Altering the Ratio of Phenazines in *Pseudomonas chlororaphis* (*aureofaciens*) Strain 30-84: Effects on Biofilm Formation and Pathogen Inhibition[▽]

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Pseudomonas chlororaphis strain 30-84 is a plant-beneficial bacterium that is able to control take-all disease of wheat caused by the fungal pathogen *Gaeumannomyces graminis* var. *tritici*. The production of phenazines (PZs) by strain 30-84 is the primary mechanism of pathogen inhibition and contributes to the persistence of strain 30-84 in the rhizosphere. PZ production is regulated in part by the PhzR/PhzI quorum-sensing (QS) system. Previous flow cell analyses demonstrated that QS and PZs are involved in biofilm formation in *P. chlororaphis* (V. S. R. K. Maddula, Z. Zhang, E. A. Pierson, and L. S. Pierson III, *Microb. Ecol.* 52:289–301, 2006). *P. chlororaphis* produces mainly two PZs, phenazine-1-carboxylic acid (PCA) and 2-hydroxy-PCA (2-OH-PCA). In the present study, we examined the effect of altering the ratio of PZs produced by *P. chlororaphis* on biofilm formation and pathogen inhibition. As part of this study, we generated derivatives of strain 30-84 that produced only PCA or overproduced 2-OH-PCA. Using flow cell assays, we found that these PZ-altered derivatives of strain 30-84 differed from the wild type in initial attachment, mature biofilm architecture, and dispersal from biofilms. For example, increased 2-OH-PCA production promoted initial attachment and altered the three-dimensional structure of the mature biofilm relative to the wild type. Additionally, both alterations promoted thicker biofilm development and lowered dispersal rates compared to the wild type. The PZ-altered derivatives of strain 30-84 also differed in their ability to inhibit the fungal pathogen *G. graminis* var. *tritici*. Loss of 2-OH-PCA resulted in a significant reduction in the inhibition of *G. graminis* var. *tritici*. Our findings suggest that alterations in the ratios of antibiotic secondary metabolites synthesized by an organism may have complex and wide-ranging effects on its biology.

Bacteria in nature exist within surface-attached communities, termed biofilms, and interact cooperatively and competitively with other members of the microbial community (reviewed in references 13, 14, 22, and 33). Biofilm communities are “microniches” that differ dramatically from surrounding conditions (33), confer resistance to deleterious agents such as antibiotics and detergents (15), and enable cells to coordinately carry out functions not typically undertaken outside of the biofilm (33). Bacteria associated with plants may form biofilms on or within plant tissues (reviewed in references 13, 33, and 41). The associations between plants and microorganisms encompass beneficial, neutral, detrimental, and pathogenic associations. The effects of deleterious or pathogenic microorganisms on plants can be ameliorated by the presence of bacteria that reduce the impact of the pathogen on the plant. Many of these beneficial bacteria (often termed biological control bacteria) produce secondary metabolites that interfere with the pathogen’s disease potential by either direct inhibition of pathogen growth, induction of plant defenses, or enhancement of plant growth (reviewed in reference 53). Secondary metabolites are chemical compounds historically defined as not being involved directly in “normal” growth, development, or reproduction. These compounds often are produced by the bacterium during the transition from exponential to stationary growth phase. Thus, plant-beneficial bac-

teria and the secondary metabolites they produce represent key ecological control points for manipulating plant-microbe interactions that promote plant health.

Among fluorescent pseudomonads that serve as biological control agents, several secondary metabolites, including phenazines (PZs), pyrrolnitrin, pyoluteorin, and 2,4-diacetylphloroglucinol, were identified as primary mechanisms of disease control. These secondary metabolites originally were classified as antibiotics due to their ability to inhibit plant pathogens on agricultural crops (reviewed in references 40, 47, 52, and 54). A general hypothesis regarding these antibiotic metabolites is that they serve a competitive function by inhibiting the growth of other microorganisms. More recently, regulatory pathways that control secondary metabolite production are being elucidated (reviewed in references 11, 40, and 51). These studies demonstrate that many bacteria utilize combinations of conserved sensory networks to control antibiotic production in response to environmental, nutritional, population, and metabolic inputs. We hypothesize that the complexity of antibiotic regulation reflects the complexity of their roles in the producing organism. In support of this hypothesis, a recent review provides evidence that secondary metabolites serve multiple roles in a number of taxonomically diverse organisms (38).

Pseudomonas chlororaphis (*aureofaciens*) strain 30-84 is a biological control bacterium that is effective against take-all disease of wheat caused by the fungal pathogen *Gaeumannomyces graminis* var. *tritici*. Strain 30-84 produces several secondary metabolites, including PZs. PZ production is the primary mechanism of pathogen inhibition and contributes to persistence of strain 30-84 in the rhizosphere (10, 28, 35, 46).

