Phenazine Production by *Pseudomonas* sp. LBUM223 Contributes to the Biological Control of Potato Common Scab

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ABSTRACT

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Common scab of potato is mainly caused by *Streptomyces scabies*. Currently, no method can efficiently control this economically important disease. We have previously determined that *Pseudomonas* sp. LBUM223 exhibits antagonistic properties toward *S. scabies* under in vitro conditions. Inhibition was mainly attributed to phenazine-1-carboxylic acid (PCA) production because an isogenic mutant of LBUM223 (*phzC*⁻), not producing PCA, was incapable of significantly reducing *S. scabies* growth. In order to understand the impact of PCA production by LBUM223 in controlling common scab under soil conditions, pot experi-

ments were performed to determine its effect on (i) reducing scab symptoms development, (ii) *S. scabies* population dynamics, and (iii) *txtA* expression in *S. scabies*, a key gene involved in thaxtomin A biosynthesis and required for pathogenesis. Symptoms were significantly reduced following inoculation with LBUM223 but not its mutant. Surprisingly, pathogen populations increased in the geocaulosphere in the presence of both wild-type and mutant strains of LBUM223; however, significant repression of *txtA* expression in *S. scabies* was only observed in the presence of PCA-producing LBUM223, not its mutant. These results suggest that, under soil conditions, PCA production by LBUM223 does not control common scab development by antibiosis but, instead, reduces *S. scabies* thaxtomin A production in the geocaulosphere, leading to reduced virulence.

Common scab is a bacterial infection of potato (*Solanum tuberosum* L.) primarily caused by *Streptomyces scabies*, which directly attacks actively growing tubers by causing necrotic lesions that reduce potato marketability (16). No method alone can currently eradicate this disease, and cultivar resistance has not yet been fully obtained, suggesting that it is not a simply inherited trait (10). The necrotic lesions, or scabs, found on tuber surfaces colonized by *S. scabies* are due to its production of thaxtomins (17). Thaxtomin A, the thaxtomin most widely secreted by *S. scabies*, is a nitrated dipeptide composed of tryptophan and phenylalanine. It inhibits cellulose synthesis in expanding plant tissues, resulting in weak cell walls (7). Thaxtomin A is produced by enzymes encoded by the genes *NOS*, *txtA*, *txtB*, and *txtC* grouped on a mobile pathogenicity island (PAI) and is regulated by the TxtR protein, which is activated by the binding of the cellulose degradation products cellobiose (21) or cellotriose (13) in the presence of suberin (19). TxtA and TxtB are responsible for the production of the N-methylated cyclic dipeptide base structure of thaxtomin A, while TxtC, a P450 monooxygenase, subsequently hydroxylates the molecule (11). A recent study has shown that thaxtomin A production correlates with the number of *txtA* and *txtC* transcripts detected by reverse-transcription quantitative polymerase chain reaction (RT-qPCR) and that thaxtomin A accumulation closely follows *txtA* and *txtC* gene expression (28).

Many of the antagonistic microorganisms inhabiting disease-suppressive soils (8) have been shown to repress plant pathogens' presence by secreting antimicrobial compounds (32), a concept known as antibiosis. Fluorescent *Pseudomonas* spp. have repeatedly been shown to be useful biocontrol agents, in part due to their capacity to produce various antimicrobial compounds, in-

cluding phenazines (5). Fluorescent *Pseudomonas* spp. have been proven present on tuber surfaces as potent antagonists against different potato pathogens (29), as well as being of significant aid in promoting tuber growth (15). Thus, inoculation of the potato rhizosphere (the soil in close contact with roots) and geocaulosphere (the soil in close contact with potato tubers) with selected antimicrobial-producing fluorescent *Pseudomonas* spp. in the aim of controlling common scab of potato appears to be an interesting biocontrol strategy.

