per gram. **B,** Standard curve obtained by plotting threshold cycle (Ct) values against the log of the numbers of pathogenic *Streptomyces* CFU in spiked soil. The standard curve is the result of three separate PCR analyses.

confidently be assigned to one of 148 described genera including *Streptomyces* (15). This means that soils could contain, on average, 3×10^7 streptomycetes per gram. Estimates of the percentage of soil streptomycetes that are plant pathogens range from less than 3 to 9% (6,17), with higher proportions being found in the rhizosphere of scabby potatoes (17,32). In an assessment of limited numbers of naturally infested soil samples from fields where common scab was found, approximately 10^3 to 10^5 CFU of pathogenic *Streptomyces* per gram of soil was found in most of the samples, with one exception in which approximately 10^6 CFU of pathogenic *Streptomyces* per gram of soil was present. These results are in good agreement with the findings of Conn et al. (6) and Wang and Lazarovits (32) on bulk soils using plating methods. The high standard deviations in Ct values observed in triplicate aliquots of some soil samples suggest that *Streptomyces* is not evenly distributed in soil. Since only 1 g soil was used for DNA extraction, it is important to ensure that adequate soil sampling strategies are used, and that DNA extraction is reproducibly executed.

Often it is difficult to distinguish common scab and powdery scab, especially in the early stages of disease development. Of seven tubers with difficult to distinguish common or powdery scab symptoms, approximately 10^1 to 10^5 pg of pathogenic *Streptomyces* DNA was detected in DNA extracts from 1 g of tuber lesion tissue. *Spongopora subterranea* was also detected in two of these tubers (X. S. Qu, and B. J. Christ, *unpublished data*), indicating the scab lesion was a mixture of common scab and powdery scab. From our experience in identifying potato diseases, it is not unusual to find common scab and powdery scab co-existing on single tubers. This PCR assay may be useful for the identification of pathogenic *Streptomyces* on scabby tubers with atypical symptoms that are difficult to diagnose visually.

A very important aspect of common scab management in potato production is the use of pathogen-free seed potatoes. Approximately 10^1 to 10^3 pg of DNA of pathogenic *Streptomyces* was detected in total DNA extracts from 1 g of tuber tissue on three out of six asymptomatic tubers. These results are in agreement with the findings of others that asymptomatic seed tubers may harbor a measurable population of pathogenic *Streptomyces* that are potential sources of inoculum of pathogenic *Streptomyces* into potato fields (32,37). The PCR assay developed in this study may serve as a sensitive and fast method to assess seed tubers.

Many factors influence common scab disease incidence, including virulence of the pathogenic bacterial strain, potato cultivar resistance, soil type, and environmental conditions, but some results suggest a relationship between the amount of pathogenic *Streptomyces* in soil and the incidence of common scab on tubers. Wang and Lazarovits (32) and Keinath and Loria (18) observed a correlation between potato common scab incidence and inoculum density of pathogenic *Streptomyces* in the rhizosphere, and Wanner (33) found a correlation between initial inoculum density and radish common scab. Accurate quantification of pathogenic *Streptomyces* in larger numbers of soil samples with subsequent determination of disease incidence under varying conditions must be accomplished to validate these results. Potentially a threshold value could be determined to indicate soils that would be at high risk for common scab disease. The method presented here makes such a study feasible.

To better understand the epidemiology of potato common scab, and to devise better measures to control the disease, it is important to use accurate methods to measure the quantity of pathogenic strains on tubers and in soils. The main advantages of our real-time PCR assay are high sensitivity, specificity, excellent efficiency, and freedom from post-PCR handling steps, which make it an ideal tool to assess target pathogens in large numbers of samples. This assay can be used for accurate detection and quantification of pathogenic *Streptomyces* in host plant tissues

and soils, which is an important step to develop an integrated approach to control common scab disease.

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