Phylogenetic Analysis of *Streptomyces* spp. Causing Potato Scab Based on 16S rRNA Sequences

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The complete 16S rRNA sequences of 12 strains of *Streptomyces* spp., including potato scab pathogens, were determined. Among the strains of *Streptomyces scabies* that were isolated from diverse geographical areas and differed in melanoid pigment production, either no difference or one difference in sequence was observed. The sequence of *S. scabies* was most similar to the sequences of *Streptomyces diastatochromogenes*, *Streptomyces bottropensis*, and *Streptomyces neyagawaensis*, which belong to the *Diastatochromogenes* group. The levels of similarity of the 16S rRNA sequences of *Streptomyces acidiscabies* and *S. scabies* were almost the same as the levels of similarity between *S. acidiscabies* and other *Streptomyces* strains. *Streptomyces* sp. strain 91-Sy-13, which was isolated in Japan from potato scab and belongs to a distinct species on the basis of phenotypic characteristics and DNA relatedness, exhibited lower levels of 16S rRNA sequence similarity with other potato scab pathogens, as well as other *Streptomyces* species. The phylogenetic tree constructed on the basis of 16S rRNA sequence data showed that the *Streptomyces* spp. that cause potato scab composed unique branches. The results of our phylogenetic analysis based on complete 16S rRNA sequences confirmed the lack of close relationships among *Streptomyces* spp. that cause potato scab. Our findings suggest that potato scab is caused by phylogenetically diverse *Streptomyces* species and that the pathogenicities of these organisms developed independently.

Scab diseases of potatoes (*Solanum tuberosum*) are caused by *Streptomyces* spp. that are widely distributed in potato-growing areas. These diseases are characterized by superficial or deep corky lesions on tubers. Depending on the *Streptomyces* strain and the environment, slightly raised lesions may occur. Although these diseases have little or no effect on total yield, the value of affected tubers is reduced, resulting in diminished marketable yields, and the content and quality of the starch in the tubers are decreased.

The taxonomic position of the bacteria that cause common scab has been confused for many years. Lambert and Loria (13) demonstrated that the majority of pathogenic streptomycetes form a distinct species consistent with the original species description and provided phenotypic criteria which differentiate this species from other species. These authors proposed that the name *Streptomyces scabies* should be revived for this pathogen. In addition, a new species, *Streptomyces acidiscabies*, which causes potato scab in soils having pH values below 5.2, has also been described (14). The acid scab symptoms caused by *S. acidiscabies*, which is distinct from *S. scabies* in phenotypic characteristics, are indistinguishable from the symptoms of common scab caused by *S. scabies*.

Additional *Streptomyces* species, including *Streptomyces setonii*, *Streptomyces griseus*, *Streptomyces tendae*, *Streptomyces aureofaciens*, and others (3, 8, 10, 13–15), have also been described as potato scab pathogens, but these organisms are usually less virulent. Furthermore, *Streptomyces* strains that cause deep-pitted scab of potato have been described by several authors (1, 4, 6, 7).

Potato scab has been a limiting factor in potato production in Japan, and *S. scabies* is the predominant species responsible for the disease. However, potato scab caused by a *Streptomyces* sp. other than *S. scabies* and *S. acidiscabies* has occurred fre-

quently in Hokkaido, the major potato-growing area in Japan (25, 27, 29, 30). Although the scab symptoms caused by this pathogen do not differ from those caused by *S. scabies*, the phenotypic characteristics of this organism clearly differ from those of other pathogens.

The purpose of this study was to determine the phylogenetic relationships among the *Streptomyces* spp. that cause potato scab and their relatives on the basis of complete 16S rRNA sequences.

MATERIALS AND METHODS

Bacterial strains. *S. scabies* ATCC 49173^T (T = type strain) (13) and ATCC 33282 and melanoid-negative strain SNS-26 (25, 28) were isolated in the United States, Hungary, and Japan, respectively. *S. acidiscabies* ATCC 49003^T has been described by Lambert and Loria (14). *Streptomyces* sp. strain 91-Sy-13 was isolated in Japan and has been described as a potato scab pathogen belonging to a distinct species on the basis of its phenotypic characteristics (25, 27). The type strains of species placed in the *Diastatochromogenes* group (5), *Streptomyces diastatochromogenes* ATCC 12309, *Streptomyces bottropensis* ATCC 25435, *Streptomyces neyagawaensis* ATCC 27449, and *Streptomyces eurythermus* ATCC 14975, were also investigated, as were a saprophyte isolated from scab lesions, *Streptomyces sampsonii* ATCC 25495^T, and the type strains of *Streptomyces* species described as less virulent pathogens, *S. setonii* ATCC 25497 and *S. tendae* ATCC 19812.

16S rRNA sequencing. The sequences of 16S rRNAs were determined by PCR (18) by using the following method. The DNA template for PCR was extracted from cells by using an InstaGene DNA purification matrix (Bio-Rad Laboratories) according to the supplier's instructions. The primers used for PCR amplification were chosen by inspecting the previously published 16S rRNA sequences of *S. griseus* subsp. *griseus* (11), *Streptomyces ambifaciens* (17), *Streptomyces lividans* (26), and *Streptomyces coelicolor* (2). The PCR primers contained the sequences of Cy5-labeled primers for cycle sequencing reactions. The 16S rRNA coding region of the total DNA was amplified by using the following three primer sets: (i) 5'-GCCAGATATGTTGATACC-3', corresponding to positions 901 to 918 of *S. griseus* subsp. *griseus* (11) flanking regions, or 5'-CGCCAGGGTTTTC CAGTCACGACTCAGGAGAGTTTGTATCC-3', corresponding to positions 4 to 21 of *S. griseus* subsp. *griseus* 16S rRNA, and 5'-TTTCACACAGGAAACAGC TATGACCTTTACGCCCAATAATTCCG-3', corresponding to the complement of positions 530 to 549; (ii) 5'-CGCCAGGGTTTTC CAGTCACGAGCAG CAGCCGCGTAATAC-3', corresponding to positions 493 to 510, and 5'-TT TCACACAGGAAACAGCTATGACGCGTCGAATTAAGCCACATG-3', corresponding to the complement of positions 924 to 943; and (iii) 5'-CGC CAGGGTTTTC CAGTCACGACAACTCAAAGGAATTGACGG-3', corre-

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sponding to positions 883 to 902, and 5'-TTTCACACAGGAAACAGCTATGA CAGAAAGGAGGTGATCCAGCC-3', corresponding to positions 1509 to 1528, or 5'-CACGTCCTTCATCGGTTCTGGTGCC-3', corresponding to the complement of positions 35 to 60 of 23S rRNA (the underlined portions of the primers correspond to the sequences of Cy5-labeled primers). PCR amplification of the target sequence was performed in a 50- μ l (total volume) reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1.5 mM MgCl₂ each deoxynucleoside triphosphate at a concentration of 200 μ M, 5 pmol of each primer, 2.5 μ l of template, and 2.5 U of *Taq* polymerase (TOYOBO). PCR was performed with a model 480 DNA thermal cycler (Perkin-Elmer Cetus) by using the following protocol: initial denaturation at 95°C for 2.5 min, followed by 35 cycles consisting of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min, and an additional extension step consisting of 72°C for 10 min. One primer of each set of primers was biotinylated, the biotinylated PCR products were immobilized on streptavidin-coated paramagnetic beads (Dynabeads M-280 streptavidin; DYNAL), and single-stranded DNA templates were prepared by following the manufacturer's instructions. Both strands were then sequenced directly by cycle sequencing by using an ALFred autocycle sequencing kit (Pharmacia Biotech) and the following Cy5-labeled primers: primer ALFred M13-40 (5'-CGCCAGGGTTT TCCAGTCACGAC-3') and reverse primer ALFred M13 (5'-TTTCACACAG GAAACAGCTATGAC-3'). The sequencing products were loaded onto a 6% polyacrylamide gel, and separation was monitored on-line with an ALFred DNA sequencer (Pharmacia Biotech).

Phylogenetic analysis. The sequences of the 16S rRNAs of *S. griseus* subsp. *griseus* (11), *S. ambifaciens* (17), *S. lividans* (26), and *S. coelicolor* (2) were obtained from the EMBL and GenBank databases for comparison. The genetic distances between sequences were estimated by using K_{nuc} values (12). A phylogenetic tree was constructed by the neighbor-joining method (19).

Nucleotide sequence accession numbers. The sequences of the strains investigated in this study have been deposited in the GenBank, EMBL, and DDBJ (DNA Data Base of Japan, Mishima, Japan) under the following accession numbers: D63862 for ATCC 49173^T, D63863 for ATCC 33282, D63864 for SNS-26, D63865 for ATCC 49003^T, D63866 for 91-Sy-13, D63867 for ATCC 12309^T, D63868 for ATCC 25435^T, D63869 for ATCC 27449^T, D63870 for ATCC 14975^T, D63871 for ATCC 25495^T, D63872 for ATCC 25497^T, and D63873 for ATCC 19812^T.

RESULTS AND DISCUSSION

The complete sequences of the 16S rRNAs of 12 strains of *Streptomyces* spp., including potato scab pathogens, were determined. The sequences from position 71 to position 210 are shown in Fig. 1. The sequences of the regions including base deletions were aligned with reference to the putative secondary structures of *S. griseus* subsp. *griseus* (11) and *S. ambifaciens* (17). A highly variable region was observed at positions 177 to 206, as shown in Fig. 1, and this region may be valuable for rapid identification of *Streptomyces* species. A matrix of the levels of sequence similarity of strains belonging to the genus *Streptomyces* is shown in Table 1, and the phylogenetic tree derived from K_{nuc} values is shown in Fig. 2. All of the levels of similarity among the *Streptomyces* species examined in this study were more than 95.4%.

When strains of *S. scabies* which were isolated in diverse geographical regions and which differed in melanoid pigment production were compared, either no different base or a single different base at position 1441 was observed. However, previous studies have shown that *S. scabies* does not form a relatively homogeneous group on the basis of DNA relatedness (9) and fatty acid and protein profiles (16). The relationships of the strains investigated in these studies should be determined in a future study.

Phenotypically, *S. scabies* is most closely related to the Diastatochromogenes group of Elesawy and Szabo (5), which is represented by the *Streptomyces cyaneus*, *Streptomyces diastaticus*, and *Streptomyces microflavus* cluster groups of Williams et al. (31). In DNA-DNA hybridization studies (9), *S. scabies* exhibited the highest levels of relatedness with *S. diastatochromogenes* and *S. bottropensis*, which represent the *S. diastaticus* group, whereas the levels of relatedness with *S. neyagawaensis* (*S. cyaneus* group) and *S. eurythermus* (*S. microflavus* group) were lower. In this study, *S. scabies* exhibited the highest levels of 16S rRNA similarity with *S. diastatochromogenes* (98.8 to

FIG. 1. Sequence alignment of 16S rRNAs encoding DNAs for 12 strains of *Streptomyces* spp., including potato scab pathogens (positions 71 to 210). The sequences are aligned with the sequence of *S. scabies* ATCC 49173^T. Only the nucleotides that differ from those of *S. scabies* ATCC 49173^T are shown; identical nucleotides are indicated by dots, and deletions are indicated by dashes. The numbers on the sequence of *S. scabies* ATCC 49173^T indicate positions from the 5' end of the 16S rRNA. Abbreviations: sca1, *S. scabies* ATCC 49173^T; sca2, *S. scabies* ATCC 33282; sca3, *S. scabies* SNS-26; aci, *S. acidiscabies*; 91Sy, *Streptomyces* sp. strain 91-Sy-13; dia, *S. diastatochromogenes*; bot, *S. bottropensis*; ney, *S. neyagawaensis*; eur, *S. eurythermus*; sam, *S. sampsonii*; set, *S. setonii*; ten, *S. tendae*; gri, *S. griseus* subsp. *griseus* (11); amb, *S. ambifaciens* (17); liv, *S. lividans* (26); coe, *S. coelicolor* (2).

98.9%), *S. bottropensis* (98.9 to 99.0%), and *S. neyagawaensis* (98.8 to 98.9%). The levels of similarity were lower with *S. eurythermus* (96.4 to 96.5%) and *S. ambifaciens* (96.3%), which are classified in the *S. microflavus* group of Williams et al. (31), and with the other *Streptomyces* spp. examined in this study (less than 97.8%).

The acid-tolerant potato scab pathogen *S. acidiscabies* can be differentiated from *S. scabies* by morphological and physiological characteristics (14) and DNA relatedness data (9). In this study, the levels of similarity between *S. acidiscabies* and all other species, including *S. setonii* and *S. tendae*, which were most similar to this species phenotypically (14), were less than 97.6%. The levels of 16S rRNA sequence similarity between *S. acidiscabies* and *S. scabies* (97.4 to 97.5%) were almost the same as the levels of similarity between *S. acidiscabies* and other *Streptomyces* species. This phylogenetic analysis showed that *S. acidiscabies*, which causes acid scab, is clearly separated from the major potato scab pathogen, *S. scabies*, which causes common scab.

Potato scab caused by a *Streptomyces* sp. other than *S. scabies* and *S. acidiscabies* has occurred frequently in Japan (25, 27, 29, 30). This organism and *S. scabies* are the predominant species responsible for the disease in Hokkaido, the major potato-growing area in Japan. The scab symptoms caused by this pathogen (represented by strain 91-Sy-13) are indistinguishable from those caused by *S. scabies*. This species is characterized by smooth grey spores in flexuous chains, no melanin production, no diffusible pigment production, usage of all International *Streptomyces* Project sugars, no degradation of xanthine, no growth at 37°C or in the presence of 7% NaCl, susceptibility to 20 μ g of streptomycin per ml and 10 IU of penicillin G per ml, and a guanine-plus-cytosine content of 65 mol% (25, 27). This organism clearly differs from *S. scabies*, *S. acidiscabies*, and other potato scab pathogens described previously (3, 6–8, 10, 13–15) in its phenotypic characteristics and

TABLE 1. Similarity matrix for 16S rRNA sequences of *Streptomyces* spp., including potato scab pathogens

Strain	% Homology with:														
	<i>S. scabies</i> ATCC 49173 ^T	<i>S. scabies</i> ATCC 33282	<i>S. scabies</i> SNS-26	<i>S. acidiscabies</i>	<i>Streptomyces</i> sp. strain 91-Sy-13	<i>S. diastatochromogenes</i>	<i>S. bottropensis</i>	<i>S. neyagawaensis</i>	<i>S. eurythermus</i>	<i>S. sampsonii</i>	<i>S. setonii</i>	<i>S. tendae</i>	<i>S. griseus</i> subsp. <i>griseus</i>	<i>S. ambofaciens</i>	<i>S. lividans</i>
<i>S. scabies</i> ATCC 49173 ^T															
<i>S. scabies</i> ATCC 33282	99.9														
<i>S. scabies</i> SNS-26	99.9	100.0													
<i>S. acidiscabies</i> ATCC 49003 ^T	97.4	97.5	97.5												
<i>Streptomyces</i> sp. strain 91-Sy-13	97.8	97.8	97.8	97.2											
<i>S. diastatochromogenes</i> ATCC 12309 ^T	98.8	98.9	98.9	97.6	97.5										
<i>S. bottropensis</i> ATCC 25435 ^T	98.9	99.0	99.0	96.9	97.2	98.4									
<i>S. neyagawaensis</i> ATCC 27449 ^T	98.8	98.9	98.9	97.6	98.1	98.6	98.5								
<i>S. eurythermus</i> ATCC 14975 ^T	96.4	96.5	96.5	96.8	96.2	96.4	96.6	96.3							
<i>S. sampsonii</i> ATCC 25495 ^T	95.9	96.0	96.0	96.8	96.9	96.0	95.4	95.9	98.4						
<i>S. setonii</i> ATCC 25497 ^T	97.1	97.2	97.2	97.2	97.4	97.0	97.5	97.2	96.4	95.7					
<i>S. tendae</i> ATCC 19812 ^T	96.5	96.5	96.5	96.9	96.2	96.3	96.7	96.1	99.0	97.9	96.6				
<i>S. griseus</i> subsp. <i>griseus</i> KCTC 9080	96.9	97.0	97.0	97.2	97.6	96.8	97.3	97.2	96.4	95.9	99.7	96.4			
<i>S. ambofaciens</i> ATCC 23877 ^T	96.3	96.3	96.3	96.5	96.1	96.2	96.5	96.1	98.8	97.8	96.3	99.0	96.1		
<i>S. lividans</i> TK21	96.6	96.7	96.7	97.0	96.1	96.5	96.8	96.3	98.9	97.5	96.7	99.6	96.5	99.0	
<i>S. coelicolor</i> A3(2)	96.3	96.4	96.4	96.7	95.8	96.2	96.5	96.0	98.6	97.2	96.5	99.3	96.3	98.7	99.6

levels of DNA-DNA relatedness (27). The characteristics obtained by performing 91 physiological and morphological tests showed that this species formed a homogeneous group that could be clearly differentiated from the other *Streptomyces* species described by Shirling and Gottlieb (20–24) and Williams et al. (31). Therefore, it will be necessary to establish a new species for this organism (unpublished data). In this study, the phylogenetic relationships between strain 91-Sy-13 and other *Streptomyces* spp., including potato scab pathogens, were investigated. The levels of similarity between strain 91-Sy-13 and *S. scabies* (97.8%) and between strain 91-Sy-13 and *S.*

acidiscabies (97.4 to 97.5%) were relatively low, and the levels of similarity between strain 91-Sy-13 and other *Streptomyces* spp., including the less virulent strains (3, 8, 10, 13–15), were less than 97.8%. These results suggest that strain 91-Sy-13 is phylogenetically distinct from *S. scabies*, *S. acidiscabies*, and other potato scab pathogens.

The phylogenetic tree constructed on the basis of 16S rRNA sequence data (Fig. 2) shows that the *Streptomyces* spp. that cause potato scab compose unique branches. This phylogenetic analysis confirmed the lack of close relationships among the *Streptomyces* spp. that cause potato scab. Our findings suggest

FIG. 2. Phylogenetic tree derived from whole 16S rRNA sequences of *Streptomyces* spp., including potato scab pathogens.

that potato scab is caused by phylogenetically diverse *Streptomyces* species and that the pathogenicities of these organisms developed independently.

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