In vitro confrontational assays previously performed in our laboratory have revealed that a fluorescent *Pseudomonas* sp. strain, LBUM223, can significantly repress the growth of *S. scabies* under in vitro conditions, and that this control appears linked to its capacity to produce phenazine-1-carboxylic acid (PCA) (26). Indeed, an isogenic mutant (*phzC*⁻) deficient in PCA production was incapable of significantly repressing the growth of the pathogen. In the present study, in order to better understand the impact of PCA production by LBUM223 on the biocontrol of common scab under soil conditions, pot experiments were performed to determine its effect on (i) reducing common scab symptom development, (ii) the population dynamics of *S. scabies* in the geocaulosphere and rhizosphere of potato, and (iii) the expression of *txtA* in *S. scabies*, a key gene involved in thaxtomin A biosynthesis, which is required for pathogenesis. We also assessed whether the application of wild-type *Pseudomonas* sp. LBUM223 or its mutant (*phzC*⁻) had an effect on overall plant growth.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Thaxtomin A-producing *S. scabies* LBUM848 was isolated from common scab lesions on a potato tuber harvested in Bloomfield, NB, Canada (27). *Pseudomonas* sp. LBUM223 was previously isolated from the rhizosphere of strawberry plants in Bouctouche, NB,

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Canada (24). An isogenic mutant of LBUM223, LBUM223 *phzC*–, incapable of producing PCA, was generated and validated as previously described (26). All strains were routinely grown in tryptic soy broth (BD, Franklin Lakes, NJ) with shaking at 25°C.

Soil and seed preparation. The soil used (sandy loam, pH 6.7) was collected from the Agriculture and Agri-Food Canada S. H. J. Michaud Research Farm (Bouctouche, NB, Canada) and sieved (4 mm). As a potting soil mix, three parts of soil were mixed with one part of sand and autoclaved twice during two consecutive days at 121°C for 1 h prior to use. Seed tubers ('Kennebec') were kept at room temperature until multiple sprouts had started to appear on each tuber and then cut in 2- to 3-cm² pieces bearing at least one sprout. Tuber pieces were left to heal for 3 days at 15°C before planting.

***S. scabies* and *Pseudomonas* sp. inocula.** The *S. scabies* inoculum was prepared in soil. Soil was placed in trays and autoclaved twice during two consecutive days (121°C for 1 h) and left for 2 days prior to use. Say solution (20 g of sucrose, 1.2 g of L-Asp, 0.6 g of K₂HPO₄, and 10 g of yeast extract [BD] per liter) was added to the trays at one-sixth of total soil volume and the trays were again autoclaved using the same conditions. A well-grown culture (for 4 days at 25°C with shaking at 200 rpm) of *S. scabies* was then used to inoculate the soil, at a ratio of 1:20. A negative control was also prepared using the autoclaved soil without adding *S. scabies*. The soil inocula (with *S. scabies* or negative control) were shaken vigorously and regularly for an incubation period of 14 days at 30°C. *S. scabies* concentration (CFU per gram of soil) was estimated by standard microbiological plating on tryptic soy agar.

For *Pseudomonas* sp. LBUM223 wild-type and isogenic mutant *phzC*– treatments, liquid inocula were prepared by growing each respective strain at 25°C for 3 days with shaking at 250 rpm. Populations were estimated using spectrophotometer readings ($\lambda = 600$ nm) and a standard curve.