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description ^a	Reference or source
<i>P. chlororaphis</i> strains		
30-84	Wild type, Rif ^r	35
30-84PCA	PCA ⁺ , 2-OH-PCA ⁻ , Rif ^r Km ^r , <i>phzO</i> ::Tn5	This study
30-84O*	30-84 containing plasmid pKM- <i>phzO</i> *	This study
30-84Z	Phz ⁻ Rif ^r <i>phzB</i> :: <i>lacZ</i> genomic fusion	36
<i>E. coli</i> strains		
DH5α	F ⁻ <i>recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1 Δ(argF-lacZYA)I169 φ80lacZ ΔM15 λ⁻</i>	GIBCO-BRL
HB101	F ⁻ <i>hsdS20(r_B⁻ m_B⁻) supE44 recA1 ara14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-5 λ⁻</i>	GIBCO-BRL
Plasmids		
pLAFR3	IncP1 <i>cos</i> ⁺ <i>rtx</i> ⁺ Tc ^r	45
pLSP18-6	pLAFR3 carrying a 9.2-kb EcoRI fragment of the 30-84 chromosome with <i>phzXYFABCD</i> and <i>phzO</i>	35
pKM- <i>phzO</i> *	pLAFR3 carrying a 5.0-kb PstI fragment of pLSP18-6 with <i>phzCD</i> and <i>phzO</i>	This study
pUC18	ColE1 Ap ^r	43
pPR510	ColE1 Cm ^r	39
pKM-38	pUC18 containing the 9.2-kb chromosomal EcoRI fragment carrying Tn5 within <i>phzO</i> ; Km ^r	This study
pKM-510-38R	pPR510 containing EcoRI-BamHI fragment of pKM-38; Km ^r	This study
pKM-510-38S	pPR510 containing BamHI-EcoRI fragment of pKM-38; Km ^s	This study
pPROBE-KT'	pVS1 replicon, p15a origin of replication, <i>gfp</i> transcriptional fusion; Km ^r	30
pHP24Ω-Cm	Contains <i>cat</i> ; Cm ^r	18
pMAL-p2X	Commercial vector containing the <i>tac</i> promoter <i>P_{tac}</i>	New England Biolabs
pLAF13-9	pLAFR3 containing a 3.5-kb BamHI-HindIII fragment with a promoterless <i>lacZ</i> gene	L. S. Pierson, unpublished
pKT2CM-GFP	pPROBE-KT' containing the 4.3-kb EcoRI fragment carrying <i>cat</i> , a 150-bp EcoRI-BamHI PCR amplicon carrying <i>P_{tac}</i> , and the 3.5-kb BamHI-HindIII fragment carrying a promoterless <i>lacZ</i> gene; Km ^r Cm ^r <i>lac</i> ⁺ <i>gfp</i> ⁺	E. A. Pierson, unpublished

^a Ap^r, Cm^r, Km^r, Rif^r, and Tc^r, resistance to ampicillin, chloramphenicol, kanamycin, rifampin, and tetracycline, respectively; Km^s, sensitivity to kanamycin.

PZ biosynthesis is tightly regulated at multiple levels, including PhzR/PhzI quorum sensing (QS) (37, 58), GacA/GacS two-component global regulation (9, 10), RpeA negative regulation (55), and RsmA posttranscriptional regulation (unpublished data). Recently, we showed that PhzR/PhzI QS mutants of strain 30-84 were defective in biofilm formation (26). Interestingly, compared to the QS mutants, a structural mutant defective only in PZ biosynthesis was equally impaired in biofilm formation (26). Genetic and biochemical complementation of the PZ mutant restored biofilm formation to wild-type levels. These results clearly demonstrated a second important role for this antibiotic secondary metabolite, i.e., involvement in the formation of biofilms.

The PZ core biosynthetic genes are conserved among pseudomonads but often differ in their terminal modifying enzymes (27). The PZ biosynthetic genes in strain 30-84 differ from those in other known pseudomonads by the presence of *phzO*, which encodes a monooxygenase (16, 27). This monooxygenase aids in conversion of the primary yellow PZ derivative phenazine-1-carboxylic acid (PCA) into the orange 2-hydroxy-PCA (2-OH-PCA). This enzymatic reaction results in the partial conversion of PCA to 2-OH-PCA. Thus, *P. chlororaphis* 30-84 produces primarily the yellow PCA (~80 to 90%) and small amounts of the orange 2-OH-PCA (~10 to 20%). We hypothesize that the types of PZs produced and the ratios in which they are produced play important roles in the competitive survival and persistence of the producing organism.

In the present study, we examined the effect of altering the ratio of PZs produced by *P. chlororaphis* 30-84 on biofilm formation and pathogen inhibition. As part of this study, we

generated derivatives of strain 30-84 that produced only PCA (strain 30-84PCA) or overproduced 2-OH-PCA (strain 30-84O*). Using flow cell assays, we showed that these PZ-altered derivatives of strain 30-84 differed from the wild type in initial attachment, mature biofilm architecture, and dispersal from biofilms. The PZ-altered derivatives of strain 30-84 also differed in their ability to inhibit the fungal pathogen *G. graminis* var. *tritici*. Our findings suggest that alterations in the ratios of antibiotic secondary metabolites produced may have diverse and possibly unforeseen effects on the biology of the organism.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains and plasmids used in this study are described in Table 1. A spontaneous rifampin-resistant derivative of *P. chlororaphis* strain 30-84 was used in all studies (35), and all mutants were derived from this parental strain. Triparental matings into strain 30-84 or its derivatives were performed as described previously (36). All strains of *P. chlororaphis* were grown at 28°C in LB medium supplemented with 5 g of NaCl/liter or in AB minimal medium supplemented with 2% Casamino Acids (AB-CAA) (58). *Escherichia coli* strains were grown at 37°C in LB with supplements. KMPE medium (35) was used for fungal inhibition assays. Antibiotics were used where appropriate at the following concentrations: for *E. coli*, ampicillin at 100 µg/ml, gentamicin at 25 µg/ml, kanamycin sulfate (Km) at 50 µg/ml, chloramphenicol (Cm) at 30 µg/ml, and tetracycline (Tc) at 25 µg/ml; for *P. chlororaphis*, gentamicin at 50 µg/ml, Km at 50 µg/ml, Tc at 50 µg/ml, Cm at 300 µg/ml, and rifampin at 100 µg/ml (58).

Construction of mutants. *P. chlororaphis* strain 30-84PCA was isolated following Tn5 mutagenesis of strain 30-84 as described previously (35). A Km^r, yellow derivative that produced only PCA was identified. To determine the location of the Tn5 insertion, chromosomal DNA from strain 30-84PCA was isolated and digested with EcoRI. The resulting fragments were cloned into pUC18, and Km^r transformants of *E. coli* strain DH5α were isolated. One transformant containing plasmid pKM-38 was selected for further analysis (Table 1). Since Tn5 contains

FIG. 1. (A) Simplified PZ biosynthetic pathway. PZs are derived from chorismate precursors, of which PCA is the primary PZ compound synthesized. Subsequently PCA is hydroxylated by the *phzO* product, a monooxygenase. (B) Liquid cultures of the PZ derivative mutants compared to wild-type 30-84. Cultures were grown in AB-CAA with selection in 96-well plates. Images of strains 30-84, 30-84O*, and 30-84PCA after 24 h are shown.

a single BamHI site, pKM-38 was digested with BamHI and EcoRI, and the resulting two fragments were cloned into BamHI- and EcoRI-digested pPR510. The resulting white Cm^r transformants were further screened for Km resistance and sensitivity. The Km^r transformants contained pKM-510-38R, pPR510 carrying the fragment of Tn5 encoding Km^r , and flanking chromosomal DNA. The Km^s transformants contained plasmid pKM-510-38S, pPR510 carrying the other fragment of Tn5, and flanking chromosomal DNA. The DNA sequences of the adjacent chromosomal DNA regions were determined using primers to the inverted repeat region of Tn5, as described previously (42). The sequences were assembled after removal of the Tn5 sequences and compared to the nucleotide database using NCBI BLAST (1).

Derivative 30-84O* was constructed by the introduction of additional copies of *phzO*, which encodes the monooxygenase responsible for the conversion of PCA into 2-OH-PCA in strain 30-84. A 5-kb PstI fragment of the PZ biosynthetic operon containing *phzO* was cloned from pLSP18-6 into pLAFR3, resulting in the isolation of pKM-*phzO**. The cloned 5-kb PstI fragment also contained part of *phzC* and *phzD*. These additional regions were included because a ~2.0-kb XbaI-PstI fragment containing only *phzO* failed to convert PCA into 2-OH-PCA in our experiments (data not shown).

To control for the effects of the Te^r -carrying pLAFR3 vector pKM-*phzO** in strain 30-84O*, we introduced pLAFR3 (no insert) into strains 30-84, 30-84Z, and 30-84PCA. The strains containing pLAFR3 were used in all assays where strains were compared to 30-84O* with appropriate antibiotic selection. For confocal laser scanning microscopy (CLSM), strain 30-84 and its derivatives also were fluorescently labeled by introduction of the plasmid pKT2CM-GFP, which contained the green fluorescent protein gene under the expression of the *tac* promoter (Table 1).

PZ quantification. PZs were extracted from strain 30-84 and derivatives as described previously (55). Briefly, cultures were grown overnight (16 to 18 h) in LB and AB-CAA to late exponential phase (optical density at 620 nm = 1.8) and cell-free supernatants prepared by centrifugation ($2,600 \times g$) for 15 min. Total PZs from cell-free supernatants were extracted with an equal volume of acidified benzene, and the benzene phase was separated and evaporated under air. Dried PZs were dissolved in 0.1 N NaOH and quantified by UV-visible spectroscopy using 0.1 N NaOH as the blank. The absorption maxima for PCA and 2-OH-PCA were measured at 367 nm and 484 nm, respectively. The relative amounts of PCA and 2-OH-PCA were calculated by multiplying their absorption maxima by their standard extinction coefficients (21, 34). PZs also were quantified from dispersing cells in flow cell effluents of 1- to 4-day-old biofilms of each 30-84 derivative. Effluent samples (50 ml) were collected in acidified benzene and the PZs extracted. The relative amounts of each PZ were calculated and standardized to the number of cells in the effluent sample. We conducted three separate experiments, each over a 4-day period.

Flow cell assay. Single-pass flow cell assays were used to visually compare biofilm formation by strain 30-84 and derivatives as described previously (26)

with slight modifications. Briefly, inocula were prepared from exponential-phase cultures grown in AB-CAA and washed twice with fresh AB-CAA. These cultures were further diluted (optical density at 620 nm = 0.4) with AB-CAA. For each treatment, a 300- μl aliquot of dilute culture was inoculated into individual flow cell chambers. After inoculation, chambers were maintained under static conditions (no flow) for 0.5 h to allow cell attachment, and then a continuous flow of fresh medium (160 $\mu\text{l}/\text{min}$) was initiated. Biofilms were visualized using an Olympus BX60 light microscope or a Nikon E800 CLSM) at various magnifications. Images were taken at multiple time intervals for up to 6 days after inoculation. At each time interval, five random images were taken for each treatment, and representative images are presented. Image stacks taken with the Nikon E800 CLSM were rendered to obtain three-dimensional images using Volocity software (Improvision Ltd., Coventry, United Kingdom). To quantify differences in biofilm architecture among mutants, biofilm parameters were calculated using ISA-2 software (6, 7). Each experiment was repeated at least twice.

Initial attachment and dispersal rates. Strain 30-84 and derivatives with altered PZ ratios were analyzed for various stages of biofilm formation, initial attachment, and dispersal rate. Initial attachment was measured by imaging biofilm establishment in the flow cell assay at early time points (45 min to 6 h) after flow initiation. Five random images were taken for each treatment using an Olympus BX60 light microscope, and the images were analyzed using ISA-2 software to calculate percent surface coverage. Experiments were repeated twice, and representative results from one experiment are presented.

Rates of dispersal from biofilms were determined by modifying the flow cell apparatus to allow collection of the effluent from each of the three flow chambers independently. Effluent samples (3 ml) were collected at 24-h intervals for 3 days. Collection of samples after 3 days was problematic because maintaining the pressure in the flow cell apparatus during sampling became more difficult due to increasing back pressure from the effluent tank. Effluent counts were determined by serial dilution plating on LB with appropriate selection. Experiments were repeated three times, and representative results from one experiment are presented.

Fungal inhibition assay. Fungal inhibition by strain 30-84 and derivatives was determined by measuring their ability to inhibit mycelial growth of *Gaeumannomyces graminis* var. *tritici* in vitro as described previously (31). Briefly, a fresh 5-mm agar plug of *G. graminis* var. *tritici* was placed in the centers of KMPE plates. After 24 h, 5- μl spots of overnight growth of the bacterial cultures to be tested were placed at the plate peripheries. Plates were incubated for 48 h, and zones of inhibition were measured in mm. Each experiment was repeated twice with three replicates.

Statistical analysis. Biofilm volumetric parameters, dispersal rates, and zones of inhibition were analyzed statistically. Means were compared among treatments (by time interval, where appropriate) using analysis of variance ($P < 0.05$)

TABLE 2. Production of PCA and 2-OH-PCA by strain 30-84 and altered PZ derivatives

Strain	PZ concn, $\mu\text{g/ml}$ (ratio to wild type) ^a		
	PCA	2-OH-PCA	Total
30-84(pLAFR3)	28.5 (1.0)	6.7 (1.0)	35.0 (1.0)
30-84PCA(pLAFR3)	34.1 (1.4)	0.0 (0.1)	34.1 (1.1)
30-84O*(pKM- <i>phzO</i> *)	18.4 (0.7)	14.6 (2.5)	33.0 (1.0)
30-84Z(pLAFR3)	0 (0)	0 (0)	0 (0)

^a PZs were quantified using the UV-visible spectral peaks for PCA and 2-OH-PCA (367 nm and 484 nm, respectively) multiplied by their respective extinction coefficients. Extractions were repeated three times, and representative data from one extraction are shown. Since total amounts of PZs differed between extractions, data for each extraction were standardized by calculating the ratio of PCA, 2-OH-PCA, or total PZs produced by each derivative relative to the wild type. Mean ratio data are given parenthetically.

and protected least-significant difference multiple-comparison tests (SAS version 8.2; SAS Institute, Inc., Cary, NC).

RESULTS

Modifying PZ production in 30-84. The PZ biosynthetic pathway is linear (Fig. 1A) and is under QS regulation (*phzR/phzI*) (36, 57). Eight genes (*phzXYFABCD*O) constitute the PZ operon of strain 30-84 (37), of which the terminal *phzO* monooxygenase is specific to *P. chlororaphis* 30-84 (16). This monooxygenase adds a hydroxyl group at the second carbon position of PCA, leading to formation of 2-OH-PCA. *P. chlororaphis* 30-84 synthesizes three PZ derivatives, i.e., PCA (~80 to 90%), 2-OH-PCA (~10 to 20%), and a small amount of the PZ derivative 2-OH-PZ (~1 to 2%) formed by spontaneous decarboxylation of 2-OH-PCA (35). To produce a derivative of strain 30-84 that produces only PCA, we constructed the *phzO* mutant 30-84PCA using Tn5 mutagenesis. Our sequence data indicated that the Tn5 was inserted 1,160 bp from the start of the 1,473-bp *phzO* gene, terminating the PZ biosynthetic path-

way at PCA production. Generation of a mutant that produced only 2-OH-PCA was not possible because 2-OH-PCA is derived from PCA. Instead we sought to enhance the conversion of PCA to 2-OH-PCA by the introduction of extra copies of *phzO* in *trans* in strain 30-84. This was achieved by cloning a 5-kb fragment of the PZ biosynthetic cluster containing *phzC*, *phzD*, and *phzO* under the control of a *lac* promoter into pLAFR3 to make pKM-*phzO**. We refer to this plasmid-containing 30-84 derivative as 30-84O*.

PZ production in derivatives 30-84PCA and 30-84O* was characterized visually and quantitatively. Compared to strain 30-84, 30-84PCA is yellow whereas 30-84O* is red when grown to late exponential phase in liquid or on solid LB or AB-CAA medium (Fig. 1B). Similar total amounts of PZ were produced by 30-84, 30-84PCA, and 30-84O* (Table 2). However, strain 30-84PCA produces only PCA, whereas strain 30-84O* produces 2.5-fold more 2-OH-PCA than the wild type. As expected, since 2-OH-PCA is derived from PCA, this strain produces a reduced amount of PCA. 30-84O* makes only 70% and 50% of the amount of PCA produced by the wild type and 30-84PCA, respectively. The PZ ratios produced by the wild type and the PZ-altered derivatives were consistent across media tested (LB and AB-CAA) (Table 2 and data not shown) and in the flow cell effluents of 1- to 4-day-old biofilms. For example, the mean percentages of PZs produced by the wild type in dispersing and LB-grown cells ($n = 14$) were 86% PCA and 14% 2-OH-PCA ($\pm 5\%$). Dispersing and LB-grown cells of 30-84PCA produced 98% PCA ($\pm 1\%$), whereas dispersing and LB grown cells of 30-84O* produced 60% PCA and 40% 2-OH-PCA ($\pm 6\%$).

Effect of altered PZ ratios on initial attachment. The early attachment phase of biofilm formation by strains 30-84, 30-84PCA, and 30-84O* was analyzed in flow cell assays by light microscopic imaging (magnification, $\times 1,000$) at early time points (Fig. 2). Five random images from each flow cell chamber were taken at 45 min and 2, 4, and 6 h after flow initiation,

FIG. 2. Effect of altered PZ ratio on the initial attachment stage of biofilm formation. The early attachment phase of biofilm formation by strains 30-84, 30-84PCA, and 30-84O* was observed and analyzed in a flow cell assay by taking light microscope images at early time points. Five random images for each treatment and time point combination were taken. Each experiment was repeated twice, and representative data are presented. Representative images were taken 45 min and 6 h after the start of medium flow. All the images were obtained at a magnification of $\times 1,000$ using an Olympus BX60 light microscope.

TABLE 3. Percentages of slide surface area covered by the 30-84 derivatives at early time points

Time	% Surface area coverage (mean $\pm$ SD) ^a by strain:		
	30-84(pLAFR3)	30-84PCA(pLAFR3)	30-84O*(pKM- <i>phzO</i> *)
45 min	1 $\pm$ 0.01	1 $\pm$ 0.01	44 $\pm$ 0.01
2 h	2 $\pm$ 0.02	1 $\pm$ 0.01	54 $\pm$ 0.02
4 h	34 $\pm$ 0.02	39 $\pm$ 0.04	56 $\pm$ 0.07
6 h	41 $\pm$ 0.02	49 $\pm$ 0.04	59 $\pm$ 0.07

^a Surface coverage area was calculated from images taken of the flow cell chambers at early time points after inoculation. Images were taken randomly with a bright-field microscope. Images were analyzed for area porosity (*P*) using ISA-2D software, and percent surface coverage ($1 - P$) was calculated from the porosity measurement. Data are based on five images. Experiments were repeated twice, and representative values from one experiment are shown.

and the images were analyzed using ISA software (6, 7) to calculate the percent surface coverage (Table 3). Images from as early as 45 min after inoculation demonstrate that 30-84O* covers more surface area than either 30-84 or 30-84PCA, and this difference in initial attachment is still apparent after 6 h (Fig. 2). The mean surface area coverage from two independent experiments for 30-84 and 30-84PCA was approximately 1% after 45 min, whereas it was already 44% for 30-84O* (Table 3). Interestingly, over the 6-h period, surface coverage of 30-84 and 30-84PCA increased from 1% to 41% and 49%, respectively, compared to a 44% to 59% increase for 30-84O*. The surface coverage by 30-84O* appeared to be more uniform than that by 30-84 and 30-84PCA, whereas the attached

FIG. 3. Effect of altered PZ ratio on biofilm architecture. Mature biofilm architecture was observed and analyzed in a flow cell assay using fluorescently labeled derivatives of strains 30-84, 30-84PCA, and 30-84O*. (A and B) Bird's-eye (A) and cross-sectional (B) three-dimensional images obtained from CLSM of 5-day-old mature biofilms of wild-type 30-84, 30-84PCA, and 30-84O*. Random Z-series image stacks were obtained using a Nikon E800 CLSM at a magnification of $\times 400$ and rendered to obtain three-dimensional images using Volocity software. (C) Basal view of the 5-day-old mature biofilms of wild-type 30-84, 30-84PCA, and 30-84O*. Images were taken using an Olympus BX60 light microscope at a magnification of $\times 100$.

TABLE 4. Biofilm volumetric parameters^a

Parameter (unit)	Value ^b for strain:		
	30-84(pLAFR3)	30-84PCA(pLAFR3)	30-84O*(pKM-phzO*)
Homogeneity (%)	79b	76b	83a
Thickness (μm) ^c	19c (1)	66a (3.5)	52b (2.7)
Porosity (%)	91b	86b	96a
Biovolume (μm ³) ^c	2.0 × 10 ⁶ b (1)	8.8 × 10 ⁶ a (4)	2.0 × 10 ⁶ b (1)

^a Mature (5-day-old) biofilm image stacks were taken using a CLSM. Biofilm volumetric parameters were calculated from the image stacks using ISA-3D software. Confocal images were taken from two experiments with a minimum of three image stacks for each treatment.

^b Average values of three image stacks from one representative experiment are shown. For each parameter, treatment values with different letters are significantly different.

^c Differences between treatments are given parenthetically as the fold difference compared to the wild type.

cells of 30-84 and 30-84PCA appeared to have a more aggregated, clustered distribution (Fig. 2).

PZ effects on biofilm architecture. Differences in the mature biofilm architecture formed by strains 30-84, 30-84PCA, and 30-84O* after 5 days were observed in flow cell assays using CLSM and light microscopy (Fig. 3). These differences in three-dimensional structure were apparent in all views (cross section, bird's eye, and basal (Fig. 3A and B) and were quantified from the volumetric parameters (homogeneity, thickness, porosity, and biovolume) (Table 4). Even after 5 days, the initial colonization patterns (seen after 45 min to 6 h) persisted in the mature biofilms. For example, strains 30-84 and 30-84PCA did not uniformly cover the attachment surface (similar to the 6-h images [Fig. 2]) and produced biofilms with mushroom-like clusters separated by open channels. Strain 30-84PCA produced a thicker, denser biofilm with significantly more cells than 30-84 or 30-84O* as determined from the thickness and biovolume parameters, respectively (Table 4). The biovolume and thickness of 30-84PCA biofilms were 4 and 3.5 times greater, respectively, than those of the wild type. In contrast, 30-84O* produced biofilms that more uniformly covered the attachment surface (similar to the 6-h images [Fig. 2]), with comparatively less structure. Biofilms of 30-84O* were significantly more homogeneous than those of either the wild type or 30-84PCA (Table 4). 30-84O* also produced a thicker (2.7 times) biofilm than 30-84; however, it was similar in cell volume. The net result is that 30-84O* produced thicker but more porous biofilms with less three-dimensional structure than the wild type.

PZ effects on bacterial dispersal from biofilms. To determine whether the PZ-altered derivatives differed from the wild type in dispersal, effluent samples were collected downstream of each flow cell and bacterial populations determined by serial dilution plating as an indicator of dispersal rates. The dispersal rates for 30-84 were ~2.5 and ~12 times higher than the dispersal rates for 30-84PCA and 30-84O*, respectively (Table 5). Dispersal from the biofilm increased very little from day 1 to day 2, but after day 2 the dispersal rates for 30-84, 30-84PCA, and 30-84O* increased 27, 16, and 13 times, respectively.

TABLE 5. Rates of dispersal of 30-84, 30-84PCA and 30-84O* from biofilms^a

Time (days)	Mean CFU/ml (10 ⁶) ^b of strain:		
	30-84(pLAFR3)	30-84PCA(pLAFR3)	30-84O*(pKM-phzO*)
1	1.74a	0.62b	0.14c
2	1.40a	1.15a	0.27c
3	46.53a	18.63b	3.60c

^a Samples of effluent from individual flow cell chambers were collected 1, 2, and 3 days after the initiation of flow, serially diluted, and plated on solid medium.

^b Data are pooled from three replicate flow chamber experiments. In each experiment, three replicate plates were counted for each sample. For each time interval, treatment values with different letters are significantly different.

Fungal inhibition assay. To determine whether the 30-84 derivatives differed in their ability to inhibit *G. graminis* var. tritici, we measured the zone of inhibition between the leading edge of the fungal mycelia and bacterial spots. 30-84 and 30-84O* produced similar zones of inhibition (8.08 mm). In contrast, 30-84PCA was significantly reduced in its ability to inhibit the fungus (fourfold-smaller zones [1.82 mm]) compared to 30-84O* and the wild type. As expected, 30-84Z, which produces no PZs, did not inhibit fungal growth.

DISCUSSION

Altering the ratio of PZs produced by strain 30-84 alters biofilm attachment. Our data suggest that more efficient conversion of PCA to 2-OH-PCA results in better initial attachment. Biofilms of 30-84O* covered a greater percentage of the flow cell surface area than those of 30-84 or 30-84PCA; this difference was evident from 45 min after initiation of flow and persisted to 6 h, when the majority of the surface was covered. Previous studies demonstrated that biofilm development occurs as a series of defined steps, including reversible attachment, irreversible attachment, microcolony formation, maturation into three-dimensional mushroom-like structures with open channels, and dispersal (22, 44). It is believed that reversible attachment takes place within a short time (i.e., seconds to minutes). In this study, the earliest time point at which we were able to reliably measure for all three treatments was 45 min after flow initiation, the period during which attachment becomes irreversible. Our data suggest that increasing 2-OH-PCA aids in irreversible attachment.

Several factors have been shown to play roles in the initial stages of biofilm formation in different bacterial systems. Factors shown to be important include flagella, type IV pili, cup fimbriae, and *sad* genes, which affect cell surface structures and properties in *Pseudomonas aeruginosa* (23, 29); production of adhesins such as LapA and LapD and regulatory domains such as GGDEF and EAL in plant-associated *Pseudomonas fluorescens* and *Pseudomonas putida* (reviewed in references 13, 20, and 23); and production of the adhesins rhicadhesin and Raps in members of the family *Rhizobiaceae* (13). Alternatively, Dietrich et al. (17) demonstrated that addition of high levels of the PZ pyocyanin to a pyocyanin-defective mutant of *P. aeruginosa* strain PAO1 induced global changes in gene expression patterns, suggesting that pyocyanin may be serving as a regulatory signal. Although the exact mechanism by which 2-OH-PCA aids in attachment is unknown, it is possible that 2-OH-PCA production acts directly as an adhesin or

that it serves as a signal inducing the expression of additional genes that are involved in initial attachment, including changes in bacterial cell surface structures or properties. It would be interesting to see if the increase in 2-OH-PCA induces the production of adhesins such as LapA, LapD, and Raps in *P. chlororaphis* 30-84.

Altering the ratio of PZs produced by strain 30-84 alters mature biofilm architecture and dispersal. Changing the ratios of PZs also produced significant changes in mature biofilm architecture. In our flow cell analysis of 5-day-old mature biofilms, we found that strain 30-84 forms a biofilm approximately 19 μm in thickness with mushroom-shaped clusters of cells. Production of only PCA resulted in substantially thicker mushroom-shaped clusters (66 μm) with a fourfold-greater cell density than the wild type. More efficient conversion of 2-OH-PCA resulted in biofilms that were thicker than those of the wild type (52 μm) with a similar cell density. These biofilms did not appear to produce the distinctive mushroom-like clusters but were more homogenous and porous than wild-type biofilms. It is interesting that changing the ratio of PZs normally produced by *P. chlororaphis* results in thicker biofilms. However, production of only PCA by strain 30-84PCA resulted in biofilms with a greater cell density, despite its having less efficient initial attachment than 30-84O*.

Differences in these architectural parameters may reflect differences in reproduction, mortality, or dispersal from the biofilm. In the present study, we looked at cell dispersal as the biofilms mature. Rates of dispersal of viable cells from the maturing biofilm were generally highest for 30-84, intermediate for 30-84PCA, and lowest for 30-84O*. The higher rate of dispersal by 30-84 may explain its production of thinner biofilms compared to 30-84PCA or 30-84O*. However, it is unclear why 30-84O*, which has the lowest dispersal rate, does not produce the thickest biofilm. Clearly, dispersal rate alone does not explain biofilm thickness.

The molecular mechanism for dispersal of sessile biofilm bacterial cells to free living planktonic cells is not well understood. Various factors or processes causing dispersal have been reported. These include sudden changes in nutrient availability (23), chelators such as EDTA (4), production of biosurfactants such as rhamnolipids in *P. aeruginosa* (8), the chemotaxis regulator BdlA in *P. aeruginosa* (32), and oxygen availability and the presence of nitric oxide (NO) in *P. aeruginosa* (5). In our current study, altered PZ ratios resulted in decreased dispersal rates in both the 30-84PCA and 30-84O* derivatives. Because PZs are known redox compounds, one possibility is that altering the PZ ratio in 30-84PCA and 30-84O* reduces nitrosative stress. If this is the case, less production of reactive nitrogen intermediates such as NO might reduce bacterial dispersal (5). Further studies are needed to determine whether these two PZ derivatives affect nitrosative stress or other known mechanisms.

Altering the ratio of PZs produced by strain 30-84 affects fungal inhibition. In the present study, we showed that loss of 2-OH-PCA production results in a significant reduction in the inhibition of the fungal pathogen *G. graminis* var. *tritici*. Similar results were reported by Toohey et al. (49), who demonstrated that 2-OH-PCA has greater antibiotic activity than PCA against an array of bacterial and fungal organisms. Interestingly, in our assay increased conversion of PCA to 2-OH-PCA by 30-84O* did not improve *G. graminis* var. *tritici* inhi-

bition significantly, compared to that by the wild type. This may indicate that the wild type already produces an optimum amount of 2-OH-PCA for fungal inhibition or, alternatively, that the 2.5-fold increase is not sufficient to produce a measurable increase in fungal inhibition.

Ecological perspectives. PZs belong to a large class of well-known heterocyclic secondary metabolites produced by a variety of bacteria (11, 19, 25, 27, 50). Over 100 natural derivatives of PZs with effects on a large variety of micro- and macroorganisms have been described (25, 50). PZs effectively control a wide range of plant pathogenic fungi and are a well-characterized mechanism of bacterial plant disease control (2, 11, 40, 51). The PZ pyocyanin also is known to be an important virulence factor in infections caused by the opportunistic human pathogen *P. aeruginosa* (reviewed in reference 38).

Although, as suggested recently by Laursen and Nielsen (25), "little is yet known about the physiological function of PZs in their natural environment," the complexity of the roles of secondary metabolites, including PZs, in the ecology and lifestyle of the organism is beginning to be recognized (17, 25; reviewed in reference 38). Similar to the case for strain 30-84, the production of PZs in other PZ-producing bacteria appears to be controlled by complex sensory networks that may include one or more QS systems, two-component regulation, posttranscriptional regulation, and other mechanisms (11, 24, 40, 51). These observations provide support for the hypothesis that the complexity of regulation of secondary metabolites reflects the complexity of their roles. This hypothesis is consistent with the evolutionary theory that metabolically costly antibiotics are subject to natural selection and are more likely to be maintained if they serve multiple functions (56).

In agriculture, antibiotic production by plant-associated microorganisms represents an environmentally compatible method of disease control. Commonly proposed approaches for improving microbial disease control include increasing secondary metabolite production, altering the structure of antibiotics produced, or introducing antibiotic pathways into other bacteria (3, 12, 48). However, our findings suggest that alterations in the ratios of antibiotic secondary metabolites produced may have wide-ranging effects on the biology of the organism. Therefore, central to intelligent manipulation of these complex interactions to improve plant health is to move beyond the conception that antibiotics function only in limiting target pathogens and to focus on the many roles antibiotics play for the producing organism and their relative importance in microbe-microbe and microbe-host interactions.

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REFERENCES

1. Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**:3389–3402.
2. Anjaiah, V., N. Koedam, B. Nowak-Thompson, J. E. Loper, M. Höfte, J. T. Tambong, and P. Cornelis. 1998. Involvement of phenazines and anthranilate in the antagonism of *Pseudomonas aeruginosa* PNA1 and Tn5 derivatives toward *Fusarium* spp. and *Pythium* spp. *Mol. Plant-Microbe Interact.* **11**: 847–854.

3. Bakker, P. A., D. C. Glandorf, M. Viebahn, T. W. Ouwens, E. Smit, P. Leeflang, K. Wernars, L. S. Thomashow, J. E. Thomas-Oates, and L. C. van Loon. 2002. Effects of *Pseudomonas putida* modified to produce phenazine-1-carboxylic acid and 2,4-diacetylphloroglucinol on the microflora of field grown wheat. *Antonie van Leeuwenhoek* **81**:617–624.
4. Banin, E., K. M. Brady, and E. P. Greenberg. 2006. Chelator-induced dispersal and killing of *Pseudomonas aeruginosa* cells in a biofilm. *Appl. Environ. Microbiol.* **72**:2064–2069.
5. Barraud, N., D. J. Hassett, S. H. Hwang, S. A. Rice, S. Kjelleberg, and J. S. Webb. 2006. Involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*. *J. Bacteriol.* **188**:7344–7353.
6. Beyenal, H., C. Donovan, Z. Lewandowski, and G. Harkin. 2004. Three-dimensional biofilm structure quantification. *J. Microbiol. Methods* **59**:395–413.
7. Beyenal, H., Z. Lewandowski, and G. Harkin. 2004. Quantifying biofilm structure: facts and fiction. *Biofouling* **20**:1–23.
8. Boles, B. R., M. Thoendel, and P. K. Singh. 2005. Rhamnolipids mediate detachment of *Pseudomonas aeruginosa* from biofilms. *Mol. Microbiol.* **57**:1210–1223.
9. Chancey, S. T., D. W. Wood, and L. S. Pierson III. 1999. Two-component transcriptional regulation of *N*-acyl-homoserine lactone production in *Pseudomonas aureofaciens*. *Appl. Environ. Microbiol.* **65**:2294–2299.
10. Chancey, S. T., D. W. Wood, E. A. Pierson, and L. S. Pierson III. 2002. Survival of GacS/GacA mutants of the biological control bacterium *Pseudomonas aureofaciens* 30-84 in the wheat rhizosphere. *Appl. Environ. Microbiol.* **68**:3308–3314.
11. Chin-Awoeng, T. F. C., G. V. Bloembergen, and B. J. J. Lugtenberg. 2003. Phenazines and their role in biocontrol by *Pseudomonas* bacteria. *New Phytologist* **157**:503–523.
12. Chin-Awoeng, T. F. C., J. E. Thomas-Oates, B. J. J. Lugtenberg, and G. V. Bloembergen. 2001. Introduction of the *phzH* gene of *Pseudomonas chlororaphis* PCL1391 extends the range of biocontrol ability of phenazine-1-carboxylic acid-producing *Pseudomonas* spp. strains. *Mol. Plant-Microbe Interact.* **14**:1006–1015.
13. Danhorn, T., and C. Fuqua. 2007. Biofilm formation by plant-associated bacteria. *Annu. Rev. Microbiol.* **61**:401–422.
14. Davey, M. E., and G. A. O'Toole. 2000. Microbial biofilms: from ecology to molecular genetics. *Microbiol. Mol. Biol. Rev.* **64**:847–867.
15. Davies, D. 2003. Understanding biofilm resistance to antibacterial agents. *Nat. Rev. Drug Disc.* **2**:114–122.
16. Delaney, S. M., D. V. Mavrodi, R. F. Bonsall, and L. S. Thomashow. 2001. *phzO*, a gene for biosynthesis of 2-hydroxylated phenazine compounds in *Pseudomonas aureofaciens* 30-84. *J. Bacteriol.* **183**:318–327.
17. Dietrich, L. E., A. Price-Whelan, A. Petersen, M. Whiteley, and D. K. Newman. 2006. The phenazine pyocyanin is a terminal signalling factor in the quorum sensing network of *Pseudomonas aeruginosa*. *Mol. Microbiol.* **61**:1308–1321.
18. Fellay, R., J. Frey, and H. Krisch. 1987. Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for *in vitro* insertional mutagenesis of gram-negative bacteria. *Gene* **52**:147–154.
19. Giddens, S. R., Y. Feng, and H. K. Mahanty. 2002. Characterization of a novel phenazine antibiotic gene cluster in *Erwinia herbicola* Eh1087. *Mol. Microbiol.* **45**:769–783.
20. Hinsia, S. M., and G. A. O'Toole. 2006. Biofilm formation by *Pseudomonas fluorescens* WCS365: a role for LapD. *Microbiology* **152**:1375–1383.
21. Jayatilake, G. S., M. P. Thornton, A. C. Leonard, J. E. Grimwade, and B. J. Baker. 1996. Metabolites from an antarctic sponge-associated bacterium, *Pseudomonas aeruginosa*. *J. Nat. Prod.* **59**:293–296.
22. Kierek-Pearson, K., and E. Karatan. 2005. Biofilm development in bacteria. *Adv. Appl. Microbiol.* **57**:79–111.
23. Klausen, M., M. Gjermansen, J. U. Kreft, and T. Tolker-Nielsen. 2006. Dynamics of development and dispersal in sessile microbial communities: examples from *Pseudomonas aeruginosa* and *Pseudomonas putida* model biofilms. *FEMS Microbiol. Lett.* **261**:1–11.
24. Lapouge, K., M. Schubert, F. H. Allain, and D. Haas. 2008. Gac/Rsm signal transduction pathway of gamma-proteobacteria: from RNA recognition to regulation of social behaviour. *Mol. Microbiol.* **67**:241–253.
25. Laursen, J. B., and J. Nielsen. 2004. Phenazine natural products: biosynthesis, synthetic analogues, and biological activity. *Chem. Rev.* **104**:1663–1686.
26. Maddula, V. S. R. K., Z. Zhang, E. A. Pierson, and L. S. Pierson III. 2006. Quorum sensing and phenazines are involved in biofilm formation by *Pseudomonas chlororaphis* (*aureofaciens*) strain 30-84. *Microb. Ecol.* **52**:289–301.
27. Mavrodi, D. V., W. Blankenfeldt, and L. S. Thomashow. 2006. Phenazine compounds in fluorescent *Pseudomonas* spp. biosynthesis and regulation. *Annu. Rev. Phytopathol.* **44**:417–445.
28. Mazzola, M., R. J. Cook, L. S. Thomashow, D. M. Weller, and L. S. Pierson III. 1992. Contribution of phenazine antibiotic biosynthesis to the ecological competence of fluorescent pseudomonads in soil habitats. *Appl. Environ. Microbiol.* **58**:2616–2624.
29. Merritt, J. H., K. M. Brothers, S. L. Kuchma, and G. A. O'Toole. 2007. SadC reciprocally influences biofilm formation and swarming motility via modulation of exopolysaccharide production and flagellar function. *J. Bacteriol.* **189**:8154–8164.
30. Miller, W. G., J. H. J. Leveau, and S. E. Lindow. 2000. Improved *gfp* and *inaZ* broad-host-range promoter-probe vectors. *Mol. Plant-Microbe Interact.* **13**:1243–1250.
31. Morello, J. E., E. A. Pierson, and L. S. Pierson III. 2004. Negative cross-communication among wheat rhizosphere bacteria: effect on antibiotic production by the biological control bacterium *Pseudomonas aureofaciens* 30-84. *Appl. Environ. Microbiol.* **70**:3103–3109.
32. Morgan, R., S. Kohn, S. Hwang, D. J. Hassett, and K. Sauer. 2006. BdlA, a chemotaxis regulator essential for biofilm dispersion in *Pseudomonas aeruginosa*. *J. Bacteriol.* **188**:7335–7343.
33. Morris, C. E., and J. M. Monier. 2003. The ecological significance of biofilm formation by plant-associated bacteria. *Annu. Rev. Phytopathol.* **41**:429–453.
34. Olson, E., and J. Richards. 1967. Structure of the orange pigment from *Pseudomonas aureofaciens*. *J. Org. Chem.* **32**:2887–2890.
35. Pierson, L. S., III, and L. S. Thomashow. 1992. Cloning and heterologous expression of the phenazine biosynthetic locus from *Pseudomonas aureofaciens* 30-84. *Mol. Plant-Microbe Interact.* **5**:330–339.
36. Pierson, L. S., III, V. D. Keppene, and D. W. Wood. 1994. Phenazine antibiotic biosynthesis in *Pseudomonas aureofaciens* 30-84 is regulated by PhzR in response to cell density. *J. Bacteriol.* **176**:3966–3974.
37. Pierson, L. S., III, T. Gaffney, S. Lam, and F. Gong. 1995. Molecular analysis of genes encoding phenazine biosynthesis in the biological control bacterium *Pseudomonas aureofaciens* 30-84. *FEMS Microbiol. Lett.* **134**:299–307.
38. Price-Whelan, A., L. E. Dietrich, and D. K. Newman. 2006. Rethinking 'secondary' metabolism: physiological roles for phenazine antibiotics. *Nat. Chem. Biol.* **2**:71–78.
39. Quigley, N. B., and P. R. Reves. 1987. Chloramphenicol resistance cloning vector based on pUC9. *Plasmid* **17**:54–57.
40. Raaijmakers, J. M., M. Vlam, and J. T. de Souza. 2002. