Genotypic and Phenotypic Characterization of *Streptomyces* Species Causing Potato Common Scab in Uruguay

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Abstract

Isolation and characterization of common scab (CS) pathogen *Streptomyces* spp. from Uruguayan potato tubers and soil samples were done in response to significant economic losses due to CS on potato in autumn 2010. Seventy of the 331 isolates were classified as pathogenic owing to their ability to induce necrosis on tuber disks and stunting of radish seedling. *Streptomyces* spp. causing CS on potato in Uruguay were found to represent a range of different species by virtue of their diverse morphological and physiological traits as well as rep-PCR, *rpoB* phylogenetic analysis, and multi-locus sequences analysis. We identified isolates primarily as *Streptomyces scabiei*, *S. acidiscabiei*, and *S. europaeiscabiei*. However, some of the pathogenic isolates still remain to be identified at

the species level. This highlights the need for improved methods for discrimination among pathogenic *Streptomyces* species. The presence of *Streptomyces* pathogenicity island (PAI) genes was analyzed, including genes encoding for thaxtomin synthetase (*txtA*, *txtB*), tomatinase (*tomA*), and a necrosis protein (*necI*). Among the isolates that were pathogenic, 50% contained the four pathogenicity genes, 33% had an atypical composition of PAI marker genes, and 17% did not contain any genes. The absence of the genes reported to be involved in thaxtomin biosynthesis (*txtA*, *txtB*) was confirmed by whole-genome sequencing of two representative strains of this group. This finding suggests the participation of other virulence factors in plant pathogenicity.

Potato common scab (CS) is a widespread disease throughout the world resulting in significant economic losses due to lesions affecting tuber surfaces. The causal agents are soil-inhabiting gram-positive bacteria belonging to the genus *Streptomyces* consisting of more than 650 species (Euzéby 2016; Loria et al. 2006). *Streptomyces* spp. play a major role in nutrient cycling and some produce antibiotics and other useful metabolites (Chamberlain and Crawford 2000). Despite the extremely high diversity of this genus, only a few species are plant pathogens. Among them, the first-described and best-characterized species, *Streptomyces scabiei*, seems to be widespread around the world; although several recent reports indicate that it is infrequently found in Europe (Bouchek-Mechiche et al. 2000; Dees et al. 2013; Flores-Gonzalez et al. 2008). Other more geographically limited species are also associated with CS. Among these are: *S. acidiscabiei*, isolated from acid soils where *S. scabiei* is normally suppressed; and *S. turgidiscabiei*, first described in Japan (Lambert and Loria 1989a; Miyajima et al. 1998). These species are considered to be newly emerging pathogens (Bignell et al. 2010; Loria et al. 2006). Other *Streptomyces* spp. that cause CS include: *S. europaeiscabiei* identified in Europe, Korea, and North America (Bouchek-Mechiche et al. 2000; Song et al. 2004;

Wanner 2009); *S. stelliscabiei* reported in France, the United States, and Germany (Bouchek-Mechiche et al. 2000; Leiminger et al. 2013; Wanner 2006); *S. luridiscabiei*, *S. puniscabiei*, and *S. niveiscabiei* isolated from Korea (Park et al. 2003); *S. caviscabiei* reported in Canada (Faucher et al. 1992; Faucher et al. 1995); and *S. reticuliscabiei* isolated from France and described by Bouchek-Mechiche et al. (2000).

CS pathogenicity is associated with production of the phytotoxic dipeptide thaxtomin A, which induces characteristic symptoms in tubers. The thaxtomin biosynthetic cluster (*txt*) is encoded in the chromosomes of *S. scabiei*, *S. turgidiscabiei*, and *S. acidiscabiei*. Furthermore, the *txt* gene cluster is localized in mobile pathogenicity islands (PAIs) (Kers et al. 2005; Huguet-Tapia et al. 2014; Zhang et al. 2016). This suggests that horizontal gene transfer likely plays a role in the acquisition of the *txt* gene cluster by *Streptomyces* spp., and consequent potential emergence of new pathogenic *Streptomyces* (Bignell et al. 2014; Loria et al. 2006). Some studies have shown that a number of *Streptomyces* isolates lacking thaxtomin A genes are also CS pathogens (Jordaán and van der Waals 2016; Pánková et al. 2012; Wanner 2004). Bignell et al. (2014) recently described other phytotoxic secondary metabolites that may contribute to CS symptom development. This might explain why isolates without thaxtomin A genes are still pathogenic and produce characteristic symptoms. Genes including *necI* and *tomA* are involved in enhancing pathogenicity, although they are not essential for symptom development (Seipke and Loria 2008; Wanner 2009). *NecI* encodes a protein inducing necrosis in plant tissue, and *tomA* encodes a virulence factor homologous to tomatinase, an enzyme that belongs to saponinases found in plant pathogenic fungi (Bukhalid et al. 1998; Seipke and Loria 2008).

Identification of *Streptomyces* isolates is very difficult owing to the numerous species described within the genus. The several sequenced genomes for *Streptomyces* are also very large, between 8 and 10 MB. The size and the high rate of recombination of the *Streptomyces* genome make species identification difficult (Doroghazi and Buckley 2010; Huguet-Tapia et al. 2016). Classical approaches for identification of *Streptomyces* spp. involve the evaluation of morphological traits, and microscopic and biochemical techniques, which are time-consuming and laborious. Although these methods contribute

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to strain characterization, they do not allow identification of isolates to the species level. In recent years, development and refinement of molecular methods offers many new possibilities for species identification. These methods include species-specific 16S rRNA gene sequencing, species-specific marker PCR methods, detection of pathogenicity determinants, and several DNA fingerprinting techniques (Guo et al. 2008; St-Onge et al. 2008; Wanner 2009). Multi-locus sequence analysis (MLSA) has become particularly relevant to this task. This method consists of phylogenetic analysis of multiple housekeeping genes (Guo et al. 2008) and it provides a good resolution to discriminate *Streptomyces* spp. (Laskaris et al. 2012; Labeda 2011).

Potato is one of the most important horticultural crops in Uruguay (MGAP and Mercado Modelo 2011). Potato production in 2010, however, experienced unusually high economic losses due to CS, corresponding to losses of 29% of total potato cultivation. The disease was very aggressive and many tubers showed pitted and raised scab lesions (Lapaz et al. 2012). Uruguayan potato producers have usually imported ca. 30% of seed tubers used in recent years (MGAP and Mercado Modelo 2011). Plant health regulations allow importation of seed tubers that pass a visual control for CS. This visual control is not rigorous enough to avoid introduction of the causal agent for CS disease, and new strategies to screen for the presence of the pathogen are clearly needed. The aim of the present study was to isolate and characterize *Streptomyces* spp. from CS lesions on potato tubers and from horticultural field soils in Uruguay. To our knowledge, an exhaustive identification and genetic characterization of *Streptomyces* spp. causing CS in Uruguay, traditionally a potato-producing country, had not been previously undertaken.

Materials and Methods

Bacterial strains and culture conditions. Five *Streptomyces* spp. reference strains representing the most important species associated with potato CS were included for phenotyping and genotyping studies: *S. scabiei* DSM41658^T (T, type strain); *S. stelliscabiei* DSM41803^T; *S. europaeiscabiei* DSM41802^T; *S. acidiscabiei* DSM41668^T; and *S. turgidiscabiei* DSM41838^T. Type strains were purchased from the DSMZ culture collection (Braunschweig, Germany). Additionally, 20 unknown *Streptomyces* spp. strains were kindly provided by Departamento de Protección Vegetal (Fitopatología), Facultad de Agronomía, Montevideo, Uruguay. These strains were isolated from potato tubers during the severe CS outbreak in 2010. *Streptomyces* isolates and reference strains were routinely cultured on yeast-malt extract agar (YME) (Shirling and Gottlieb 1966) at 28°C and maintained as spore suspensions in 20% glycerol at -70°C.

Isolation of *Streptomyces* spp. from necrotic lesions on potato tubers. Potato tubers (61 samples) and imported seed potato (13 samples) showing symptoms of CS were obtained from potato growers and farm advisors. Samples were obtained from main potato-growing areas in Uruguay (San José, Canelones, and Rocha) in 2010, 2011, and 2012.

Isolation of *Streptomyces* spp. from scab lesions was performed using a modified method of Loria et al. (2010). Tubers were washed, surface-sterilized with 1.0% sodium hypochlorite (NaOCl) for 1 min, and rinsed with sterile distilled water. A portion of lesion was macerated in 2 ml sterile distilled water. Dilutions of macerated tissue were spread on NPPC (nystatin, polymyxin, penicillin, and cycloheximide) water agar (Loria et al. 2010) and incubated at 28°C for 10 to 14 days. Colonies characteristic of *Streptomyces* were transferred onto fresh YME and incubated at 28°C for 5 to 7 days. Subsequent transfer to fresh medium was done to obtain pure cultures.

Isolation of *Streptomyces* spp. from soil. Soil samples were collected from various potato fields in San José, Uruguay. Isolation of *Streptomyces* spp. from soil was done essentially as described by Loria et al. (2010). Air-dried soil (10 g) was added to 100 ml sterile distilled water, and agitated on a shaker for 10 min. Five successive tenfold dilutions of the soil suspension were made using sterile water and 0.1 ml of the three most dilute suspensions were spread uniformly on NPPC water agar. Plates were incubated at 28°C for 7 to 10 days and characteristic powdery *Streptomyces* colonies were transferred to fresh

medium. Pure cultures were obtained through repeated culturing of single colonies on YME medium.

Pathogenicity tests. Pathogenicity was tested on tuber slices as described previously by Loria et al. (1995). Cultures of test strains were grown on YME agar medium for 5 to 7 days at 28°C, and agar plugs from the sporulating colonies were transferred onto disks of excised tuber tissue. Disks were incubated at 28°C in the dark and evaluated for the presence of necrosis after 5 days.

In addition, pathogenicity was tested using a radish-seedling bioassay as described previously by Fyans et al. (2016), with some modifications. *Streptomyces* isolates were cultured in 5 ml of TSB medium on a shaker at 150 rpm for 2 to 3 days at 28°C. For both tuber slice and radish pathogenicity testing, *S. scabiei* DSM41658 was included as positive control and sterile water was used as negative control. The pathogenicity tests were done two times for each isolate.

Morphological and physiological characterization of *Streptomyces* isolates. Morphological and physiological characterization was performed according to the International *Streptomyces* Project (ISP) (Loria et al. 2010; Shirling and Gottlieb 1966). YME (ISP2) agar was used to assess colony and spore color. Tyrosine agar (ISP7) was used to determine production of melanin. Water agar cultures were used to determine spore-chain type by direct microscopic examination. Carbon utilization medium (ISP9) was used to assess utilization of raffinose. Utilization of D-glucose was also evaluated as positive control of the assay. Carbon source solutions were sterilized by filtration and added to autoclaved ISP9 medium to 1% final concentration (w/v). Plates were incubated at 28°C for up to 10 days.

DNA isolation. Genomic DNA from *Streptomyces* isolates was extracted according to Sambrook and Russell (2001). The quantity of extracted DNA was measured by Nanodrop ND-100 (Nanodrop Technologies, Stone, UK) and final DNA concentrations were adjusted to 50 ng/μl (by dilution) and stored at -20°C before use.

PAI marker genes. Genomic DNA from reference strains and *Streptomyces* spp. isolates was analyzed by PCR for the presence of genes characteristic of the *S. turgidiscabiei* PAI as described by Kers et al. (2005). Amplifications were performed using specific primer pairs: TxtAB1/TxtAB2 (Wanner 2006) and TxtA1/TxtA2 for the *txtA* and *txtB* genes (Wanner 2004); Tom3/Tom4 for the *tomA* gene (Wanner 2006); and Nf/Nr for the *nec1* gene (Bukhalid et al. 1998) (Table 1).

PCR analyses were done in 25 μl containing 1× Buffer, 1.0 to 2.0 mM MgCl₂ (Wanner 2006), 200 μM each dNTP, 12.5 pmol each primer, 1.0 U of *Taq* DNA polymerase (recombinant) (Thermo Scientific, Carlsbad, CA), and 50 ng of DNA template. The time and temperature conditions for PCRs were done as described by Wanner (2006). Amplified DNA fragments were separated on 1.5% agarose gel and analyzed after staining with ethidium bromide.

Genome sequencing and analysis. Whole-genome sequencing of strains ST1015 and ST1020 was performed with the illumina MiSeq platform. We obtained around 20 million 100-bp paired-end reads for each genome. These produce around 100× coverage. Reads were assembled using Spades 3.5 software (Bankevich et al. 2012). Resulting contigs were annotated using prokka annotation pipeline (Seemann 2014). The contigs were used as a nucleotide sequence database for BLAST searching and the sequences of the virulent factors were used as queries.

Genotyping using rep-PCR. Genetic diversity of pathogenic *Streptomyces* isolated in Uruguay was determined by fingerprinting techniques rep-PCR (BOX and ERIC-PCR), using primers BOXA1R and ERIC2/ERIC1R, respectively (Louws et al. 1995) (Table 1). The analysis was done with 70 pathogenic isolates, five reference *Streptomyces* strains (*S. acidiscabiei* DSM41668, *S. scabiei* DSM41658, *S. turgidiscabiei* DSM 41838, *S. europaeiscabiei* DSM 41802, and *S. stelliscabiei* DSM 41803) and *Ralstonia solanacearum* strain UY031 (Siri et al. 2011) as outgroups.

PCR reactions were performed in 25 μl containing 1× Buffer, 6mM of MgCl₂, 200 μM of dNTP, 25 pmol of each primer, 10% v/v dimethylsulfoxide (DMSO), 2.0 U of *Taq* polymerase (recombinant) (Thermo Scientific) and 25 to 140 ng of DNA template.

The amplification cycle conditions were based on Clark et al. (1998) with some modifications, with an initial denaturation step at

95°C for 5 min; followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and extension at 65°C for 4 min; and a final extension step at 65°C for 10 min. The resulting products were analyzed by electrophoresis using 2% (w/v) agarose gels with 0.5 µg/ml ethidium bromide and photographed under UV light. Gel images were analyzed with the software GelCompar 4.2 (Kortrijk, Belgium). The differences between the patterns generated were established by the presence (1) or absence (0) of an amplification product. A similarity matrix was constructed from the binary data with Dice's coefficients and dendrogram was generated with UPGMA (Unweighted Pair-Group Method using arithmetic Average) clustering algorithm. Data from ERIC and BOX-PCR were combined for strain comparisons.

Analysis of the *rpoB* gene. RNA polymerase beta subunit (*rpoB*) gene was amplified by PCR using primer pair SRPOF1/SRPOR1 (Kim et al. 2004) (Table 1). The *rpoB* amplifications were done in 25 µl containing 1× Buffer, 1.5 mM MgCl₂, 250 µM each dNTP, 12.5 pmol each primer, 1.25 U of *Taq* DNA polymerase (recombinant) (Thermo Scientific), 50 ng of DNA template. The amplification cycle conditions were based on Kim et al. (2004) with some modifications, using an initial denaturation step at 95°C for 3 min and an annealing temperature of 56°C. Amplified DNA fragments were separated on 1.5% agarose gel and visualized with ethidium bromide under UV light.

Multi locus sequence analysis (MLSA). The characterization by MLSA was conducted using four housekeeping genes: *rpoB* (RNA polymerase beta subunit); *atpD* (ATP synthase beta subunit); *recA* (recombinase A); and *trpB* (tryptophan synthetase subunit B). PCR amplifications were performed using primers previously described by Guo et al. (2008) (Table 1). The PCR mix (25µl) contained 1× Buffer, 3 mM of MgCl₂, 200 µM of dNTP, 10 pmol of each primer, 10% v/v dimethylsulfoxide (DMSO), 1.25 U of *Taq* DNA polymerase (recombinant) (Thermo Scientific), and 50 ng of DNA template. The MLSA genes were amplified using thermal cycling conditions described by Guo et al. (2008) with some modification in annealing temperatures (Table 1). Amplified DNA fragments were separated on 1.5% agarose gels and visualized with ethidium bromide under UV light.

Amplicon purification, gene sequencing and sequence editing. Amplicons were purified and sequenced in both directions by the Macrogen Service (Geumcheon-gu, Seoul). Sequences were edited

and assembled using Geneious Pro 4.8.5 software (Biomatters, Auckland, New Zealand) and deposited in the GenBank database. Sequences were compared with the GenBank nr database using the BLAST algorithm (Altschul et al. 1997).

Phylogenetic analyses. Phylogenetic analysis of *rpoB* was conducted using sequences of an uninterrupted 306-bp internal fragment of a subset of 52 pathogenic *Streptomyces* isolates chosen as representative of different morphological and physiological characteristics present among the 70 strains and the following reference strains: *S. stelliscabiei* DSM41803; *S. scabiei* DSM41658; *S. acidiscabies* DSM41668; and *S. turgidiscabies* DSM41838. The sequences of an additional five reference *Streptomyces* strains (*S. scabiei* ATCC49173, *S. acidiscabies* ATCC49003, *S. turgidiscabies* ATCC700248, *S. europaeiscabiei* CFBP4497, and *S. ipomoeae* DSM40383) and *S. flavidofuscus* DSM43111, used as an outgroup, were obtained from public databases and were also included in the analysis. All sequences were aligned using ClustalW in MEGA 5.0 software (Tamura et al. 2011).

For MLSA, sequences of 28 pathogenic *Streptomyces* isolates from Uruguay with different characteristics were analyzed. The sequences of 11 pathogenic *Streptomyces* spp. deposited in GenBank were also included in the analysis: *S. scabiei* 87.22; *S. acidiscabies* 84-104; *S. europaeiscabiei* NRRL B-24443; *S. luridiscabiei* LMG 21390; *S. niveiscabiei* NRRL B-24457; *S. puniscabiei* NRRL B-24456; *S. cavaresabiei* AS 4.1836; *S. reticuliscabiei* NRRL B-24446; *S. stelliscabiei* NRRL B-24447; *S. turgidiscabies* NRRL B-24078; and *S. ipomoeae* NRRL B-12321 (causing scab in sweet potato). Sequences of *Streptomyces flavidofuscus* AS 4.1617 was included as an outgroup for the phylogenetic analysis. All sequences of each gene were concatenated and then aligned using ClustalW in MEGA 5.0 software (Tamura et al. 2011). Sequences were concatenated following the alphabetic order of the genes, ending in a large sequence of 2111 bp (*atpD*, *recA*, *rpoB*, and *trpB*).

Phylogenetic trees for individual genes and the tree constructed from concatenated sequences alignment of four genes were inferred using the neighbor-joining (Saitou and Nei 1987), and maximum-parsimony (Fitch 1972) methods. The distance (percent divergence) was calculated between all pairs of sequences based on the alignment. The neighbor-joining and maximum-parsimony methods were done using MEGA 5.0 (Tamura et al. 2011). The resultant neighbor-joining

Table 1. PCR primers and conditions used for *Streptomyces* isolated in Uruguay

Gene/region	Primers	Nucleotide sequence (5'-3')	Amplicon size (bp)	Annealing temperature (°C)
PAI markers genes				
<i>nec1</i>	Nf	ATGAGCGCGAACGGAAGCCCCGGA	700	60
	Nr	GCAGGTCGTCACGAAGGATCG		
<i>txtA</i>	TxtA1	CACGTACGCGCAGTTCAATG	398	48
	TxtA2	AGATGATGTAGGCGGGACTC		
<i>txtAB</i>	TxtAB1	CCACCAGGACCTGCTCTTC	385	48
	TxtAB2	TCGAGTGAGCCTCACAGATG		
<i>tomA</i>	Tom3	GAGGCGTTGGTGGAGTTCTA	392	55
	Tom4	TTGGGGTTGTACTCCTCGTC		
Phylogenetic marker gene				
<i>rpoB</i>	SRPOF1	TCGACCACTTCGGCAACCGC	352	56
	SRPOR1	TCGATCGGGCACATGCGGCC		
MLSA				
<i>atpD</i>	atpDPF	GTCGGCGACTTCACCAAGGGCAAGGTGTTCAACACC	998	61
	atpDPR	GTGAAGTCTGTGGCGACGTGGGTGTTCTGGGACAGGAA		
<i>recA</i>	recAPF	CCGCRCTCGCACAGATTGAACGSCAATTC	913	65
	recAPR	GCSAGGTCGGGGTTGCTCTTSAGGAAGTTGCG		
<i>rpoB</i>	rpoBPF	GAGCGCATGACCACCCAGGACGTGAGGC	994	65
	rpoBPR	CCTCGTAGTTGTGACCCTCCACGGCATGA		
<i>trpB</i>	trpBPF	GCGCGAGGACCTGAACCACACCGGCTCACACAAGATCAACA	822	67
	trpBPR	TCGATGGCCGGGATGATGCCCTCGGTGCGCGACAGCAGGC		
rep-PCR				
BOX elements	BOXA1R	CTACGGCAAGGCGACGCTGACG	-	50
ERIC elements	ERIC2	AAGTAAGTGACTGGGGTGAGCG	-	50
	ERIC1R	ATGTAAGCTCCTGGGGATTAC		

tree and topology were evaluated using 1000 bootstrap resamplings (Hillis and Bull 1993).

Results

CS pathogens were collected from 2010 through 2012 from fields located in the main potato-production regions in Uruguay. Tubers sampled in this survey exhibited diverse symptoms, ranging from mild, superficial, to deep-pitted lesions (Fig. 1). A total of 331 *Streptomyces* isolates were obtained from both necrotic tuber lesions and soils.

Pathogenicity. Only 70 *Streptomyces* spp. isolates of the 331 were determined to be pathogenic, including 45 isolates from necrotic lesions on potato tubers and 25 isolates from soil samples (Table 2). The 70 pathogenic isolates were designated based on a plant pathogenic phenotype on potato tuber slices and radish seedlings (Fig. 2). All pathogenic isolates were tested for presence of genes *txtA*, *txtB*, *nec1*, and *tomA*, which are characteristic of the *Streptomyces* PAI (Table 2). The thaxtomin biosynthesis genes *txtA* and *txtB* were present in 77% of the pathogenic isolates. Among the isolates that were pathogenic, 50% contain the four pathogenicity genes and 17% did not contain any genes for which they were tested.

Sequence scanning of virulent factors in draft genomes of ST1020 and ST1015. In order to confirm the PCR results and corroborate the absence of virulence factors among the 17% of isolates, we selected two representative strains, ST1020 and ST1015, and we conducted a whole-genome sequencing of these strains. Our objective was to obtain draft genomes with high coverage (100x) that could be used for gene content analysis. Whole genome sequencing and assembly produced 1,569 contigs for strain ST1015 and 1,428 contigs for strain ST1020. The N50 of strain ST1015 and ST1020 were 14,127 bp and 17,382 bp, respectively. Resulting contigs were used as a nucleotide sequence database for BLAST searching. The sequences of the virulent factors were used as queries; there were no hits for any of the virulence factors among the draft genomes. The results confirmed the absence of known virulence factors in ST1020 and ST1015.

Morphological and physiological traits. A total of 331 isolates formed colonies phenotypically characteristic of *Streptomyces* spp. when grown on yeast malt extract (YME) media. Seventy pathogenic isolates and five reference strains were compared for colony and spore color en masse. Appearance and colony and spore color varied among isolates on YME media. Pathogenic isolates had colony colors ranging from cream to brown and produced spores ranging in color from white and light gray to dark gray. Based on spore and colony color, melanin production, and raffinose utilization, six different phenotypes were found: G/Be/M/R; G/Be/m/NT; G/Br/M/R; W/Be/M/NT; W/Br/m/r; and W/Br/M/NT (Table 2). These morphological and physiological traits, though consistent with previous studies (Wanner 2006), did not provide sufficient information to distinguish *Streptomyces* isolates at the species level.

Genotyping using rep-PCR. BOX and ERIC-PCR techniques were employed to analyze the genetic variability among 70 isolates of *Streptomyces* spp. causing potato common scab in Uruguay and five reference strains. The duplicate trees of each isolate exhibited similar topologies on both rep-PCR techniques. A high genetic diversity among the isolates of pathogenic *Streptomyces* spp. can be seen

in the combined dendrogram (Fig. 3). This method allowed differentiation of the five reference strains used: *S. acidiscabiei*; *S. scabiei*; *S. turgidiscabiei*; *S. europaeiscabiei*; and *S. stelliscabiei*. The cluster analysis of banding patterns revealed that some isolates cluster in proximity to reference strains. Ten isolates were associated with the reference strain of *S. acidiscabiei*. Fifteen isolates were associated with *S. scabiei* and four with *S. turgidiscabiei*. The isolate ST1229 was clustered with the reference strain of *S. europaeiscabiei* and the isolate ST1218 with *S. stelliscabiei*. Finally, 39 isolates were not associated with any *Streptomyces* spp. for which they were tested.

Phylogenetic analysis of *rpoB*. The *rpoB* phylogenetic analysis revealed genetic variation among the 52 pathogenic *Streptomyces* isolates representing distinct morphological and physiological phenotypes and different genetic profiles determined by rep-PCR analysis. In the tree shown in Figure 4, Group I (10 isolates) clustered close to *S. acidiscabiei* reference strains. The Group II (15 isolates) form a distinct cluster related to Group I. Group III (12 isolates) clustered with or in close proximity to *S. scabiei* and *S. stelliscabiei*. Group IV consisted of five isolates that are in the same cluster with *S. europaeiscabiei* and *S. turgidiscabiei*. On the other hand, 10 isolates did not cluster closed to any of the reference *Streptomyces* strains used in this analysis.

Multi locus sequence analysis (MLSA). MLSA analysis based on the comparison of partial sequences of the four housekeeping genes was performed for 28 isolates of *Streptomyces* spp. representing the different groups formed in the phylogenetic analysis of *rpoB* gene. The analysis allows differentiation of all *Streptomyces* pathogenic species. MLSA confirms the high genetic variability among the pathogenic *Streptomyces* spp. isolated in Uruguay. Overall, a good correlation was found with the previous groups identified by *rpoB* phylogenetic analysis, although a better level of discrimination between the different *Streptomyces* spp. was achieved by MLSA. The phylogenetic tree based on the concatenated sequences formed the same four congruent groups distinguished previously (Fig. 5). Group I (nine isolates) associated with the reference strain of *S. acidiscabiei* supported with a *bootstrap* of 100. Group II (eight isolates) associated with the reference strain of *S. niveiscabiei* with a *bootstrap* of 79, making up a distinct cluster close to that of Group I represented by *S. acidiscabiei*. Group III (five isolates) associated with the reference strain of *S. scabiei* with a *bootstrap* of 100. The isolate ST1229 from Group IV cluster with the reference strain of *S. europaeiscabiei* with a *bootstrap* of 99. Five of the 28 isolates did not cluster with any of the *Streptomyces* reference strain. In the present study, an isolate was assigned to a particular species if it was strongly associated in the same cluster to the corresponding reference strain, sharing high sequence identity (>99%) along the concatenated partial sequences of *atpD*, *recA*, *rpoB*, and *trpB* genes (see Table 2).

Discussion

This study constitutes the first extensive characterization of CS-inciting *Streptomyces* spp. isolated from infected tubers and soils in Uruguay. The unusually significant economic losses due to the spread of CS in Uruguay in autumn 2010 prompted an urgent need to fully understand the CS pathogen complex in as much detail as

Fig. 1. CS symptom classification on potato tubers used in this study: left, superficial lesions; middle, raised lesions; and right, pitted lesions.

possible (Lapaz et al. 2012). *Streptomyces* spp. causing CS on potato in Uruguay were comprised of a range of different species, distinguished by diverse morphological and physiological properties as well as by rep-PCR, *rpoB* sequence analysis, and multi locus sequences analysis. Seventy pathogenic *Streptomyces* spp. were characterized in the present study: 10 could be assigned to *S. acidiscabies* (Group I); 12 to *S. scabiei* (Group III); 1 isolate as *S. europaeiscabiei* (Group IV); and 15 isolates clustered in proximity to *S. neiveiscabiei* (Group II) (Table 2). Thirty-two of the 70 isolates analyzed could not be assigned to any of the five principal CS species investigated here. These isolates may be related to other pathogens of described species within the genus *Streptomyces* and were not evaluated further in this survey.

A high genetic diversity among the pathogenic *Streptomyces* spp. isolated in Uruguay can be seen in the combined dendrogram of (Fig. 3). Phylogenetic analysis based on *rpoB* sequences was reported as a powerful tool for phylogenetic analysis and species differentiation within the genera *Streptomyces* and *Kitasatospora* (Kim et al. 2004). However, our results revealed that *rpoB* phylogeny is not reliable, indicating that *rpoB* alone is not enough for *Streptomyces* spp. identification. The phylogenetic tree obtained by *rpoB* sequence comparison showed low bootstrap support and did not infer the correct evolutionary relationships among *Streptomyces* spp. For example, it has been reported that *S. europaeiscabiei* is closely related to *S. scabiei* (Bouchek-Mechiche et al. 2000); however, in Figure 4, *S. europaeiscabiei* is close to *S. turgidiscabies* but distant from

S. scabiei. In addition, *S. scabiei* and *S. stelliscabiei* were in the same cluster in the *rpoB* sequence analysis, suggesting that the *rpoB* fragment used in this study was not generally suitable for distinguishing all *Streptomyces* spp. causing CS. Phylogenetic relationships of the tree, constructed from the concatenated sequence alignment of four housekeeping genes (2111 bp) (Fig. 5), largely agreed with those inferred from analysis of *rpoB* gene sequences (Fig. 4), but bootstrap support for these groupings was significantly higher. In addition, MLSA analysis allowed us to differentiate *S. scabiei* and *S. stelliscabiei* species and to identify isolates such as *S. scabiei*, which were previously identified as *S. scabiei*/*S. stelliscabiei* by phylogenetic analysis of *rpoB*. These isolates had morphological and physiological characteristics similar to type strains of *S. scabiei* and *S. stelliscabiei*. They produced gray spores, melanin on tyrosine agar medium, and utilized raffinose as a carbon source (Table 2). *Streptomyces scabiei* is worldwide in its distribution and is dominant in the United States (Dees and Wanner 2012).

The identity of isolates previously clustered with *S. acidiscabies* by rep-PCR were confirmed through partial sequencing of *rpoB* gene and MLSA. These isolates had morphological and physiological characteristics similar to those of *S. acidiscabies* type strain DSM41668. They produced white spores, did not produce melanin on tyrosine agar, and did not utilize raffinose as a carbon source. *Streptomyces acidiscabies* was first isolated from low pH soils in the United States (Lambert and Loria 1989a) and was later found

Table 2. Phenotypic and genetic characterization of *Streptomyces* spp. isolates predicted to cause CS on potato in Uruguay and reference strains

Isolate/type strain	Genera/species ^a	Origin		Sample		Morphological and physiological traits				PAI marker genes ^e			Analysis ^f
		Year	Location	Source of isolates	Symptom	Spore color ^b	Colony color ^b	Melanin production ^c	Raffinose utilization ^d	<i>txtAB</i>	<i>necI</i>	<i>tomA</i>	
ST103	Sac	2010	San José	Tubers	Pitted	W	Br	m	r	+	+	+	rep/ <i>rpoB</i> /MLSA
ST104	Sac	2010	San José	Tubers	Pitted	W	Br	m	r	+	+	+	rep/ <i>rpoB</i>
ST105	Sac	2010	San José	Tubers	Pitted	W	Br	m	r	+	+	+	rep/ <i>rpoB</i> /MLSA
ST106	Sac	2010	San José	Tubers	Pitted	W	Br	m	r	+	+	+	rep/ <i>rpoB</i> /MLSA
ST107	Ssp	2010	San José	Tubers	Pitted	W	Br	m	r	–	+	–	rep/ <i>rpoB</i> /MLSA
ST108	Ssp	2010	San José	Tubers	Pitted	W	Br	m	r	–	–	–	rep/ <i>rpoB</i> /MLSA
ST109	Ssp	2010	San José	Tubers	Pitted	W	Br	m	r	–	–	–	rep/ <i>rpoB</i> /MLSA
ST1010	Ssp	2010	San José	Tubers	Pitted	W	Br	m	r	+	+	–	rep/ <i>rpoB</i>
ST1011	Ssp	2010	San José	Tubers	Pitted	W	Br	m	r	+	–	–	rep/ <i>rpoB</i> /MLSA
ST1012	Ssp	2010	San José	Tubers	Pitted	W	Br	m	r	–	–	–	rep/ <i>rpoB</i>
ST1013	Ssp	2010	San José	Tubers	Pitted	W	Br	m	r	–	–	–	rep/ <i>rpoB</i> /MLSA
ST1014	Ssp	2010	San José	Tubers	Pitted	W	Br	m	r	–	–	–	rep/ <i>rpoB</i>
ST1015	Ssp	2010	Canelones	Tubers	Pitted	W	Br	m	r	–	–	–	rep/ <i>rpoB</i> /MLSA
ST1016	Ssp	2010	Canelones	Tubers	Pitted	W	Br	m	r	–	+	–	rep/ <i>rpoB</i>
ST1017	Ssp	2010	Canelones	Tubers	Pitted	W	Br	m	r	–	–	–	rep/ <i>rpoB</i>
ST1018	Ssp	2010	Canelones	Tubers	Pitted	W	Br	m	r	+	+	–	rep/ <i>rpoB</i> /MLSA
ST1019	Ssp	2010	Canelones	Tubers	Pitted	W	Br	m	r	–	–	–	rep/ <i>rpoB</i>
ST1020	Ssp	2010	Canelones	Tubers	Pitted	W	Br	m	r	–	–	–	rep/ <i>rpoB</i> /MLSA
ST1021	Ssp	2010	Canelones	Tubers	Pitted	W	Br	m	r	–	–	–	rep/ <i>rpoB</i>
ST111	Sac	2011	San José	Tubers	Superficial	W	Br	m	r	+	+	+	rep/ <i>rpoB</i> /MLSA
ST112	Sac	2011	San José	Tubers	Superficial	W	Br	m	r	+	+	+	rep/ <i>rpoB</i> /MLSA
ST113	Sac	2011	Rocha	Tubers	Raised	W	Br	m	r	+	+	+	rep/ <i>rpoB</i> /MLSA
ST114	Sac	2011	Rocha	Tubers	Superficial	W	Br	m	r	+	+	+	rep/ <i>rpoB</i> /MLSA
ST115	Sac	2011	Rocha	Tubers	Raised	W	Br	m	r	+	+	+	rep/ <i>rpoB</i> /MLSA
ST116	Sac	2011	San José	Tubers	Pitted	W	Br	m	r	+	+	+	rep/ <i>rpoB</i> /MLSA
ST119	Ssp	2011	San José	Soil		W	Be	M	NT	+	+	+	rep/ <i>rpoB</i> /MLSA
ST1113	Ssc	2011	San José	Tubers	Raised	G	Br	M	R	+	+	+	rep/ <i>rpoB</i> /MLSA
ST1114	Ssc	2011	San José	Tubers	Pitted	G	Br	M	R	+	+	+	rep/ <i>rpoB</i>
ST1116	Ssp	2011	Seed	Tubers		W	Be	M	NT	+	–	+	rep
ST1118	Ssp	2011	San José	Soil		W	Be	NT	NT	+	+	–	rep/ <i>rpoB</i>
ST1119	Ssp	2011	San José	Soil		W	Be	m	NT	–	–	–	rep/ <i>rpoB</i>
ST1121	Ssp	2011	San José	Soil		G	Br	M	NT	+	+	–	rep

(continued on next page)

^a Ssp. = *Streptomyces* spp., Ssc = *S. scabiei*, Stu = *S. turgidiscabies*, Sac = *S. acidiscabies*, Seu = *S. europaeiscabiei*, and Sst = *S. stelliscabiei*.

^b G = gray, W = white, Br = brown, and Be = beige.

^c M = production of melanin, m = No production of melanin, and NT = not tested.

^d R = Utilization of raffinose, and r = no utilization of raffinose.

^e Presence (+) or absence (–) of genes associated with the *Streptomyces* PAI.

^f rep = BOX and ERIC-PCR, *rpoB* = Phylogenetic analysis of *rpoB*, and MLSA = multi locus sequence analysis. Words in bold type indicate the method/s used for species assignment for each *Streptomyces* isolate. In this study, an isolate was assigned to a particular species if it was strongly associated in the same cluster to the corresponding reference strain, sharing high sequence identity (>99%) along the concatenated partial sequences of *atpD*, *recA*, *rpoB*, and *trpB* genes. Some strains not analyzed by MLSA were identified based on rep-PCR or *rpoB* phylogeny, only if they were strongly associated with a reference strain or with an isolate assigned previously by MLSA.

in Canada, China, Japan, Korea, the UK, and Uruguay (Dees and Wanner 2012; Lapaz et al. 2012).

Group II is associated with the reference strain of *S. niveiscabiei* in MLSA, reported in Korea (Park et al. 2003). This group is near Group I in both phylogenetic analyses. The isolate ST1229 was identified as *S. europaeiscabiei* using the MLSA approach and presented similar phenotypical characteristics to said species. This constituted the first report of this pathogenic species in Uruguay. *Streptomyces europaeiscabiei* is dominant in Europe (Bouček-Mechiche et al. 2000; Dees et al. 2013; Flores-Gonzalez et al. 2008; Leiminger et al. 2013), but has also been reported in Canada, Korea, and the United States (Song et al. 2004; Wanner 2006). Phylogenetic analyses and rep-PCR showed a vast diversity of pathogenic isolates that have not been identified yet. This would suggest that there may be emerging pathogens that cause common scab, possibly due to the horizontal transference of PAIs from pathogenic to saprophytic *Streptomyces* spp. (Bukhalid et al. 2002; Healy et al. 1999; Kers et al. 2005). Based on the results obtained from this work, the best method would be MLSA (Labeda 2011), as it allows the distinction of each *Streptomyces* spp. causing common scab.

The isolates investigated in this study were obtained from lesions with varied symptoms, ranging from superficial to raised or pitted lesions (Fig. 1). There was no correlation, however, between a particular assigned species and a given type of symptom. Both *S. acidiscabies* and *S. scabiei* were identified in samples of tissue with,

e.g., superficial, raised, or pitted scab symptoms; these results are in accordance with previous studies indicating that symptoms were not species-specific (Dees et al. 2013; Loria et al. 1997). Rather, severity of symptoms seems to be influenced by inoculum level in the soil, susceptibility of the potato cultivar, as well as virulence of the pathogen strain (Loria et al. 1997).

The presence of PAI marker genes in Uruguayan *Streptomyces* isolates was found to be highly variable. Six of eight possible combinations of the four PAI markers were found among the 70 pathogenic isolates. Among the isolates that were pathogenic, 50% have

Fig. 2. Pathogenicity assays on different plant hosts. **A**, a potato tuber slices bioassay; and **B**, a radish seedling bioassay. Negative control (on the top), nonpathogenic phenotype (middle), and plant-pathogenic phenotype (on the bottom).

Table 2. (continued from preceding page)

Isolate/type strain	Genera/species ^a	Origin		Sample		Morphological and physiological traits				PAI marker genes ^e			Analysis ^f
		Year	Location	Source of isolates	Symptom	Spore color ^b	Colony color ^b	Melanin production ^c	Raffinose utilization ^d	txtAB	necI	tomA	
ST1123	Ssp	2011	San José	Soil		G	Br	m	NT	+	–	–	rep
ST1124	Ssp	2011	San José	Soil		G	Br	m	NT	+	+	+	rep/rpoB
ST1125	Ssp	2011	San José	Soil		G	Br	M	NT	+	+	+	rep/rpoB
ST1127	Ssp	2011	San José	Soil		G	Br	m	NT	+	+	–	rep
ST1128	Ssp	2011	San José	Soil		W	Br	m	NT	+	+	+	rep
ST1131	Ssp	2011	San José	Soil		G	Br	M	NT	+	–	+	rep/rpoB
ST1133	Ssp	2011	San José	Soil		W	Br	m	NT	–	–	–	rep/rpoB
ST1134	Ssp	2011	San José	Soil		G	Br	NT	NT	+	+	+	rep/rpoB/MLSA
ST1135	Ssp	2011	San José	Soil		W	Be	M	NT	+	–	–	rep
ST1136	Ssp	2011	San José	Soil		W	Br	M	NT	+	–	+	rep
ST1137	Ssp	2011	San José	Soil		G	Br	NT	NT	–	–	+	rep
ST1140	Ssp	2011	Seed	Tubers	Raised	G	Br	M	R	+	+	+	rep/rpoB/MLSA
ST1142	Ssp	2011	San José	Soil		G	Br	M	NT	+	+	–	rep
ST121	Ssp	2012	San José	Tubers	Pitted	W	Br	m	r	+	–	+	rep/rpoB/MLSA
ST123	Ssp	2012	San José	Tubers	Pitted	G	Be	M	R	+	–	+	rep/rpoB
ST124	Ssc	2012	San José	Tubers	Superficial	G	Br	M	R	+	+	+	rep/rpoB/MLSA
ST125	Ssc	2012	San José	Tubers	Pitted	G	Br	M	R	+	+	+	rep/rpoB
ST126	Ssc	2012	San José	Tubers	Superficial	G	Br	M	R	+	+	+	rep/rpoB
ST127	Ssc	2012	San José	Tubers	Pitted	G	Br	M	NT	+	+	+	rep/rpoB/MLSA
ST128	Ssc	2012	San José	Tubers	Pitted	G	Br	M	R	+	+	+	rep/rpoB
ST129	Ssc	2012	San José	Tubers	Pitted	G	Br	M	R	+	+	+	rep/rpoB/MLSA
ST1210	Ssc	2012	San José	Tubers	Pitted	G	Br	M	R	+	+	+	rep/rpoB
ST1211	Ssp	2012	San José	Soil		G	Br	M	NT	+	–	+	rep
ST1212	Ssp	2012	San José	Soil		W	Be	M	NT	+	+	+	rep
ST1213	Ssp	2012	San José	Soil		G	Br	m	NT	+	+	+	rep
ST1214	Ssp	2012	San José	Soil		G	Br	m	NT	+	+	+	rep
ST1218	Ssp	2012	San José	Soil		W	Br	m	NT	+	+	+	rep/rpoB/MLSA
ST1221	Ssp	2012	San José	Soil		G	Br	m	NT	+	–	–	rep
ST1222	Ssp	2012	San José	Soil		W	Be	m	NT	–	+	–	rep
ST1224	Ssp	2012	San José	Soil		G	Br	m	NT	+	+	–	rep
ST1225	Ssp	2012	San José	Soil		G	Br	m	NT	+	–	–	rep
ST1227	Ssp	2012	San José	Tubers	Superficial	G	Be	M	NT	+	+	+	rep/rpoB
ST1228	Ssp	2012	San José	Tubers	Superficial	G	Be	m	NT	+	–	–	rep
ST1229	Seu	2012	San José	Tubers	Pitted	G	Br	M	R	+	+	+	rep/rpoB/MLSA
ST1230	Ssc	2012	San José	Tubers	Superficial	G	Br	M	R	+	+	+	rep/rpoB
ST1232	Ssc	2012	San José	Tubers	Superficial	G	Br	M	R	+	+	+	rep/rpoB/MLSA
ST1234	Ssp	2012	San José	Tubers	Superficial	G	Br	M	R	+	+	+	rep
ST1236	Ssc	2012	San José	Tubers	Superficial	G	Br	M	R	+	+	+	rep/rpoB
DSM41658	Ssc					G	Br	M	R	+	+	+	rep/rpoB
DSM41803	Sst					G	Br	M	R	+	+	+	rep/rpoB
DSM41802	Seu					G	Br	M	R	+	+	+	rep
DSM41668	Sac					W	Br	m	r	+	+	+	rep/rpoB
DSM41838	Stu					G	Be	m	R	+	+	+	rep/rpoB

Fig. 3. Combined dendrogram based on repetitive sequence polymerase chain reaction (BOX/ERIC-PCR) fingerprints of 70 *Streptomyces* spp. isolates from Uruguay created using unweighted pair-group method using arithmetic averages (UPGMA) clustering of Dice coefficient values. Reference strains included in the dendrogram are DSM41658, DSM41803, DSM41802, DSM41668, DSM41838, and *Ralstonia solanacearum* strain UY031 as an outgroup (see Table 2 for details of the isolates).

Fig. 4. Phylogenetic relationships between 52 *Streptomyces* spp. Uruguayan isolates and reference strains (see Table 2 for details on isolates and strains) based on partial nucleotide sequences (306 bp) of the gene *rpoB*. The tree was constructed using the neighbor-joining method. Percentages at nodes represent levels of bootstrap support from 1000 resampled datasets. Bootstrap values less than 50% are not shown. *Streptomyces flavidofuscus* DSM 43111 was used as outgroup. The bar represents a phylogenetic distance of 0.01.

Fig. 5. Neighbor-joining tree based on concatenation of partial sequences of *atpD*, *recA*, *rpoB*, and *trpB* genes (2111pb) for 28 *Streptomyces* spp. isolates from Uruguay, 10 pathogenic reference strains, and *S. flavidofuscus* AS 4.1617 as an outgroup. Only bootstrap values higher than 50% are displayed at each node. The bar represents a phylogenetic distance of 0.02.

an atypical composition of PAI marker genes. We found that *necI* was missing from more than one of the pathogenic isolates tested. The tomatinase homolog-encoding gene *tomA* was absent in some pathogenic isolates, which was also consistent with previous studies showing that not all CS-inducing *Streptomyces* spp. are *tomA*+ (Wanner 2006). Both virulence-related genes, *necI* and *tomA*, have been reported to be present in many CS-inducing *Streptomyces* strains, but not in all, and they are not required for pathogenicity (Seipke and Loria 2008; Wanner 2009). *Streptomyces* lacking *txtAB* genes in 16 isolates were still able to cause pathogenicity. There have been only a few reports of pathogenic isolates that do not produce thaxtomins (Cao et al. 2012; Fyans et al. 2016; Pánková et al. 2012; Wanner 2004) and isolates seem to be geographically restricted. Unknown pathogenicity factors in addition to thaxtomins were recently suggested by comparative genomic analyses of *S. scabiei* 87-22 genome sequence (GenBank Accession No. NC_013929) (Bignell et al. 2010).

Twelve isolates that lacked all four pathogenicity marker genes were able to induce necrosis on potato slices and stunting of radish seedling (Table 2). The absence of pathogenicity genes was determined by PCR and confirmed by genomes sequencing analysis of two representative strains of this group. Jordaan and van der Waals (2016) reported pathogenic isolates with these characteristics. It is possible that these isolates have novel pathogenicity factors. Genetic variation regarding the presence or absence of genes associated with the *Streptomyces* PAI element can be expected in that it is known to be horizontally transferred as a unit or in modules by site-specific recombination (Huguet-Tapia et al. 2014; Zhang et al. 2016). It is also known that pathogenicity and virulence-related gene clusters in *S. turgidiscabies* have multiple origins, and that a given PAI consists of a mosaic of regions that may undergo independent evolution (Aittamaa et al. 2010). The heterogeneous composition of PAI, and the fact that species are constantly evolving, may contribute to the emergence of new pathogenic species.

This study shows that diversity of CS-inciting species in Uruguay is greater than previously thought and may include new, not previously reported *Streptomyces* spp. that are associated with CS. Genome sequencing studies for more CS strains isolated in Uruguay are ongoing. It is expected that their genomic analysis could contribute to clarification of the interesting results about the pathogenic behavior of these strains.

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