Growth chamber experiments. The experimental set-up consisted of nine replicates of each of the following treatments: (i) no inoculum (control), (ii) LBUM223, (iii) LBUM223 *phzC*–, (iv) *S. scabies* (no antagonist), (v) *S. scabies* + LBUM223, and (vi) *S. scabies* + LBUM223 *phzC*–. The entire experiment was repeated a second time. For pots requiring *S. scabies*, the *S. scabies* soil inoculum was blended with the potting soil mix to obtain $\approx 5 \times 10^7$ CFU/g of soil, and a total of 1 kg of soil was added per 6-in. pot. For pots not requiring *S. scabies*, the same amount of negative control soil inoculum was used. For antagonist treatments, tubers were first dipped in a bacterial culture of either LBUM223 or LBUM223 *phzC*–; then, the rest of the culture was subsequently added to the pot around the seed tuber to obtain a final concentration of 4×10^7 CFU/g of soil. Negative controls received the same volume of water. Tubers (one per pot) were planted at a depth of ≈ 5 cm. Pots were placed in the growth chamber (Conviron CMP5000 series; Controlled Environments, Winnipeg, MB, Canada) using a completely randomized design, and were exposed to 16 h of daylight at 23°C and 8 h of darkness at 18°C. After 8 weeks, the photoperiod was reduced to 10 h to favor tuber growth. Plants were watered daily or every other day as needed with 100 ml of tap water and fertilized once most plants had broken soil and again after 3, 7, and 10 weeks with a water-soluble fertilizer (all-purpose fertilizer 20-20-20, N:P:K). Destructive harvesting of three replicates per treatment occurred at 5, 10, and 15 weeks. Shoots, roots, tubers, and rhizosphere and geocaulosphere soil were sampled. At each sampling time, the plants and tubers were shaken to remove loosely adhering soil, and the soil remaining on the roots and the tubers was sampled and labeled as rhizosphere and geocaulosphere, respectively. No tubers were present at week 5 and, consequently, no potato geocaulosphere was harvested at that time. Total plant weight, consisting of shoot and root combined weight without tubers, was

determined prior to freezing all soil and plant samples in liquid nitrogen and then storing at –80°C. Disease severity was assessed by estimating the percentage of common scab lesion coverage on each harvested tuber. Values were averaged for all tubers originating from the same plant.

Extraction of DNA and RNA from soil. Rhizosphere and geocaulosphere soil samples were lyophilized prior to nucleic acid extractions. DNA was extracted using the UltraClean Soil DNA Isolation Kit (Mobio, Carlsbad, CA) following the manufacturer's instructions, except for the vortex mixing step that was substituted with the use of a FastPrep (QBiogene, MP Bio-medicals, Santa Ana, CA) at a speed of 5.5 ms^{–1} during 45 s. Extracted DNA was diluted 1:10 prior to qPCR analyses. The same samples were also submitted to RNA extraction using the protocol described by Bürgmann (4), using a FastPrep as the homogenizing apparatus and resuspending the final RNA pellet in 100 μ l of diethylpyrocarbonate (DEPC)-treated water. Resuspended RNA was submitted to a DNase treatment (TURBO DNA-free, Ambion; Applied Biosystems, Foster City, CA), using 20 μ l of the provided 10 $\times$ buffer, 3 μ l of enzyme, 100 μ l of RNA, and 77 μ l of DEPC-treated water. A 45-min incubation at 37°C was done prior to performing a chloroform extraction and the final pellet was resuspended in 50 μ l of DEPC-treated water. A second DNase treatment was performed following the manufacturer's instructions (TURBO DNA-free, Ambion; Applied Biosystems), with a second-digest buffer (200 mM Tris-HCl, 100 mM MgCl₂, and 5 mM CaCl₂, pH 7.5) and use of the provided inactivation reagent, resulting in a final volume of 65 μ l. Prior to qPCR analyses, 7.9 μ l of RNA was retro-transcribed using the reverse *txtA* primer (26) and the TaqMan Reverse Transcription Reagents Kit (Applied Biosystems).

qPCR and RT-qPCR analysis of *txtA*. The primers and TaqMan probe targeting *txtA* have previously been described (26) and were purchased from Integrated DNA Technologies (Coralville, IA) and Applied Biosystems, respectively. The DNA and cDNA obtained from the rhizosphere and geocaulosphere soil samples were amplified using the TaqMan PCR Core Reagent Kit (Applied Biosystems) and an ABI 7500 RealTime PCR System (Applied Biosystems). Each 25 μ l reaction was prepared following the manufacturer's instructions and contained 100 nM TaqMan probe and 200 nM each primer. qPCRs were performed in triplicate and included no-template controls and a standard curve, determined by serial dilutions of a known number of plasmids containing *txtA*, to obtain absolute gene copy numbers. Plasmid standards were developed as previously described (6).

