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Interaction between 2,4-Diacetylphloroglucinol- and Hydrogen Cyanide-Producing *Pseudomonas brassicacearum* LBUM300 and *Clavibacter michiganensis* subsp. *michiganensis* in the Tomato Rhizosphere

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ABSTRACT We have previously demonstrated that inoculation of tomato plants with 2,4-diacetylphloroglucinol (DAPG)- and hydrogen cyanide (HCN)-producing *Pseudomonas brassicacearum* LBUM300 could significantly reduce bacterial canker symptoms caused by *Clavibacter michiganensis* subsp. *michiganensis*. In this study, in order to better characterize the population dynamics of LBUM300 in the rhizosphere of tomato plants, we characterized the role played by DAPG and HCN production by LBUM300 on rhizosphere colonization of healthy and *C. michiganensis* subsp. *michiganensis*-infected tomato plants. The impact of *C. michiganensis* subsp. *michiganensis* presence on the expression of DAPG and HCN biosynthetic genes in the rhizosphere was also examined. *In planta* assays were performed using combinations of *C. michiganensis* subsp. *michiganensis* and wild-type LBUM300 or DAPG (LBUM300Δ*phlD*) or HCN (LBUM300Δ*hcnC*) isogenic mutant strains. Populations of LBUM300 and *phlD* and *hcnC* gene expression levels were quantified in rhizosphere soil at several time points up to 264 h postinoculation using culture-independent quantitative PCR (qPCR) and reverse transcriptase quantitative PCR (RT-qPCR) TaqMan assays, respectively. The presence of *C. michiganensis* subsp. *michiganensis* significantly increased rhizospheric populations of LBUM300. In *C. michiganensis* subsp. *michiganensis*-infected tomato rhizospheres, the populations of wild-type LBUM300 and strain LBUM300Δ*hcnC*, both producing DAPG, were significantly higher than the population of strain LBUM300Δ*phlD*. A significant upregulation of *phlD* expression was observed in the presence of *C. michiganensis* subsp. *michiganensis*, while *hcnC* expression was only slightly increased in the mutant strain LBUM300Δ*phlD* when *C. michiganensis* subsp. *michiganensis* was present. Additionally, biofilm production was found to be significantly reduced in strain LBUM300Δ*phlD* compared to the wild-type and LBUM300Δ*hcnC* strains.

IMPORTANCE The results of this study suggest that *C. michiganensis* subsp. *michiganensis* infection of tomato plants contributes to increasing rhizospheric populations of LBUM300, a biocontrol agent, as well as the overexpression of the DAPG biosynthetic operon in this bacterium. The increasing rhizospheric populations of LBUM300 represent one of the key factors in controlling *C. michiganensis* subsp. *michiganensis* in tomato plants, as DAPG-producing bacteria have shown the ability to decrease bacterial canker symptoms in tomato plants.

KEYWORDS *Clavibacter michiganensis*, DAPG, HCN, *Pseudomonas*, gene expression, rhizosphere, tomato

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Pseudomonads are well-studied plant-growth-promoting rhizobacteria (PGPR) that have long been recognized for their role in biological control of different plant pathogens (1–5). The adaptability of certain strains to inhabit the rhizosphere, the root surface, and sometimes the roots themselves is indispensable to their disease suppression capacities (6, 7). Their ability to produce different antimicrobial compounds has also been identified as another key factor for controlling plant pathogens (1, 4). Some beneficial pseudomonad strains are able to produce antimicrobial metabolites such as 2,4-diacetylphloroglucinol (DAPG) and hydrogen cyanide (HCN), which have been demonstrated to play a central role in pseudomonads' biocontrol capacities in different agrosystems (1, 4). DAPG, for instance, has broad-spectrum activity against a variety of fungi, bacteria, and nematodes (8). It can trigger an induced systemic resistance response in plants (9–11), stimulate root exudation (12), enhance root branching (13), and even stimulate plant-beneficial activities in other non-*Pseudomonas* PGPR (14). While HCN is rarely the sole antagonistic compound responsible for biocontrol in *Pseudomonas* strains and its effectiveness is not as important or as broad spectrum as DAPG, *Pseudomonas* strains producing DAPG generally also produce HCN (15). HCN has been studied for its antifungal properties and its involvement in disease suppression such as black root rot of tobacco (15).

Pseudomonas spp. have been extensively studied for their ability to protect plants from pathogenic fungi and oomycetes (16), but fewer studies have focused on their ability to suppress bacterial pathogens. *Clavibacter michiganensis* subsp. *michiganensis* is a Gram-positive actinomycete causing bacterial canker, the most destructive bacterial disease in tomato, for which no truly efficient chemical or biological control method exists. *C. michiganensis* subsp. *michiganensis* spreads through the xylem vessels, eventually colonizing the whole plant and causing systemic infection, which leads to unilateral wilting of leaves, development of canker lesions, tissue disintegration, and plant death (17). We previously reported (18) that under *in planta* conditions, development of bacterial canker disease in tomato was reduced by inoculation with *Pseudomonas brassicacearum* LBUM300, which is able to produce both DAPG and HCN (19), but not by inoculation with its *phlD*- or *hcnC*-deficient isogenic mutant counterparts. This suggests that the production of both antimicrobial metabolites contributes to reducing disease development and that *P. brassicacearum* LBUM300 may have the potential to be used as a biocontrol agent for bacterial canker.

In addition to biocontrol traits, the selection of rhizosphere-competent strains is essential to achieve efficient biological control of pathogens. Biofilms are communities of microbes living together in a self-produced extracellular matrix binding the cells to one another and adhering them to a surface (20). Biofilms are considered to be an important colonization strategy in various environments, including the rhizosphere, as the biofilm matrix is able to help protect the cells from desiccation and other stresses (21, 22). Most *Pseudomonas* isolates are able to form biofilms (21).

While pseudomonads are well known for their ability to protect plants from pathogens, the impact of pseudomonads' antimicrobial metabolite production on their own rhizosphere competency in the absence and especially in the presence of plant pathogens is not well documented. As most biocontrol agent-pathogen interaction studies generally focus on the ability of a potential biocontrol strain to suppress the growth of a specific plant pathogen and reduce disease symptom development, the biocontrol agent's behavior in the system is often overlooked. Previous studies have focused on the impact of soil factors, host genotype, and specific microbial genetic traits on rhizosphere colonization by *Pseudomonas* spp. (4, 23, 24). However, the role played by antimicrobial metabolite production in rhizosphere colonization remains controversial (25–28).

While it was demonstrated in our laboratory that *P. brassicacearum* LBUM300 is capable of significantly reducing disease symptoms of bacterial canker caused by *C. michiganensis* subsp. *michiganensis* (18), it remains unknown how the capacity to produce DAPG and HCN in the absence/presence of *C. michiganensis* subsp. *michiganensis* might impact rhizosphere colonization by *P. brassicacearum* LBUM300. Fur-

TABLE 1 Nucleotide sequences of the primers and TaqMan probes designed and used in this study

Target gene	Name of primer or probe[a]	Sequence (5′ → 3′)	Product size (bp)	Reference
phlD	300phlD28 (F)	GAGCGAAGCCGGGAACAT	73	This study
	300phlD100 (R)	TACAGGCCCGCTGTCGAA		
	300phlD52 (P)	CGTGGTGGTCTTCGACGT		
hcnC	hcnCfwd423 (F)	CCTGCCCCAGTCGTTCTTT	60	52
	hcnCrev482 (R)	TGCAACTGCGGATACATTGC		
	hcnC443 (P)	ATTTCGCCTTGCAGTCC		

[a]F, forward; R, reverse; P, probe.

thermore, it is unclear how the presence of *C. michiganensis* subsp. *michiganensis* might impact regulation of gene expression responsible for DAPG and HCN production.

In this study, the effects of inoculation with wild-type *P. brassicacearum* LBUM300 as well as two isogenic mutant strains incapable of producing DAPG or HCN (*P. brassicacearum* LBUM300Δ*phlD* and LBUM300Δ*hcnC*, respectively) on the population dynamics of *P. brassicacearum* LBUM300 and DAPG and HCN gene expression in the rhizosphere of healthy and *C. michiganensis* subsp. *michiganensis*-infected tomato plants were studied using quantitative PCR (qPCR) assays. Finally, the biofilm production of each *Pseudomonas* strain under study was measured *in vitro* and the plant-growth-promoting capacity of the three strains was also evaluated by measuring tomato root and shoot weight in the presence and absence of *C. michiganensis* subsp. *michiganensis*.

RESULTS

Primer design and evaluation. The *phlD* primers and the probe designed in this study (Table 1) targeted a 73-bp region that remained uninterrupted during mutagenesis in wild-type and mutant strains of *P. brassicacearum* LBUM300 in order to enable absolute quantification of gene copy number in all strains, which was used as an indication of bacterial population numbers. The qPCR amplification of DNA extracted from nonsterile soil inoculated with *P. brassicacearum* LBUM300 (*phlD* and *hcnC* targets), LBUM300Δ*phlD* (*phlD* and *hcnC* targets), or LBUM300Δ*hcnC* (*phlD* target only) as well as DNA from pure cultures generated a single band of the appropriate size when DNA was run by conventional agarose gel electrophoresis. No amplification product of *phlD* or *hcnC* was obtained from DNA extracted from uninoculated soil (data not shown). For standard curves, a linear relation was observed between log copy numbers of cloned *phlD* and *hcnC* fragments and qPCR crossing points ($R^2 = 0.998$ to 1.000) over 8 orders of magnitude ranging from 10^8 to 10^1 copies per qPCR, with amplification efficiencies ranging from 98% to 102%. The results obtained showed that in the soil system used in this study, 1:10 soil DNA dilutions were able to overcome the inhibition due to the presence of inhibitory compounds in soil while obtaining copy numbers representative of the amount of bacterial cells that were added to the soil (data not shown).

Population of *Pseudomonas* spp. in the rhizosphere of tomato plants. Absolute gene copy numbers were obtained by qPCR amplification of the *phlD* gene fragment and used to represent cell populations of *P. brassicacearum* LBUM300, LBUM300Δ*phlD*, or LBUM300Δ*hcnC* per gram of rhizosphere soil, as *phlD* is a single-copy gene in this *Pseudomonas* strain (29). In all treatments, populations generally decreased over time following inoculation ($P < 0.0001$). At the last harvest point, *Pseudomonas* species populations in treatments coinoculated with *C. michiganensis* subsp. *michiganensis* were maintained around 10^5 cells per g soil or higher, while treatments without the pathogen showed generally lower populations. Inoculation of the plant with the pathogen had an overall significant effect on *Pseudomonas* species populations in the rhizosphere ($P < 0.0001$). As shown in Fig. 1, analyses performed to evaluate the effects of different inoculation treatments indicated that when wild-type LBUM300 or

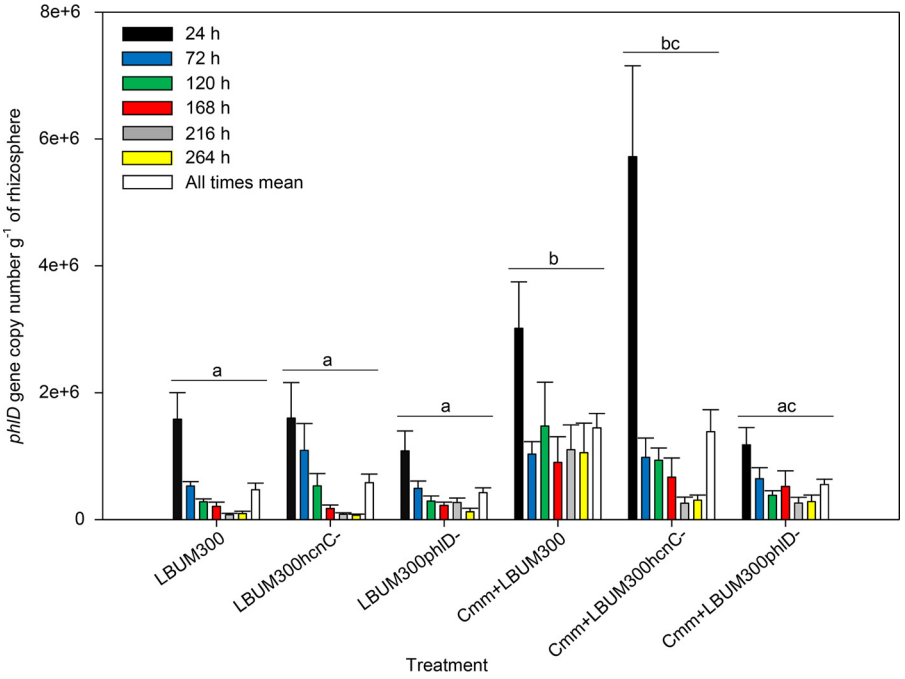

FIG 1 Population of wild-type *P. brassicacearum* LBUM300 and isogenic mutant strains LBUM300Δ*phlD* and LBUM300Δ*hcnC* in the rhizosphere of tomato plants, represented by the *phlD* gene copy number detected per gram of soil at 24 h, 72 h, 120 h, 168 h, 216 h, and 264 h postinoculation using qPCR ($n = 8$ for each treatment). The mean of all times within each treatment is indicated by a white bar, and treatments showing different letters are significantly different ($P < 0.05$). Error bars are standard errors of the means.

LBUM300Δ*hcnC* was coinoculated with *C. michiganensis* subsp. *michiganensis*, their populations were significantly increased ($P < 0.05$) in comparison to treatments inoculated with *Pseudomonas* spp. alone. Populations of LBUM300Δ*phlD* when coinoculated with *C. michiganensis* subsp. *michiganensis* were not significantly different from those of LBUM300Δ*phlD* inoculated alone but were significantly lower than the wild-type LBUM300 coinoculated with *C. michiganensis* subsp. *michiganensis* ($P < 0.05$).

Expression of *phlD* and *hcnC* genes in the rhizosphere of tomato plants. Transcripts of both *phlD* and *hcnC* genes were successfully detected from rhizosphere soil samples by reverse transcriptase quantitative PCR (RT-qPCR). Gene expression of *phlD* and *hcnC* was calculated as the ratio of transcript copy number to *phlD* DNA copy number as a way of describing transcription activity independently of potential cell number fluctuations.

As shown in Fig. 2, inoculation of tomato plants with only wild-type *P. brassicacearum* LBUM300 or strain LBUM300Δ*hcnC* led to similar levels of *phlD* gene expression. However, pathogen inoculation had a significant effect on *phlD* gene expression ($P < 0.05$). When plants were coinoculated with the pathogen and wild-type *P. brassicacearum* LBUM300 or LBUM300Δ*hcnC*, expression of *phlD* was significantly increased in comparison to treatments where *C. michiganensis* subsp. *michiganensis* was absent. Time postinoculation also had a significant effect on gene expression ($P < 0.05$), although no interaction between time and treatment was observed. No data are presented for plants inoculated with LBUM300Δ*phlD*, since *phlD* was disrupted and this rendered the organism incapable of gene expression and DAPG production.

While statistical analysis also revealed a significant effect of pathogen inoculation on *hcnC* gene expression, it was demonstrated that *hcnC* expression was significantly upregulated only when the Δ*phlD* strain of *P. brassicacearum* LBUM300 was coinoculated with *C. michiganensis* subsp. *michiganensis* (Fig. 3). No statistical difference was observed between treatments when wild-type LBUM300 or LBUM300Δ*phlD* was inoculated alone or when *C. michiganensis* subsp. *michiganensis* was inoculated in combination with the wild-type *P. brassicacearum* LBUM300, capable of DAPG and HCN

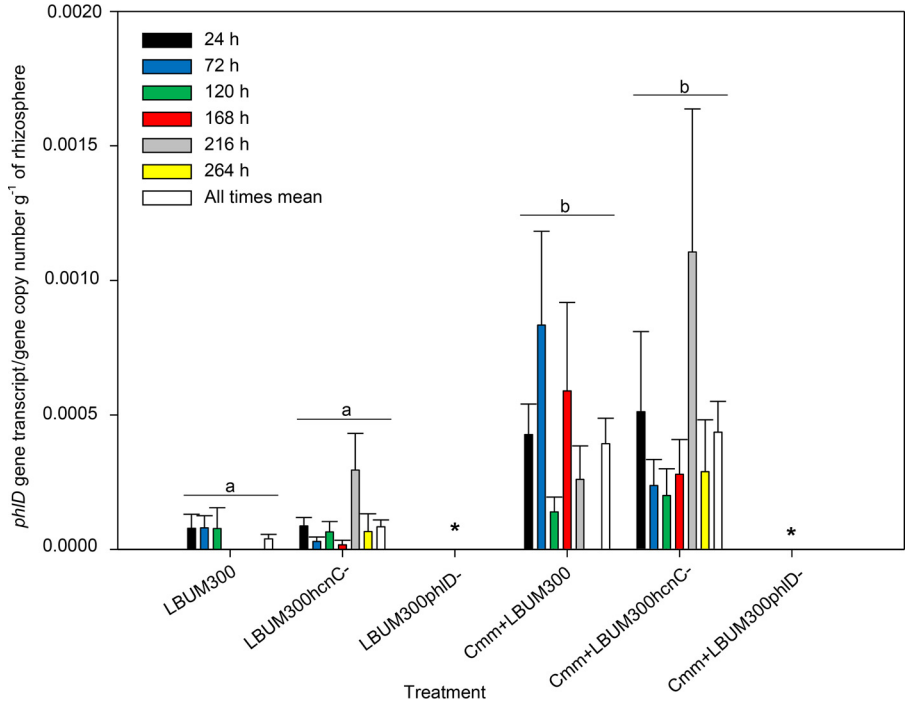

FIG 2 Transcriptional activity of the *phlD* gene in wild-type *P. brassicacearum* LBUM300 and strain LBUM300Δ*hcnC* in the rhizosphere of tomato plants detected at 24 h, 72 h, 120 h, 168 h, 216 h, and 264 h postinoculation using RT-qPCR ($n = 8$ for each treatment). *phlD* gene transcripts were normalized to *phlD* gene copy number per gram of rhizosphere soil. Strain LBUM300Δ*phlD*, carrying a disrupted *phlD* gene and incapable of *phlD* expression and DAPG production, is represented by an asterisk. The mean of all times within each treatment is indicated by a white bar, and treatments showing different letters are significantly different ($P < 0.05$). Error bars are standard errors of the means.

production. Time postinoculation had a significant effect ($P < 0.05$), with a general increase in *hcnC* expression over time in most treatments, but no interaction between time and treatment was detected. No data are presented for plants inoculated with strain LBUM300Δ*hcnC*, since *hcnC* was disrupted and this rendered the organism incapable of gene expression and HCN production.

Biofilm production by the three *P. brassicacearum* strains under study. As shown in Fig. 4, the measured absorbance of wild-type LBUM300 and strain LBUM300Δ*hcnC*, both able to produce DAPG, was significantly higher than the absorbance of strain LBUM300Δ*phlD*, indicating a greater biofilm production. These results suggest that LBUM300's ability to produce DAPG leads to a greater biofilm production. HCN did not show an effect on biofilm formation.

Influence of *C. michiganensis* subsp. *michiganensis* and *P. brassicacearum* inoculations on tomato root and shoot weights. As shown in Tables 2 and 3, the presence or absence of *P. brassicacearum* LBUM300 or its mutant strains alone did not significantly alter tomato root or shoot weight in comparison to the control, with values ranging between 0.81 g and 0.97 g and between 2.37 g and 2.66 g, respectively. However, infection with *C. michiganensis* subsp. *michiganensis* had a significantly negative impact ($P < 0.05$) on these two growth parameters in comparison to the control or to plants inoculated with only *Pseudomonas* spp., with root and shoot weights dropping to 0.24 g and 0.76 g, respectively. The symptoms observed on the plants inoculated with *C. michiganensis* subsp. *michiganensis* were similar to those reported previously (18).

A priori contrasts also revealed that shoot and root weights of plants coinoculated with the pathogen were able to regain levels similar to those of the uninfected control plants and did not differ significantly regardless of whether *C. michiganensis* subsp. *michiganensis* was coinoculated with wild-type *P. brassicacearum* LBUM300 or with strain LBUM300Δ*phlD* or LBUM300Δ*hcnC*.

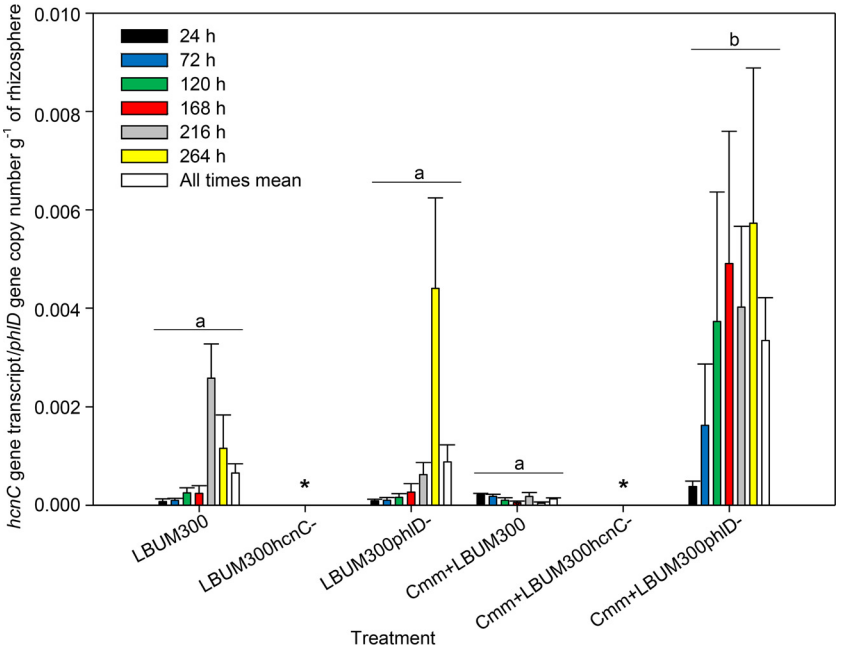

FIG 3 Transcription activity of the *hcnC* gene in wild-type *P. brassicacearum* LBUM300 and strain LBUM300Δ*phlD* in the rhizosphere of tomato plants detected at 24 h, 72 h, 120 h, 168 h, 216 h, and 264 h postinoculation using RT-qPCR ($n = 8$ for each treatment). *hcnC* gene transcripts were normalized to *phlD* gene copy number per gram of rhizosphere soil. Strain LBUM300Δ*hcnC*, carrying a disrupted *hcnC* gene and incapable of *hcnC* expression and HCN production, is represented by an asterisk. The mean of all times within each treatment is indicated by a white bar, and treatments showing different letters are significantly different ($P < 0.05$). Error bars are standard errors of the means.

DISCUSSION

Very few studies thus far have specifically looked at the impact of antimicrobial metabolite production on rhizosphere competency, and little is known about the contribution that these metabolites may have on the colonization and survival of the producing strains in the rhizosphere. In this study, we demonstrated that the inability to produce DAPG or HCN by *P. brassicacearum* LBUM300 did not influence its colonization of the tomato rhizosphere in the absence of the bacterial pathogen *C. michiganensis* subsp. *michiganensis*. However, this was not the case in the presence of *C. michiganensis* subsp. *michiganensis*, where the population dynamic of *P. brassicacearum* LBUM300 was clearly influenced by its capacity to produce DAPG. A higher abundance

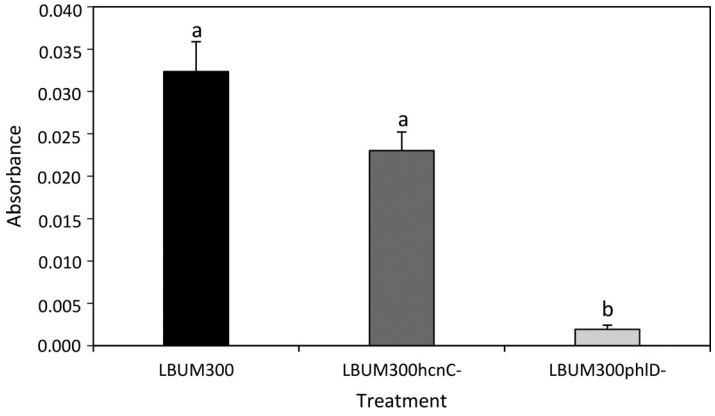

FIG 4 Biofilm production by wild-type *P. brassicacearum* LBUM300 and strains LBUM300Δ*hcnC* and LBUM300Δ*phlD* after 48 h of growth ($n = 16$ for each treatment). All treatments showing different letters are significantly different ($P < 0.05$). Error bars are standard errors of the means.

TABLE 2 Root and shoot weight of tomato plants grown in nonsterile agricultural soil under growth chamber conditions at 4 weeks postinoculation treated with combinations of *P. brassicacearum* strains and *C. michiganensis* subsp. *michiganensis*

Treatment[a]	Wt (g) at wk 4	
	Roots	Shoots
None (control)	0.97	2.54
C. michiganensis subsp. *michiganensis*	0.24	0.76
P. brassicacearum LBUM300	0.89	2.66
P. brassicacearum LBUM300Δ*hcnC*	0.87	2.37
P. brassicacearum LBUM300Δ*phlD*	0.92	2.42
C. michiganensis subsp. *michiganensis* + LBUM300	0.67	1.94
C. michiganensis subsp. *michiganensis* + LBUM300Δ*hcnC*	0.76	1.68
C. michiganensis subsp. *michiganensis* + LBUM300Δ*phlD*	0.89	2.39

[a]$n = 8$ for each treatment.

of strains able to produce DAPG was found in the rhizosphere of tomato when *C. michiganensis* subsp. *michiganensis* was present, and this effect was not seen for the non-DAPG-producing strain LBUM300Δ*phlD*, whose population did not differ significantly from those seen in treatments without the pathogen. These results strongly suggest that DAPG antibiotic production contributes to the ecological fitness of *P. brassicacearum* LBUM300 in the rhizosphere of tomato and that this is somehow related to the presence of *C. michiganensis* subsp. *michiganensis*.

Previous works have shown conflicting results as to the effect of DAPG production on a strain's rhizocompetence. Production of DAPG in *Pseudomonas fluorescens* P32 increased colonization capacity on wheat roots in natural soils in comparison to the parental strain, which produced HCN and siderophores but not DAPG (30). Additionally, a DAPG overproducer (due to a *phlF* gene mutation) was able to colonize the rhizosphere of tomato plants at a higher rate than the wild type (31). In contrast, the loss of DAPG production did not reduce the ability of *P. fluorescens* F113 to colonize and persist in the rhizosphere of sugar beets in short-term (27 days) and long-term (270 days) experiments (26, 32). Another study demonstrated that the inability to synthesize DAPG or HCN metabolites did not reduce the persistence of *P. fluorescens* CHA0 in the rhizosphere of wheat (33).

It has been previously noted that the presence of root pathogens can support larger populations of bacteria, presumably attributed to excess substrate availability due to root leakage of nutrients as a result of disease (28, 34). This alone does not explain why introduced *P. brassicacearum* strains specifically colonized the *C. michiganensis* subsp. *michiganensis*-infested rhizosphere to a higher extent only if these strains were capable of DAPG production. With respect to pathogen presence, Mazzola and colleagues observed that in the presence of *Gaeumannomyces graminis* var. *tritici*, a fungal pathogen responsible

TABLE 3 Resulting *P* values obtained by mixed ANOVAs using *a priori* comparisons between treatments of *P. brassicacearum* strains and *C. michiganensis* subsp. *michiganensis* at 4 weeks postinoculation on root and shoot weight of tomato plants[a]

Treatments compared	*P* value	
	Roots	Shoots
Control versus *C. michiganensis* subsp. *michiganensis*	**0.001**	**0.003**
Control versus LBUM300, LBUM300Δ*hcnC*, and LBUM300Δ*phlD*	0.656	0.541
C. michiganensis subsp. *michiganensis* versus LBUM300, LBUM300Δ*hcnC*, and LBUM300Δ*phlD*	**0.000**	**0.002**
Control versus *C. michiganensis* subsp. *michiganensis* + LBUM300, *C. michiganensis* subsp. *michiganensis* + LBUM300Δ*hcnC*, and *C. michiganensis* subsp. *michiganensis* + LBUM300Δ*phlD*	0.229	0.159
C. michiganensis subsp. *michiganensis* versus *C. michiganensis* subsp. *michiganensis* + LBUM300, *C. michiganensis* subsp. *michiganensis* + LBUM300Δ*hcnC*, and *C. michiganensis* subsp. *michiganensis* + LBUM300Δ*phlD*	**0.003**	**0.016**
C. michiganensis subsp. *michiganensis* + LBUM300 versus *C. michiganensis* subsp. *michiganensis* + LBUM300Δ*hcnC* and *C. michiganensis* subsp. *michiganensis* + LBUM300Δ*phlD*	0.358	0.533
C. michiganensis subsp. *michiganensis* + LBUM300Δ*hcnC* versus *C. michiganensis* subsp. *michiganensis* + LBUM300Δ*phlD*	0.491	0.857

[a]Significant values ($P < 0.05$) are in bold; $n = 8$ for each treatment.

for take-all disease in wheat, phenazine-producing and -nonproducing strains maintained similar populations (28). In contrast to our results with DAPG producers, the population of the phenazine-producing strain was significantly higher than its nonproducing counterpart in the absence of the pathogen. Others have noted that the presence of root pathogens can lead to larger populations of *phlD*-positive *Pseudomonas* spp. (DAPG producers), for instance, in maize and cucumber infected with *Pythium ultimum* (35, 36), in bean infected with *Rhizoctonia solani* (37), and in wheat infected with *G. graminis* var. *tritici* (34, 38), although none of these studies clearly demonstrated the specific role of DAPG production in root colonization. Clearly, the results of this study and those available in the existing literature suggest that different responses could be expected depending on the antimicrobial metabolite produced, the host plant, and the pathogen present. Our results indicate that the capacity to produce DAPG allows a better establishment of *P. brassicacearum* LBUM300 in the rhizosphere of tomato in the presence of the bacterial pathogen *C. michiganensis* subsp. *michiganensis*.

We found that the presence of the tomato pathogen *C. michiganensis* subsp. *michiganensis* influenced expression of the *phlD* and *hcnC* genes, involved in the synthesis of DAPG and HCN, respectively. This clearly suggests that the bacterial pathogen *C. michiganensis* subsp. *michiganensis* has an effect on DAPG and HCN biosynthesis. Previous reports have shown that the expression of *phlA* in *P. fluorescens* CHA0 was significantly increased in the rhizosphere of bean in the presence of the fungal pathogen *Rhizoctonia solani* (37) and in the rhizosphere of maize and cucumber infected with *Pythium ultimum* (35). Other fungal pathogens, such as *Fusarium oxysporum*, have been found to repress *phlA* gene expression and DAPG production in the rhizosphere of wheat (39). While most studies have focused on the effects of fungal plant pathogens, we demonstrate here that the presence of a bacterial plant pathogen caused increases in *phlD* gene expression. The ability or inability to produce HCN did not have an effect on *phlD* gene expression.

In contrast to DAPG, very little information regarding expression of HCN biosynthetic genes in the plant rhizosphere is available, even though most DAPG producers also produce HCN. We report that expression of the *hcnC* gene was increased in the presence of *C. michiganensis* subsp. *michiganensis* in comparison to treatments without the pathogen, but only in the non-DAPG-producing strain LBUM300Δ*phlD*. These results indicate that the inability to produce DAPG clearly had an effect on *hcnC* expression. We demonstrated in an earlier study that the presence of the fungal pathogen *Verticillium dahliae* had a significant stimulatory effect on *hcnC* expression in the rhizosphere of strawberry compared to treatments in which the pathogen was absent (40). However, the increased expression was observed only in wild-type *P. brassicacearum* LBUM300, as isogenic mutants were not used. Jamali and colleagues monitored the expression of the *hcnA* gene in *P. fluorescens* CHA0 in the rhizosphere of bean infected with the pathogen *R. solani* under gnotobiotic conditions (37). An increase in *hcnA* gene expression per gram of rhizosphere was observed; however, this was attributed to an increase in root colonization by CHA0, since *hcnA* expression in individual cells was not significantly enhanced by the presence of *R. solani* (37).

We can further conclude that the upregulation of the *phlD* and *hcnC* genes was not attributable to an increase in colonization since all gene expression data for both genes were normalized to the *phlD* gene copy number in the rhizosphere. Hence, the results obtained here may be an indication that the presence of *C. michiganensis* subsp. *michiganensis* stimulates production of DAPG by *P. brassicacearum* LBUM300 in the rhizosphere of tomato and that when the strain is unable to produce DAPG, it may try to compensate through production of HCN. However, it is known that production of both metabolites by *P. brassicacearum* LBUM300 is necessary to reduce populations of *C. michiganensis* subsp. *michiganensis* in the rhizosphere of tomato and to reduce the development of bacterial canker (18).

The biofilm results showed that the ability to produce DAPG by *P. brassicacearum* LBUM300 and *P. brassicacearum* LBUM300Δ*hcnC* leads to higher biofilm production

than that of the mutant deficient in DAPG production. The ability to produce HCN did not influence biofilm production. Phenazines are known to significantly contribute to biofilm maturation *in vitro* and on plants (22); however, very few studies have shown an effect of DAPG on biofilm formation and the results have been contradictory to date. A study by Combes-Meynet et al. (41) showed that when adding DAPG to a culture of *Azospirillum* sp. strain Sp245-Rif, the biofilm production increased with increasing DAPG concentrations. They also observed that the expression of the *ppk* gene, which contributes to cell motility and biofilm formation (42, 43), was upregulated by DAPG (41). However, Powers et al. (20) demonstrated that subinhibitory concentrations of DAPG were able to inhibit *Bacillus subtilis* biofilm formation. These results indicate that DAPG can affect biofilm formation; however, the mechanism of action and the reasons leading to a positive or negative effect are clearly not known.

With regard to plant growth promotion, conflicting results on the potential implication of antimicrobial metabolite production are reported in the literature. In our experiments, the presence of the wild-type or mutant strains of *P. brassicacearum* LBUM300 did not significantly increase root or shoot weight or the general growth or vigor of tomato plants at 4 weeks postinoculation. Therefore, we can conclude that *P. brassicacearum* LBUM300 does not display general growth-promoting activities on tomato. Similarly, no significant difference in shoot or root weight was observed when pea plants were inoculated with the wild type and with non-DAPG producers (44, 45). However, in other studies, the inoculation of healthy pea plants with the DAPG producer *P. fluorescens* F113 or a DAPG-overproducing strain resulted in greater root weight than in plants inoculated with a non-DAPG-producing mutant strain (46).

Results following the coinoculation of plants with *C. michiganensis* subsp. *michiganensis* and any of the wild-type or mutant strains of *P. brassicacearum* LBUM300 indicated that the plants had significantly greater root and shoot weights than plants inoculated with the pathogen alone. Similar positive effects on shoot and/or root weight were observed when pea plants were inoculated with the pathogen *Pythium* and DAPG-producing strains of *P. fluorescens* (strain F113, CHA0, or Q2-87), but no plant growth promotion by DAPG producers was detected in healthy plants (47). In another study (48), similar results were obtained in wheat inoculated with the DAPG producer *P. fluorescens* Pf29 and/or *G. graminis* var. *tritici*, but in the absence of the pathogen, the presence of the DAPG-producing pseudomonads significantly increased root weight compared to the control plants. Our results suggest that regardless of the capacity to produce DAPG or HCN, inoculation of tomato plants with *P. brassicacearum* LBUM300 reduced the negative impact caused by the pathogen on root and shoot weight. The mechanisms involved, however, remain unknown.

In conclusion, our results demonstrated that the tomato bacterial pathogen *C. michiganensis* subsp. *michiganensis* had an effect on rhizosphere colonization by *P. brassicacearum* LBUM300 and that the capacity to produce DAPG by *P. brassicacearum* LBUM300 contributed to rhizosphere competency whereas this was not the case for HCN. While expression of both *phlD* and *hcnC* genes was detected in all treatments, upregulation of *phlD* was noted only in the presence of the pathogen and that of *hcnC* occurred only when the gene for production of DAPG was disrupted. Although *P. brassicacearum* LBUM300 cannot be considered a true tomato plant growth promoter, its presence in the rhizosphere attenuates the host plant's root and shoot weight decrease due to the presence of *C. michiganensis* subsp. *michiganensis*. This effect is, however, not associated with the inherent capacity to produce DAPG or HCN and remains to be characterized.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *P. brassicacearum* LBUM300 carrying functional *phl* and *hcn* operons was previously isolated from the rhizosphere of strawberry plants in Bouctouche, NB, Canada (19). Isogenic mutant strains of *P. brassicacearum* LBUM300 deficient in DAPG production (*P. brassicacearum* LBUM300Δ*phlD*) or HCN production (*P. brassicacearum* LBUM300Δ*hcnC*) were previously developed by site-directed mutagenesis of *phlD* and *hcnC* genes using the suicide plasmid pKNOCK-Gm^r

(18). The pathogenic *C. michiganensis* subsp. *michiganensis* strain LMG 5644 was obtained from the Microbiology Laboratorium of the University of Ghent (Belgium). All bacterial strains were maintained and grown routinely in tryptic soy broth (TSB) or agar (TSA) at 25°C. Prior to *in planta* assays, strains were grown at 25°C in TSB with continuous shaking at 200 rpm for approximately 24 h and 48 h for *P. brassicacearum* strains and *C. michiganensis* subsp. *michiganensis*, respectively.

Biofilm assay. Biofilm formation was assessed in the three *P. brassicacearum* strains according to the methods described by Arseneault et al. (49). Briefly, *P. brassicacearum* strains (wild-type LBUM300, LBUM300Δ*hcnC*, and LBUM300Δ*phlD*) were grown at 25°C for 48 h in TSB. An optical density at 600 nm (OD_{600}) reading was performed to ensure equal concentrations among all samples. Cultures were diluted 1:10 in M9 minimal salts medium with Casamino Acids (BD), supplemented with 0.2% (wt/vol) glucose and 1 mM $MgSO_4$, of which 100 μl was plated in each well of a 96-well polystyrene cell culture plate (Thermo Scientific, Waltham, MA). Plates were incubated without shaking at 25°C for 48 h, after which biofilm formation was assessed. The medium was removed, and wells were washed once with water. Each well was stained with 125 μl of 0.1% crystal violet for 10 min, washed 5 times with water, air dried, and destained for 10 min using 200 μl of 95% ethanol. The OD_{600} was quantified using a plate reader (Varioskan; Thermo Scientific) after pipetting 125 μl from each destained well into a new well. For each culture, in addition to a control with medium only (blank), 16 replicate wells were used.

***In planta* assays.** Seeds of *Solanum lycopersicum* cv. Scotia (Vesey's Seeds, York, Canada) were first germinated on watered compressed peat disks (Vesey's Seeds) and incubated for 24 days in a growth chamber (Conviron, Winnipeg, Canada) under controlled conditions (12-h light/12-h dark photoperiod, 25°C, 80% humidity). Plantlets were then transplanted into 4-inch-diameter pots and filled with sieved nonsterile agricultural field soil collected in Bouctouche, NB, Canada. The soil was stored at 4°C until use and characterized as a gleyed podzolic gray luvisol, a subgroup of the Canadian System of Soil Classification (50), pH 5.2, consisting of 62% sand, 25% silt, 13% clay, and 2.6% organic matter.

To prepare for root and soil inoculation, *P. brassicacearum* and *C. michiganensis* subsp. *michiganensis* cells were grown to late stationary phase as described above in TSB and harvested by centrifugation at 5,000 × *g* for 5 min at 4°C. Cells were washed twice, resuspended in 1× phosphate-buffered saline (PBS; 25 mM phosphate buffer, 125 mM NaCl, pH 7.4), and adjusted to 10^9 CFU ml^{-1} and 2 × 10^9 CFU ml^{-1} for the three *P. brassicacearum* strains and *C. michiganensis* subsp. *michiganensis*, respectively. At transplantation, root systems of plantlets were briefly dipped in the following *P. brassicacearum* suspensions: LBUM300, LBUM300Δ*phlD*, LBUM300Δ*hcnC*, or 1× PBS solution alone ("none-control"). An additional 5 ml of the respective *P. brassicacearum* solution and 5 ml of the *C. michiganensis* subsp. *michiganensis* solution (or 5 ml of 1× PBS for the none-control) were inoculated in the soil/root area of each plantlet. The plantlets were grown in a growth chamber (Conviron) (16-h light/8-h dark photoperiod with alternating 25°C/20°C thermoperiod, 100% humidity) and watered daily or when needed.

The experimental setup consisted of 6 harvesting dates (24 h, 72 h, 120 h, 168 h, 216 h, and 264 h), 8 treatments (LBUM300, LBUM300Δ*phlD*, LBUM300Δ*hcnC*, *C. michiganensis* subsp. *michiganensis*, *C. michiganensis* subsp. *michiganensis* plus LBUM300, *C. michiganensis* subsp. *michiganensis* plus LBUM300Δ*phlD*, *C. michiganensis* subsp. *michiganensis* plus LBUM300Δ*hcnC*, and control), and 4 biological replicates per time and treatment, for a total of 192 samples. An additional identical set of plants comprising all treatments and biological replicates were kept until 4 weeks postinoculation in order to weigh roots and shoots once symptoms of disease had developed. The whole experiment was repeated twice.

Plant harvest. Plants and rhizosphere soil were destructively sampled at 24 h, 72 h, 120 h, 168 h, 216 h, and 264 h postinoculation, for a total of 32 plants per time point. Rhizosphere soil and plant roots were immediately frozen in liquid nitrogen and stored at −80°C until use. Rhizosphere soil was later lyophilized using a ModulyoD freeze dryer (Thermo Fisher Scientific, Waltham, MA, USA), and plant roots were crushed in liquid nitrogen and stored at −80°C until microbial DNA and RNA extractions. After 4 weeks of growth postinoculation, eight plants per treatment were harvested and plant roots and shoots were weighed.

Nucleic acid extraction. Total DNA and RNA were coextracted from 0.5 g rhizosphere soil samples as previously described (51) with the following modification: 15% polyethylene glycol (PEG) 6000 was used instead of 30% PEG 6000. Samples were kept on ice during the whole procedure, and final extracts were resuspended in 50 μl of DNase- and RNase-free diethyl pyrocarbonate (DEPC)-treated water (Fisher Scientific, Mississauga, Canada) and stored at −80°C. Total DNA and RNA were also extracted from 0.1 g of crushed root samples using the DNeasy plant minikit (Qiagen, Mississauga, Canada) and the RNeasy plant minikit (Qiagen), respectively, by following the manufacturer's protocol. Final extracts were resuspended in DNase- and RNase-free DEPC-treated water (Fisher Scientific); RNA samples were stored at −80°C, and DNA samples were stored at −20°C. For all samples, DNA and RNA quantity and quality were evaluated using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

DNase treatment and reverse transcription. To eliminate contaminating DNA, RNA samples extracted from rhizosphere soil were treated with 6 U and subsequently 3 U of Turbo DNA-free DNase I enzyme as suggested by the manufacturer (Life technologies, Burlington, Canada). RNA from plant roots were subjected to an on-column DNase digestion as indicated by the manufacturer of the RNeasy plant minikit (Qiagen) and subsequently to 6 U and 3 U of DNase I enzyme (Life Technologies). Immediately after DNase treatments, 7.9-μl subsamples of each DNase-treated extract were used as a template for gene-specific reverse transcription using 200 nM appropriate reverse primers (Table 1) and TaqMan reverse transcription reagents according to the manufacturer protocol (Life Technologies).

Primer and probe design for qPCR. The design of RT-qPCR primers and a TaqMan probe based on the *hcnC* sequence of *P. brassicacearum* LBUM300 was previously described (Table 1) (52). The design of

primers and a TaqMan probe for the *phlD* gene was performed using the PrimerExpress 3.0 software (Applied Biosystems, Foster City, CA) based on the *phlD* gene sequence of *P. brassicacearum* LBUM300 (GenBank accession number DQ788986). Because the designed primers and probe for the *phlD* gene were developed in a region that was not interrupted during mutagenesis in LBUM300, LBUM300Δ*phlD*, and LBUM300Δ*hcnC*, the same set could be used for DNA quantification of the three strains. Specificity was confirmed through a BLASTn search in the NCBI database and through qPCR amplification on DNA extracted from nonsterile field soil samples uninoculated and inoculated with each of the wild-type and mutant *P. brassicacearum* strains under study in addition to DNA extracted from pure cultures (data not shown). All TaqMan probes were labeled with a 6-carboxyfluorescein (6-FAM) reporter dye at the 5′ end and an MGBNFQ quencher dye at the 3′ end (Applied Biosystems). The primers were custom synthesized by Integrated DNA Technologies (Coralville, IA, USA). The sequences of the primers and probe are listed in Table 1.

Standard curves for absolute quantification of *hcnC* and *phlD* genes. A standard curve for *hcnC* was prepared by cloning a 60-bp PCR amplicon into the TOPO-TA 3,956-bp plasmid (Invitrogen, Burlington, Canada). For *phlD*, a 73-bp PCR amplicon was cloned into the 2,976-bp pKRX plasmid (National Institute of Genetics, Mishima, Japan). Plasmid copy numbers were quantified using a spectrophotometer (NanoDrop Technologies), and the gene copy numbers were calculated according to the molar mass derived from the plasmid and amplicon lengths. Dilutions of the known concentrations of plasmid DNA containing the appropriate inserts were made to generate standard curves ranging from 10^8 to 10^1 gene copies per qPCR mixture obtained by 10-fold dilutions.

Because the *phlD* primers and probe were compatible with a region that was not interrupted during mutagenesis in LBUM300, LBUMΔ300*phlD*, and LBUM300Δ*hcnC*, the *phlD* standard curve was used for the absolute quantification of *phlD* gene copy numbers in the three strains. This standard curve was also used to quantify *phlD* mRNA transcripts, while *hcnC* mRNA transcripts were quantified using the *hcnC* standard curve. Gene expression data of *phlD* and *hcnC* were presented as ratios of mRNA transcripts normalized to the *phlD* DNA gene copy number. Standard curves were always run in triplicate, and amplification efficiency (*E*) was calculated from the slope of the standard curve using the equation $E = 10^{(-1/\text{slope})} - 1$.

Quantitative PCR. qPCR targeting DNA and cDNA from *hcnC* and *phlD* genes (Table 1) was performed using a Bio-Rad CFX Connect real-time PCR detection system and the iTaq universal probe supermix kit (Bio-Rad Laboratories, Mississauga, Canada). Each qPCR mixture contained 6 μl of template DNA (diluted 1 in 10 in DEPC-treated water following extraction), cDNA, or DNase-treated RNA (no RT control) and was prepared with 10 μl (1×) of iTaq universal probe supermix, 0.8 μl (200 nmol/liter) of appropriate reverse and forward primers, 1.2 μl (100 nmol/liter) of appropriate probe, and 1.1 μl of sterile double-distilled water (ddH_2O), for a total volume of 20 μl. The cycling conditions were 95°C for 2 min followed by 50 cycles of 95°C for 5 s and 60°C for 30 s. Negative-control reactions were performed during each qPCR run by replacing DNA with sterile ddH_2O. All qPCRs were replicated 3 times.

Statistical analysis. Statistical analyses were performed using the SAS Statistical Software version 9.2 (SAS institute Inc., 1992). Mixed analyses of variance (ANOVAs) were carried out on the qPCR data and were followed by *a posteriori* comparisons using Tukey's tests at a 5% significance level. An analysis was performed on all the data to examine the effects of time, the presence of the pathogen, and the presence of the *P. brassicacearum* strain. This was followed by a second analysis between different treatments grouping all sampling dates without factoring in the presence of the pathogen. The biofilm data were analyzed by ANOVA to examine the effect of the *P. brassicacearum* strain on biofilm formation, followed by *a posteriori* comparisons using Tukey's test at a 5% significance level. Mixed ANOVAs using *a priori* contrasts between appropriate treatments were carried out on the shoot and root weight data. To meet the requirements of the tests, the qPCR data used in the mixed ANOVA analysis were either log or rank transformed whereas the biofilm data and the week 4 shoot weight data were both rank transformed.

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REFERENCES

1. Haas D, Défago G. 2005. Biological control of soil-borne pathogens by fluorescent pseudomonads. Nat Rev Microbiol 3:307–319. https://doi.org/10.1038/nrmicro1129.
2. Maurhofer M, Baehler E, Notz R, Martinez V, Keel C. 2004. Cross talk between 2,4-diacetylphloroglucinol-producing biocontrol pseudomonads on wheat roots. Appl Environ Microbiol 70:1990–1998. https://doi.org/10.1128/AEM.70.4.1990-1998.2004.
3. Raaijmakers JM, Vlami M, de Souza JT. 2002. Antibiotic production by bacterial biocontrol agents. Antonie Van Leeuwenhoek 81:537–547. https://doi.org/10.1023/A:1020501420831.
4. Weller DM. 2007. *Pseudomonas* biocontrol agents of soilborne pathogens: looking back over 30 years. Phytopathology 97:250–256. https://doi.org/10.1094/PHYTO-97-2-0250.
5. Weller DM, Raaijmakers JM, Gardener BBM, Thomashow LS. 2002. Mi-

crobial populations responsible for specific soil suppressiveness to plant pathogens. Annu Rev Phytopathol 40:309–348. https://doi.org/10.1146/annurev.phyto.40.030402.110010.
6. Couillerot O, Pringent-Combaret C, Caballero-Mellado J, Moenne-Loccoz Y. 2009. *Pseudomonas fluorescens* and closely-related fluorescent pseudomonads as biocontrol agents of soil-borne phytopathogens. Lett Appl Microbiol 48:505–512. https://doi.org/10.1111/j.1472-765X.2009.02566.x.
7. Lugtenberg BJJ, Dekkers LC. 1999. What makes *Pseudomonas* bacteria rhizosphere competent? Environ Microbiol 1:9–13. https://doi.org/10.1046/j.1462-2920.1999.00005.x.
8. Weller DM, Landa BB, Mavrodi OV, Schroeder KL, de la Fuente L, Bankhead SB, Molar RA, Bonsall RF, Mavrodi DV, Thomashow LS. 2007. Role of 2,4-diacetylphloroglucinol-producing fluorescent *Pseudomonas* spp. in the defense of plant roots. Plant Biol 9:4–20. https://doi.org/10.1055/s-2006-924473.
9. Iavicoli A, Boutet E, Buchala A, Metraux JP. 2003. Induced systemic resistance in *Arabidopsis thaliana* in response to root inoculation with *Pseudomonas fluorescens* CHA0. Mol Plant Microbe Interact 16:851–858. https://doi.org/10.1094/MPMI.2003.16.10.851.
10. Siddiqui IA, Shahid Shaukat S. 2003. Suppression of root-knot disease by *Pseudomonas fluorescens* CHA0 in tomato: importance of bacterial secondary metabolite, 2,4-diacetylphloroglucinol. Soil Biol Biochem 35:1615–1623. https://doi.org/10.1016/j.soilbio.2003.08.006.
11. Weller DM, Mavrodi DV, van Pelt JA, Pieterse CM, van Loon LC, Bakker PAMH. 2012. Induced systemic resistance in *Arabidopsis thaliana* against *Pseudomonas syringae* pv. tomato by 2,4-diacetylphloroglucinol-producing *Pseudomonas fluorescens*. Phytopathology 102:403–412. https://doi.org/10.1094/PHYTO-08-11-0222.
12. Phillips DA, Fox TC, King MD, Bhuvaneswari TV, Teuber LR. 2004. Microbial products trigger amino acid exudation from plant roots. Plant Physiol 136:2887–2894. https://doi.org/10.1104/pp.104.044222.
13. Brazelton JN, Pfeufer EE, Sewat TA, Gardener BBM, Coenen C. 2008. 2,4-Diacetylphloroglucinol alters plant root development. Mol Plant Microbe Interact 21:1349–1358. https://doi.org/10.1094/MPMI-21-10-1349.
14. Combes-Meynet E, Pothier JF, Moënne-Loccoz Y, Prigent-Combaret C. 2011. The *Pseudomonas* secondary metabolite 2,4-diacetylphloroglucinol is a signal inducing rhizoplane expression of *Azospirillum* genes involved in plant-growth promotion. Mol Plant Microbe Interact 24:271–284. https://doi.org/10.1094/MPMI-07-10-0148.
15. Rezzonico F, Zala M, Keel C, Duffy B, Moenne-Loccoz Y, Défago G. 2007. Is the ability of biocontrol fluorescent pseudomonads to produce the antifungal metabolite 2,4-diacetylphloroglucinol really synonymous with higher plant protection? New Phytol 173:861–872. https://doi.org/10.1111/j.1469-8137.2006.01955.x.
16. Sharifi-Tehrani A, Zala M, Natsch A, Moënne-Loccoz Y, Défago G. 1998. Biocontrol of soil-borne fungal plant diseases by 2,4-diacetylphloroglucinol-producing fluorescent pseudomonads with different restriction profiles of amplified 16S rDNA. Eur J Plant Pathol 104:631–643. https://doi.org/10.1023/A:1008672104562.
17. Eichenlaub R, Gartemann KH. 2011. The *Clavibacter michiganensis* subspecies: molecular investigation of gram-positive bacterial plant pathogens. Annu Rev Phytopathol 49:445–464. https://doi.org/10.1146/annurev-phyto-072910-095258.
18. Lanteigne C, Gadkar VJ, Wallon T, Novinscak A, Filion M. 2012. Production of DAPG and HCN by *Pseudomonas* sp. LBUM300 contributes to the biological control of bacterial canker of tomato. Phytopathology 102:976–973. https://doi.org/10.1094/PHYTO-11-11-0312.
19. Paulin MM, Novinscak A, St-Arnaud M, Goyer C, DeCoste NJ, Privé J-P, Owen J, Filion M. 2009. Transcriptional activity of antifungal metabolite-encoding genes *phlD* and *hcnBC* in *Pseudomonas* spp. using qRT-PCR. FEMS Microbiol Ecol 68:212–222. https://doi.org/10.1111/j.1574-6941.2009.00669.x.
20. Powers MJ, Sanabria-Valentín E, Bowers AA, Shank EA. 2015. Inhibition of cell differentiation in *Bacillus subtilis* by *Pseudomonas protegens*. J Bacteriol 197:2129–2138. https://doi.org/10.1128/JB.02535-14.
21. Ude S, Arnold DL, Moon CD, Timms-Wilson T, Spiers AJ. 2006. Biofilm formation and cellulose expression among diverse environmental *Pseudomonas* isolates. Environ Microbiol 8:1997–2011. https://doi.org/10.1111/j.1462-2920.2006.01080.x.
22. Danhorn T, Fuqua C. 2007. Biofilm formation by plant-associated bacteria. Annu Rev Microbiol 61:401–422. https://doi.org/10.1146/annurev.micro.61.080706.093316.
23. Landa BB, Mavrodi OV, Raaijmakers JM, Gardener BBM, Thomashow LS, Weller DM. 2002. Differential ability of genotypes of 2,4-diacetylphloroglucinol-producing *Pseudomonas fluorescens* strains to colonize the roots of pea plants. Appl Environ Microbiol 68:3226–3237. https://doi.org/10.1128/AEM.68.7.3226-3237.2002.
24. Lugtenberg BJJ, Dekkers LC, Bloemberg GV. 2001. Molecular determinants of rhizosphere colonization by *Pseudomonas*. Annu Rev Phytopathol 39:461–490. https://doi.org/10.1146/annurev.phyto.39.1.461.
25. Athukorala SNP, Fernando WGD, Rashid KY, de Kievit T. 2010. The role of volatile and non-volatile antibiotics produced by *Pseudomonas chlororaphis* strain PA23 in its root colonization and control of *Sclerotinia sclerotiorum*. Biocontrol Sci Technol 20:875–890. https://doi.org/10.1080/09583157.2010.484484.
26. Carroll H, Moenne-Loccoz Y, Dowlong DN, Ogara F. 1995. Mutational disruption of the biosynthesis genes-coding for the antifungal metabolite 2,4-diaceylphloroglucinol does not influence the ecological fitness of *Pseudomonas fluorescens* F113 in the rhizosphere of sugar beets. Appl Environ Microbiol 61:3002–3007.
27. Ghirardi S, Dessaint F, Mazurier S, Corberand T, Raaijmakers JM, Meyer JM, Dessaux Y, Lemanceau P. 2012. Identification of traits shared by rhizosphere-competent strains of fluorescent pseudomonads. Microb Ecol 64:725–737. https://doi.org/10.1007/s00248-012-0065-3.
28. Mazzola M, Cook RJ, Thomashow LS, Weller DM, Pierson LS. 1992. Contribution of phenazine antibiotic biosynthesis to the ecological competence of fluorescent pseudomonads in soil habitats. Appl Environ Microbiol 58:2616–2624.
29. Novinscak A, Gadkar VJ, Joly DL, Filion M. 2016. Complete genome sequence of *Pseudomonas brassicacearum* LBUM300, a disease-suppressive bacterium with antagonistic activity toward fungal, oomycete, and bacterial plant pathogens. Genome Announc 4(1):e01623-15. https://doi.org/10.1128/genomeA.01623-15.
30. Zhou HY, Wei HL, Liu XL, Wang Y, Zhang LQ, Tang WH. 2005. Improving biocontrol activity of *Pseudomonas fluorescens* through chromosomal integration of 2,4-diacetylphloroglucinol biosynthesis genes. Chin Sci Bull 50:775–781.
31. Zhou T-T, Li C-Y, Chen D, Wu K, Shen Q-R, Shen B. 2014. *phlF*$^-$ mutant of *Pseudomonas fluorescens* J2 improved 2,4-DAPG biosynthesis and biocontrol efficacy against tomato bacterial wilt. Biol Control 78:1–8. https://doi.org/10.1016/j.biocontrol.2014.07.006.
32. Fenton AM, Stephens PM, Crowley J, Ocallaghan M, Ogara F. 1992. Exploitation of gene(s) involved in 2,4-diacetylphloroglucinol biosynthesis to confer a new biocontrol capability to a *Pseudomonas* strain. Appl Environ Microbiol 58:3873–3878.
33. Natsch A, Keel C, Pfirter HA, Haas D, Défago G. 1994. Contribution of the global regulator gacA to persistence and dissemination of *Pseudomonas fluorescens* biocontrol strain CHA0 introduced into soil microcosms. Appl Environ Microbiol 60:2553–2560.
34. Raaijmakers JM, Weller DM. 1998. Natural plant protection by 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp. in take-all decline soils. Mol Plant Microbe Interact 11:144–152. https://doi.org/10.1094/MPMI.1998.11.2.144.
35. Notz R, Maurhofer M, Schnider-Keel U, Duffy B, Haas D, Défago G. 2001. Biotic factors affecting expression of the 2,4-diacetylphloroglucinol biosynthesis gene *phlA* in *Pseudomonas fluorescens* biocontrol strain CHA0 in the rhizosphere. Phytopathology 91:873–881. https://doi.org/10.1094/PHYTO.2001.91.9.873.
36. Rotenberg D, Joshi R, Benitez M-S, Chapin LG, Camp A, Zumpetta C, Osborne A, Dick WA, McSpadden Gardener BB. 2007. Farm management effects on rhizosphere colonization by native populations of 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp. and their contributions to crop health. Phytopathology 97:756–766. https://doi.org/10.1094/PHYTO-97-6-0756.
37. Jamali F, Sharifi-Tehrani A, Lutz MP, Maurhofer M. 2009. Influence of host plant genotype, presence of a pathogen, and coinoculation with *Pseudomonas fluorescens* strains on the rhizosphere expression of hydrogen cyanide and 2,4-diacetylphloroglucinol biosynthetic genes in *P. fluorescens* biocontrol strain CHA0. Microb Ecol 57:267–275. https://doi.org/10.1007/s00248-008-9471-y.
38. McSpadden Gardener BB, Weller DM. 2001. Changes in populations of rhizosphere bacteria associated with take-all disease of wheat. Appl Environ Microbiol 67:4414–4425. https://doi.org/10.1128/AEM.67.10.4414-4425.2001.
39. Notz R, Maurhofer M, Dubach H, Haas D, Défago G. 2002. Fusaric acid-producing strains of *Fusarium oxysporum* alter 2,4-diacetylphloroglucinol biosynthetic gene expression in *Pseudomonas fluorescens* CHA0

in vitro and in the rhizosphere of wheat. Appl Environ Microbiol 68:2229–2235. https://doi.org/10.1128/AEM.68.5.2229-2235.2002.
40. DeCoste NJ, Gadkar VJ, Filion M. 2010. *Verticillium dahlia* alters *Pseudomonas* spp. populations and HCN gene expression in the rhizosphere of strawberry. Can J Microbiol 56:906–915. https://doi.org/10.1139/W10-080.
41. Combes-Meynet E, Pothier JF, Moënne-Loccoz Y, Prigent-Combaret C. 2011. The *Pseudomonas* secondary metabolite 2,4-diacetylphloroglucinol is a signal inducing rhizoplane expression of *Azospirillum* genes involved in plant-growth promotion. Mol Plant Microbe Interact 2:271–284. https://doi.org/10.1094/MPMI-07-10-0148.
42. Brown MRW, Kornberg A. 2008. The long and short of it—polyphosphate, PPK and bacterial survival. Trends Biochem Sci 33:284–290. https://doi.org/10.1016/j.tibs.2008.04.005.
43. Daniels R, Reynaert S, Hoekstra H, Verreth C, Janssens J, Braeken K, Fauvart M, Beullens S, Heusdens C, Lambrichts I, De Vos DE, Vanderleyden J, Vermant J, Michiels J. 2006. Quorum signal molecules as biosurfactants affecting swarming in *Rhizobium etli*. Proc Natl Acad Sci U S A 103:14965–14970. https://doi.org/10.1073/pnas.0511037103.
44. Naseby DC, Lynch JM. 1998. Impact of wild-type and genetically modified *Pseudomonas fluorescens* on soil enzyme activities and microbial population structure in the rhizosphere of pea. Mol Ecol 7:617–625. https://doi.org/10.1046/j.1365-294x.1998.00367.x.
45. Naseby DC, Pascual JA, Lynch JM. 1999. Carbon fractions in the rhizosphere of pea inoculated with 2,4-diacetylphloroglucinol producing and non-producing *Pseudomonas fluorescens* F113. J Appl Microbiol 87:173–181. https://doi.org/10.1046/j.1365-2672.1999.00809.x.
46. Naseby DC, Lynch JM. 2001. Effect of 2,4-diacetylphloroglucinol producing, overproducing, and nonproduction *Pseudomonas fluorescens* F113 in the rhizosphere of pea. Microb Ecol 42:193–200.
47. Naseby DC, Way JA, Bainton NJ, Lynch JM. 2001. Biocontrol of *Pythium* in the pea rhizosphere by antifungal metabolite producing and non-producing *Pseudomonas* strains. J Appl Microbiol 90:421–429. https://doi.org/10.1046/j.1365-2672.2001.01260.x.
48. Chapon A, Guillerm A-Y, Delalande L, Lebreton L, Sarniquet A. 2002. Dominant colonisation of wheat roots by *Pseudomonas fluorescens* Pf29A and selection of the indigenous microflora in the presence of the take-all fungus. Eur J Plant Pathol 108:449–459. https://doi.org/10.1023/A:1016099707119.
49. Arseneault T, Goyer C, Filion M. 2016. Biocontrol of potato common scab is associated with high *Pseudomonas fluorescens* LBUM223 populations and PCA biosynthetic transcripts accumulation. Phytopathology 106:963–970. https://doi.org/10.1094/PHYTO-01-16-0019-R.
50. Agriculture and Agri-Food Canada. 1998. The Canadian system of soil classification, 3rd ed. NRC Research Press, Ottawa, ON, Canada.
51. Paulin MM, Nicolaisen MH, Sørensen J. 2011. (R,S)-dichlorprop herbicide in agricultural soil induces proliferation and expression of multiple dioxygenase-encoding genes in the indigenous microbial community. Environ Microbiol 13:1513–1523. https://doi.org/10.1111/j.1462-2920.2011.02456.x.
52. DeCoste NJ, Gadkar VJ, Filion M. 2011. Relative and absolute quantitative real-time PCR-based quantifications of *hcnC* and *phlD* gene transcripts in natural soil spiked with *Pseudomonas* sp. strain LBUM300. Appl Environ Microbiol 77:41–47. https://doi.org/10.1128/AEM.01387-10.