Pathogenicity of *Streptomyces scabies* Mutants Altered in Thaxtomin A Production

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ABSTRACT

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To investigate the role of thaxtomin A in the pathogenicity of *Streptomyces scabies*, mutants altered in thaxtomin A production were obtained by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis. Mutants of *S. scabies* EF-35 could be differentiated according to levels of thaxtomin production. Mutants M1, M8, and M19 produced 2 to 20 times less thax-

tomin A in oat bran medium than did EF-35. M1 and M19 were deficient in tryptophan catabolism. Thaxtomin production was reduced by about 300 times in mutant M16, which was a glutamic acid auxotroph. No thaxtomin A was detected in M13 culture supernatant. This mutant had a normal growth rate, was prototrophic, and catabolized tryptophan. Pathogenicity of mutants was tested on radish and potato. Mutants M1, M8, and M19 were pathogenic but, in most cases, less virulent than EF-35. M13 and M16 were nonpathogenic. These results suggest that thaxtomin A is an important pathogenicity determinant in *S. scabies*.

Phytotoxins are produced by several plant-pathogenic bacteria, such as *Pseudomonas tolaasii* (3), *P. syringae* pv. *syringae* (31), *P. syringae* pv. *phaseolicola* (28), *P. syringae* pv. *tagetis* (22,25), and *P. syringae* pv. *glyciniae* (27). These compounds elicit a variety of symptoms including wilting, chlorosis, necrosis, water-soaking, and growth abnormalities. In 1989, a new group of phytotoxins associated with plant-pathogenic streptomycetes was discovered (16) and called thaxtomin. Thaxtomins consist of 4-nitroindol-3-yl-containing 2,5-dioxopiperazines, and they were first isolated from common scab lesions induced by *Streptomyces scabies* on potato (*Solanum tuberosum* L.) tissues. Thaxtomin A, the major phytotoxin produced by *S. scabies*, is composed of two modified *N*-methylated amino acids, α -hydroxy-*m*-tyrosine plus 4-nitro-tryptophan (15,18). Thaxtomin A is produced by *S. scabies* on potato tissues and also in some culture media containing plant extracts such as oatmeal broth (1) or potato peel broth (8). Thaxtomins are not produced in minimal or rich media without plant extracts (20).

Evidence for a crucial role of thaxtomin A in pathogenesis continues to accumulate (10,17-20). Application of purified thaxtomin A on potato mini-tubers causes necrosis of treated tissues (18). Thaxtomin A also inhibits growth of seedlings such as radish (*Raphanus sativus* L.), alfalfa (*Medicago sativa* L.), cauliflower (*Brassica oleracea* L.), colza (*Brassica napus* L.), and turnip (*Brassica rapa* L.) (19). Plant tissues treated with thaxtomin A show thickening of their cell walls and hypertrophy (19). Furthermore, King et al. (17) established a positive correlation between pathogenicity of *S. scabies* and thaxtomin production. Loria et al. (20) also suggested that symptom severity on potato tubers correlates with the amount of thaxtomin A produced by different potato scab-inducing streptomycetes. Healy et al. (10) recently showed that mutants of *S. scabies* defective in thaxtomin A production are nonpathogenic on radish seedlings.

Thaxtomin biosynthesis is not limited to *S. scabies*; other streptomycetes causing potato scab such as *S. acidiscabies* (17) and *S. caviscabies* (8), as well as the causal agent of soil rot of sweet pota-

to, *S. ipomoeae* (12), produce thaxtomin. Thaxtomin production thus appears as a common feature of plant-pathogenic streptomycetes.

In this work, the involvement of thaxtomin A in the pathogenicity of *S. scabies* strain EF-35 was analyzed. EF-35 was isolated in Québec, Canada, from a scab lesion on a potato tuber (5). This strain causes common scab not only on potato, but also on carrot, sugar beet, turnip, parsnip, and radish (7). EF-35 produces an average of 2.2 μ g of thaxtomin A per 1 mg of cells (dry weight) in oat bran broth. Following *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) mutagenesis, mutants of *S. scabies* EF-35 altered in thaxtomin A production were obtained and briefly characterized, and their pathogenic behavior was analyzed on both radish seedlings and potato tubers.

MATERIALS AND METHODS

Chemical mutagenesis and selection of mutants altered in thaxtomin A production. NTG mutagenesis was carried out on spores of *S. scabies* EF-35 (7) as described by Hopwood et al. (11). Following mutagenesis, *S. scabies* spores were diluted, plated on yeast malt extract agar (29), and grown for 5 days at 30°C. Developing colonies were transferred to oat bran agar for detection of thaxtomin production. Oat bran agar was prepared by boiling 40 g of oat bran per 1 liter of water for 15 min. Insoluble matter was removed by centrifuging 15 min at 10,000 $\times$ g. Supernatant was kept and 2% agar was added. After 6 days of incubation at 30°C, production of thaxtomin was detected by the presence of a yellowish halo surrounding the colonies, and recorded. Colonies with no detectable halo were restreaked twice on fresh oat bran agar.

Production and purification of thaxtomin A. Overnight cultures of *S. scabies* EF-35 and of mutants, grown with shaking (300 rpm) at 30°C in 25 ml of tryptic soy broth (TSB) (Difco Laboratories, Detroit), were centrifuged in graduated tubes. The volume of each pellet was measured, and these were then diluted in five volumes of fresh TSB. An aliquot of 80 μ l of each fresh cell suspension was used to inoculate 25 ml of oat bran broth, and the cultures were incubated with shaking (300 rpm) at 30°C for 8 days. After centrifugation of the bacterial cultures, the pellets were dried overnight in an oven at 80°C and weighed; thaxtomin A was extracted from supernatants using an equal volume of ethyl acetate. The ethyl acetate phase containing thaxtomin A was concentrated

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on a Büchi Rotavapor R-114 (Büchi Labortechnik AG, Flawil, Switzerland). The resulting material was redissolved in ethyl acetate and separated by thin-layer chromatography on glass plates pre-coated with 0.25 mm of silica gel 60, using chloroform/methanol (9:1) as migration solvents. Yellow products with an R_f of 0.27 (thaxtomin A) were eluted from the silica gel using chloroform/methanol (7:3). Thaxtomin extraction experiments were carried out on three different cultures for each strain.

Quantification of thaxtomin A. The level of thaxtomin A production between the wild-type strain and the selected mutants was compared using a sensitive test as described below. Thaxtomin A was quantified by high-performance liquid chromatography (HPLC) using a Varian LC5500 liquid chromatograph (Varian Chromatography Systems, Walnut Creek, CA) equipped with a Water's C18 column (10- μ m particle size, 3.9 mm $\times$ 125 mm; Waters Corporation, Milford, MA). Thaxtomin A was eluted from the column with a 25 to 50% acetonitrile gradient for 20 min at a flow rate of 1.3 ml/min and monitored at 249 nm using a UV detector. UV spectroscopic analyses were performed with an LKB spectrophotometer (model Ultrospec III; LKB Biochrom, Cambridge) using the extinction coefficient of thaxtomin A (249 nm, ϵ 15070) determined by King et al. (15). A standard curve was established using dilutions of a known quantity of purified thaxtomin A.

Characterization of mutants. The ability of each mutant to sporulate was tested by spreading 1 ml of a 3-day-old TSB culture of the mutant or the wild-type strain EF-35 onto SLM3 agar (10 g of starch, 5 ml of corn steep liquor [Sigma Chemical Co., St. Louis], 3 g of CaCO_3 , 12 mg of Fe_2SO_4 , and 20 g of agar per liter). The presence of spores was recorded after incubation for 7 days at 30°C.

Minimal medium, used to test bacterial growth, consisted of 10 g of glucose, 12 g of agar, 0.5 g of K_2HPO_4 , 0.2 g of MgSO_4 , and 0.01 g of FeSO_4 per 1 liter of water (10). Amino acids or $(\text{NH}_4)_2\text{SO}_4$ were used as a nitrogen source and were added to minimal medium at a final concentration of 0.1 and 0.2%, respectively. Growth on minimal media was observed after incubation for 7 days at 30°C.

To compare the growth rate, cell suspensions of each mutant and wild-type strain EF-35 were adjusted to the same concentration and were used to inoculate three flasks containing 300 ml of TSB. Bacteria were grown with shaking (300 rpm) at 30°C for 45 h. At intervals, 10-ml aliquots of the cultures were removed and centrifuged to determine the dry mass of mycelium as described above.

Pathogenicity tests. Pathogenicity of *S. scabies* EF-35 and of mutants was tested on *Solanum tuberosum* cv. Kennebec according to Faucher et al. (5). Potato tubers were harvested after 8 weeks and examined for common scab symptoms. The number of tubers presenting symptoms was recorded. The coverage of common scab lesions on each infected tuber was estimated visually.

Pathogenicity tests were also carried out on radish seedlings as described by Leiner et al. (19) with the following modifications. Seeds of radish cv. Cherry Belle were sterilized 5 min with 0.26%

NaOCl and were then rinsed twice with sterile water. The seeds were then germinated in the dark for 24 h in petri dishes containing 15 g of agar per 1 liter of water. Individual seedlings were transferred to the top of an agar slant (1.5%) in 50-ml tubes. Bacterial inocula used to infect radish seedlings were prepared as follows: bacteria were grown with shaking (300 rpm) in TSB for 2 days at 30°C. Cultures were centrifuged in graduated tubes, and the volume of the pellets was measured. Bacterial mycelia were resuspended in a volume of fresh TSB equivalent to five volumes of pellet, and these cell suspensions were used as inocula. An inoculum (200 μ l) of *S. scabies* wild-type strain EF-35 or of each mutant was applied to radish seedlings, whereas 200 μ l of sterile TSB was added to control seedlings. These seedlings were incubated in a growth chamber at 23°C on a 16-h photoperiod. Seedlings were grown under Gro Lux wide-spectrum fluorescent lighting (General Electric, Cleveland) with a quantum flux density of 175 $\mu\text{E m}^{-2} \text{s}^{-1}$. After incubation for 6 days, radish seedlings were removed from the tubes and their lengths measured. The difference between the length of uninoculated controls and treated seedlings was divided by the length of controls to evaluate growth inhibition.

A single pathogenicity test comprised five replicates. All pathogenicity tests experiments were repeated three times.

RESULTS

Chemical mutagenesis and selection of mutants altered in thaxtomin A production. Six thousand colonies of *S. scabies* strain EF-35 were recovered from the NTG mutagenesis, and about 1.5% of them were auxotrophic mutants. These 6,000 colonies were screened for thaxtomin production on oat bran agar. Thaxtomins are yellow compounds (15). Production of thaxtomin by *S. scabies* on oat bran agar can be detected by the presence of a yellowish halo surrounding colonies. Five mutants (M1, M8, M13, M16, and M19) produced no detectable yellowish halo on oat bran agar. Selected mutants were then compared with the wild-type strain EF-35 for thaxtomin production in oat bran broth. The wild-type strain produced approximately 2,240 ng of thaxtomin A per 1 mg of dried cells after incubation for 8 days in oat bran broth. Mutants were differentiated according to level of thaxtomin A production. Mutants M1, M8, and M19 produced 2 to 20 times less thaxtomin A than did the wild-type strain EF-35. Thaxtomin A production for mutants M1, M8, and M19 ranged from 227 to 1045 ng/mg (Table 1).

M16 produced 355 times less thaxtomin A than did the wild-type strain EF-35. A small amount of thaxtomin A (6.3 ng/mg) was recovered in the supernatant of mutant M16 after incubation for 8 days (Table 1). M13 was the only mutant producing no detectable amount of thaxtomin A after incubation for 8 days in oat bran broth (Table 1).

Characterization of mutants. As did the wild-type strain, all mutants except M16 grew on minimal medium containing an in-

TABLE 1. Pathogenicity on radish seedlings and on potato tubers of *Streptomyces scabies* and of mutants altered in thaxtomin A production

Strain or treatment	Amount of thaxtomin A (ng/mg of dried mycelium) ^w	Pathogenicity on ^x		
		Radish seedlings	Potato tubers	Surface of infected tubers covered by lesions (%)
		Growth inhibition (%)	Infected potato tubers (%)	
EF-35	2239.2 a ^y	72 a ^y	76 a ^z	20 a ^z
M8	1045.4 b	42 bc	22 c	4 bc
M19	309.5 c	42 bc	37 bc	4 bc
M1	227.3 c	59 ab	54 ab	9 b
M16	6.3 d	26 cd	0 d	0 c
M13	0.0 d	27 cd	0 d	0 c
Noninoculated medium	0.0 d	0.0 d	0 d	0 c

^w Amount of thaxtomin A produced in oat bran broth. Results are the mean of three replications.

^x The results of three independent pathogenicity test experiments are combined, because there was no significant difference between experiments for the same treatment variable ($P < 0.05$). A single experiment comprised five replicates.

^y Numbers accompanied by the same letters are not significantly different ($P < 0.05$, t test).

^z Numbers accompanied by the same letters are not significantly different ($P < 0.05$, series of $2 \times 2 \chi^2$ test).

organic nitrogen source (Table 2). Mutant M16 did not grow on a minimal medium containing $(\text{NH}_4)_2\text{SO}_4$ as the sole nitrogen source. However, the growth of this auxotrophic mutant was restored when glutamic acid was supplied to minimal medium (Table 2). Glutamine also restored growth of M16 on minimal medium, but the growth remained poor compared with that of the wild-type strain. Although M1 and M19 could grow on minimal medium containing $(\text{NH}_4)_2\text{SO}_4$, these mutants did not grow on minimal media when tryptophan was used as the sole nitrogen source (Table 2). By contrast, they could utilize other amino acids such as L-alanine, L-glutamic acid, L-phenylalanine, L-serine, and L-tyrosine (Table 2).

Growth curves in TSB were similar for wild-type strain EF-35 and for all mutants (data not shown). All mutants except M16 sporulated on SLM3 (Table 2). However, addition of 0.1% L-glutamic acid to SLM3 restored the ability to sporulate in M16.

Pathogenicity tests on radish seedlings. Radish seedlings inoculated with mutants M1, M8, and M19 or with wild-type strain EF-35 showed growth inhibition ranging from 42 to 72% when compared with that of noninoculated controls. EF-35 and M1 were the most virulent strains, causing 72 and 59% growth inhibition, respectively. M8 and M19 were less virulent than EF-35 on radish seedlings. Indeed, radish seedlings inoculated with mutants M8 and M19 were significantly taller than those inoculated with the wild-type strain, yet they remained shorter than the noninoculated controls. Even though mutants M13 and M16 inhibited growth by 27 and 26%, respectively, the differences in lengths between noninoculated and inoculated seedlings was not significantly different for these two mutants (Table 1).

Pathogenicity tests on potato tubers. *S. scabies* strain EF-35 induced common scab on 76% of the progeny tubers, and the lesions covered an average of 20% of the tuber surface (Table 1). Mutant M1 caused common scab symptoms on only 54% of the progeny tubers; however, the difference in infection incidence between EF-35 and M1 was not significantly different ($P < 0.05$). Nevertheless, M1 was shown to be significantly less virulent than the wild-type strain; an average of only 9% of the tuber surface was covered by scab lesions for M1 as compared with 20% for EF-35 (Table 1). M19 and M8 were also significantly less virulent than the wild-type strain; the percentage of infected tubers and the coverage of scab lesions being reduced by factors of 2.0 to 3.5 and of 5.0, respectively (Table 1). Mutants M13 and M16, which showed the greatest reduction in thaxtomin A production, were nonpathogenic on potato (Table 1).

DISCUSSION

Studies have suggested that thaxtomin A acts as an important pathogenicity determinant in plant-pathogenic *Streptomyces* spp. (17–20). To further investigate the role of thaxtomin A in pathogenicity, we analyzed the pathogenic behavior of *S. scabies* mutants altered in thaxtomin A production. Mutants were selected for the absence of a yellowish halo around colonies growing on oat bran agar. Most of the selected mutants still produced thaxtomin A, even

though no yellowish halo was detected on oat bran agar. Opacity of the oat bran medium probably prevents the detection of faint halos. Nevertheless, oat bran agar appears to be a good screening medium, because all selected mutants were severely reduced in thaxtomin A production and because melanoid pigments (9) that could mask thaxtomins were not produced by *S. scabies* in oat bran medium.

Mutants producing up to 20 times less thaxtomin A than the wild-type strain were all pathogenic on potato, as well as on radish seedlings. In these mutants, reduction in thaxtomin A biosynthesis coincided with a reduced virulence on potato. For mutants M8 and M19, the decrease in the amount of thaxtomin A produced in oat bran broth also coincided with reduced virulence on radish seedlings. Radish seedlings thus appear to be a useful tool to screen for pathogenic *S. scabies*. This system could be considered a useful alternative to using potato tubers to screen for plant-pathogenic streptomycetes, especially because testing pathogenicity on potato is time-consuming and requires important greenhouse or growth chamber space.

No strict correlation was found between virulence and thaxtomin A production. For example, M8 produced about three times more thaxtomin A than did M1 and M19, but was less virulent on plants. However, thaxtomin production in oat bran broth might not necessarily reflect the in planta production of thaxtomin on potato tubers or radish seedlings. In a previous study, Loria et al. (20) suggested that differential virulence of two *S. scabies* strains reflected the level of thaxtomin A production in oatmeal broth. However, strains analyzed in the study of Loria et al. (20) did not share the same genetic background. Consequently, the possibility that differential virulence is due to pathogenicity determinants other than thaxtomin A should not be excluded. Other factors, such as phytohormones (21) and hydrolytic enzymes (4,23), might also be involved in the disease process.

Mutants M16 and M13 were the most severely affected in thaxtomin A production. Mutant M16 produced 355 times less thaxtomin A than did the wild-type strain EF-35 and thaxtomin production could not be detected in mutant M13. These mutants were nonpathogenic on both potato tubers and radish seedlings. This confirms the work of Healy et al. (10), who previously showed that mutants defective in thaxtomin A were nonpathogenic. In contrast to other phytotoxins, such as coronatin (2,26) and tabtoxin (33), which contribute to symptom development but are not essential to pathogenicity, thaxtomin A seems to play an essential role in pathogenicity.

The biosynthetic pathway of thaxtomin A has not been elucidated yet. However, King and Lawrence (13,14) suggested that precursors of the biosynthesis are phenylalanine and 4-nitro-tryptophan. The phenotype of mutants M1 and M19, both deficient in tryptophan catabolism, suggests that an enzyme involved in tryptophan catabolism may also be required for thaxtomin A biosynthesis. Other *Streptomyces* spp. are known to produce secondary metabolites for which a modified tryptophan is a precursor molecule. Actinomycin (32), streptonigrin (30), staurosporine (24), and thiostrepton (6) are examples of such secondary metabolites.

TABLE 2. Characterization of *Streptomyces scabies* EF-35 and of mutants affected in thaxtomin A production

Strain	Sporulation ^w	Nitrogen sources utilized							
		$(\text{NH}_4)_2\text{SO}_4$	Alanine	Glutamic acid	Glutamine	Serine	Phenylalanine	Tryptophan	Tyrosine
EF-35	+ ^x	+	+	+	+	+	+	+	+
M1	+	+	+	+	+	+	+	— ^y	+
M8	+	+	+	+	+	+	+	+	+
M13	+	+	+	+	+	+	+	+	+
M16	—	—	—	+	± ^z	—	—	—	—
M19	+	+	+	+	+	+	+	—	+

^w Sporulation was tested on SLM3 medium.

^x + means that growth or sporulation was recorded.

^y — means that no growth or sporulation was recorded.

^z ± means that scarce growth was recorded.

M16 is an auxotrophic mutant and, like other *Streptomyces* auxotrophic mutants (34), it is affected in its sporulation ability. Addition of glutamic acid to minimal medium and, to a lesser extent, addition of glutamine, restored its ability to grow on minimal medium and to sporulate. Since glutamic acid acts as an α -amino group donor in the biosynthesis of several amino acids, it is likely that glutamic acid will influence the synthesis of a product derived from amino acid metabolism, such as thaxtomin A.

Similarly to M8, M13 grew normally, was not auxotrophic, and could catabolize tryptophan. Nevertheless, M13 lost the ability to produce a detectable amount of thaxtomin, suggesting that some of the genes responsible for thaxtomin A biosynthesis are not involved in tryptophan catabolism. The decrease in thaxtomin production for M8 and M13 suggests that the enzyme encoded by the mutated gene was less efficiently produced or somehow impaired.

Given the mutagenesis procedure, it cannot be totally excluded that some mutants could carry multiple mutations that could affect thaxtomin production independently of auxotrophy and loss of pathogenicity. However, mutant phenotypes suggest that amino acid metabolism is closely linked to thaxtomin A biosynthesis. Mutants M1 and M19 appear to carry mutations in genes involved in tryptophan catabolism, while M16 carries a mutation in a gene involved in the biosynthesis of an amino acid. The functions of the mutated genes in M8 and M13 are still unknown, but they may be involved in the regulation of thaxtomin production or in some biochemical step specific to thaxtomin synthesis.

This study represents a first step to localize and analyze the genes of *S. scabies* involved in the biosynthesis of thaxtomins and in the regulation of thaxtomin production. The mutants obtained in this study will be useful tools to study the biochemistry and genetics of thaxtomin biosynthesis.

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