Statistical analyses. Two-factor analyses of variance (time and treatment) were performed with subsequent Tukey-Kramer adjustment to determine significance ($P < 0.05$). Mixed models were used to include a random factor, which removes variation between different replications of experiments and allows the analysis of both experiments together ($n = 6$). The interaction between factors was verified and, if an interaction occurred, analyses were completed per time point. The absence of an interaction confirmed that the trend observed was the same at all time points, which means that the effect of a particular treatment is true at all times combined. Population and expression data required log transformations to achieve suitable homogeneity and normality, while plant weight required no transformation. Each harvested pot at a given time represented an independent experimental unit. Measures obtained from technical replicates originating from the same unit were averaged prior to statistical analysis. The SAS Statistical Analysis Software (v. 9.2; SAS Institute, Cary, NC) was used for all analyses.

RESULTS

Growth promotion of potato plants. Regardless of the presence or absence of *S. scabies*, treatments with *Pseudomonas* sp.

LBUM223 or its isogenic mutant (*phzC*⁻) significantly increased ($P < 0.001$) total plant weight in 10-week-old plants (Table 1).

Biological control of common scab. Common scab symptoms were assessed on tubers harvested at weeks 10 and 15 (Fig. 1). The severity of symptoms significantly increased from week 10 to 15 ($P < 0.05$). At both time points, treatment with *Pseudomonas* sp. LBUM223 significantly reduced scab symptoms ($P < 0.01$) by ≈ 40 to 45% compared with tubers inoculated with *S. scabies* alone or in combination with LBUM223 *phzC*⁻. Scab symptoms were also sporadically found on stolons and underground stems when infection was severe (results not shown).

Soil populations of *S. scabies*. Absolute quantification of the *txtA* gene by qPCR in the geocaulosphere and rhizosphere soil samples enabled the quantification of *S. scabies* cell number. In the rhizosphere, there were no differences in *S. scabies* populations between the different treatments at all times (Fig. 2A). *S. scabies* populations were statistically different at all time points ($P < 0.01$), with populations increasing two- to threefold from week 5 to week 10 and decreasing slightly at week 15. There was no interaction between time and treatment factors. In the geocaulosphere, treatments with LBUM223 (wild type or mutant) significantly increased *S. scabies* populations ($P < 0.05$), at all times, by approximately twofold (Fig. 2B). There was no significant difference in populations over time and no interaction between time and treatment factors.

Expression of *txtA* by *S. scabies* in soil. Geocaulosphere and rhizosphere soil *txtA* gene expression values were obtained by dividing total transcript copy numbers detected by RT-qPCR by *S. scabies* cell number determined by qPCR. In the rhizosphere, the application of LBUM223 or LBUM223 *phzC*⁻ had no effect on *txtA* expression in *S. scabies* at all times (Fig. 3A). There was a significant difference in expression values between week 5 and other time points ($P < 0.01$), with the expression at week 5 being generally higher. There was no interaction between time and treatment factors. In the geocaulosphere, when compared with *S. scabies* alone, the application of the PCA-producing LBUM223 significantly reduced the expression of *txtA* by *S. scabies* ($P < 0.005$), at both 10 and 15 weeks (Fig. 3B), by as much as 6.7-fold at week 15. Inoculation with LBUM223 *phzC*⁻ had no effect on *txtA* gene expression when compared with the other treatments. There was no significant change in expression over time and no interaction between time and treatment factors.

DISCUSSION

This study aimed at determining the effect of PCA production by *Pseudomonas* sp. LBUM223 on potato common scab and its pathogen, *S. scabies*, under soil conditions. Results showed that inoculating potato plants with PCA-producing LBUM223 significantly reduced potato common scab symptoms by almost twofold when compared with plants inoculated with *S. scabies* alone, whereas inoculation with the LBUM223 isogenic *phzC*⁻ mutant did not result in any symptom reduction. Therefore, the ability of LBUM223 to produce PCA seems essential in reducing disease development. Quantification of pathogen populations in the rhizosphere and geocaulosphere soils and characterization of the

expression of *txtA*, a key biosynthetic gene involved in the production of thaxtomin A, were done to better understand the mechanisms of action potentially involved in common scab disease reduction by LBUM223. Surprisingly, inoculation with LBUM223 (wild type or mutant) increased *S. scabies* populations in the geocaulosphere, suggesting that PCA production was not acting by antibiosis against the pathogen. The reason for this increase is unknown and needs to be further investigated. However, the presence of the wild-type PCA-producing LBUM223 in the geocaulosphere significantly repressed *txtA* expression in *S. scabies*, whereas the LBUM223 isogenic mutant (*phzC*⁻) did not. The previous demonstration of a strong correlation existing between *txtA* expression and thaxtomin production (28) suggests that the decrease in *txtA* gene expression observed here could result in a decrease in thaxtomin A production in the geocaulosphere. Because it has previously been shown that a positive relationship exists between the quantity of thaxtomin A produced by *S. scabies* and the severity of common scab symptoms observed (14), the decrease in thaxtomin A production suggested here may account, at least in part, for the reduced symptoms observed when potato plants were treated with PCA-producing LBUM223. This significant difference was only found in the geocaulosphere and not in the rhizosphere. This seems logical considering that the geocaulosphere is adjacent to the tuber surface, the site of action of the pathogen, whereas the roots are generally not affected by common scab.

The mechanism involved in decreasing *txtA* gene expression in response to PCA production is not yet characterized and, although many studies have identified molecules that may enhance thaxtomin production, only a few studies have identified repressors of thaxtomin biosynthesis (2,18). It has previously been shown that phenazines are redox molecules capable of interfering with the electron transport chain in different organisms, and there are also some reports of phenazines actively interacting with double-stranded DNA, inhibiting DNA-dependent RNA synthesis to

Fig. 1. Tuber disease coverage. Each pot represents an independent experimental unit for which the percentage of scab coverage was averaged for all tubers belonging to the same plant. Values are presented as mean $\pm$ standard error of the mean ($n = 6$ for each treatment at each time). Because an interaction occurred between time and treatment factors, the different treatments were analyzed per time point. Significant differences between treatments are presented ($P < 0.01$) using the letters a and b for week 10 and the letters x and y for week 15.

TABLE 1. Growth-promoting capacity of LBUM223 and LBUM223 *phzC*⁻

Treatment	Total plant weight (g) $\pm$ SEM ^z
Control	84.47 $\pm$ 2.64 a
LBUM223 <i>phzC</i> ⁻	103.62 $\pm$ 5.35 b
LBUM223	103.98 $\pm$ 4.32 b
<i>Streptomyces scabies</i>	88.60 $\pm$ 2.19 a
<i>S. scabies</i> + LBUM223 <i>phzC</i> ⁻	100.55 $\pm$ 4.83 b
<i>S. scabies</i> + LBUM223	102.84 $\pm$ 5.26 b

^z Letters indicate statistically significant differences ($P < 0.001$) ($n = 6$); SEM = standard error of the mean.

Fig. 2. Soil populations of *Streptomyces scabies*, shown as the number of *txtA* gene copies per gram of soil in **A**, potato rhizosphere and **B**, potato geocaulosphere. Values are presented as mean $\pm$ standard error of the mean ($n = 6$). Because no interaction was obtained between time and treatment factors, horizontal lines were used to group all time points per treatment. Therefore, for all time points combined, significant differences between treatments ($P < 0.05$) are presented using different letters.

varying extents (23). Therefore, it appears logical that PCA might directly affect the expression of genes involved in thaxtomin production but would most probably modify *S. scabies*' expression profile in a more general manner, altering the expression of numerous genes. This would have to be validated using a transcriptomics approach to determine large-scale changes in gene expression in response to PCA production by LBUM223. Another possibility is that PCA production by LBUM223 may heighten plant defense responses which, in turn, may modulate *S. scabies*' expression profile. A type of phenazine, pyocyanin, has been shown to elicit an induced systemic resistance (ISR) response in tomato, another solanaceous species (1). The potential of PCA as an ISR elicitor would have to be validated in our current potato system.

In addition to reducing common scab symptoms, *Pseudomonas* sp. LBUM223 was able to promote plant growth by increasing total plant weight, regardless of its ability to produce PCA. The mechanisms accounting for this growth promotion are currently unknown but may include facilitating nutrient mobilization or the production of growth-inducing phytohormones, such as indole-3-acetic acid (30). No increase in total potato yield was observed when disease-free plants were inoculated with LBUM223 (wild type or mutant). Considering that, in this pot experiment, plants were generally limited in the space available for tuber growth, different results may be obtained in a field setting or under other nonrestrictive environmental conditions.

This study is not the first demonstration that antagonistic bacteria can reduce common scab of potato in planta. Greenhouse assays have previously shown that *Bacillus* sp. Sunhua, producing the antibiotics iturin A and macrolactin A, was able to significantly reduce the severity of common scab (9), suggesting that the production of different antibiotics may contribute to controlling the disease. A recent study has also assessed the ability of several fluorescent *Pseudomonas* spp. to control potato common scab in

the field in combination with a vermicompost soil amendment (25). In this study, one strain of *Pseudomonas mosselii* was able to significantly reduce symptoms only when combined with vermicompost. However, the mechanisms involved were not elucidated. Other research groups have also successfully shown common scab disease reduction using nonpathogenic *Streptomyces* spp. *S. diastatochromogenes* strain PonSSII and the antagonistic (avirulent) *S. scabies* strain PonR have been shown to reduce disease symptoms for many years in field-pot trials (20). *S. melanosporofaciens* was also shown capable of reducing symptoms in the field (3). These strains, being of the same genus as the pathogen, have the advantage of niche competition as a mechanism of biocontrol (12) but have the disadvantage of being able to acquire the pathogenicity island responsible for causing the disease through horizontal gene transfer (22).

Among the different groups of antagonistic bacteria that have been studied thus far as potential biocontrol agents against *S. scabies*, each with their advantages and disadvantages, *Pseudomonas* spp. are of high interest. This is mainly due to their excellent capacity for colonizing the rhizosphere and geocaulosphere, their large nutritional plasticity, and their ease and rapidity of growth under controlled conditions (31). This can positively impact our capacity to formulate, produce, and utilize these organisms at a reasonable cost in an agricultural context.

The underlying mechanisms involved in controlling common scab of potato by PCA-producing *Pseudomonas* sp. LBUM223 have been partly investigated in this study, and suggest a novel mode of action for PCA, through alteration of the transcriptional activity of at least one key gene required for *S. scabies* pathogenesis. However, this interaction occurring between *Pseudomonas* sp. LBUM223 and *S. scabies* in the geocaulosphere will need to be further characterized in order to fully understand what PCA specifically targets in the pathogen that leads to repressing *txtA* expression and reducing virulence.

Fig. 3. Expression of *txtA* by *Streptomyces scabies* in soil, shown as the total number of *txtA* transcripts divided by total *S. scabies* cell number in **A**, potato rhizosphere and **B**, potato geocaulosphere. Values are presented as mean $\pm$ standard error of the mean ($n = 6$). Because no interaction was obtained between time and treatment factors, horizontal lines were used to group all time points per treatment. Therefore, for all time points combined, significant differences between treatments ($P < 0.05$) are presented using different letters.

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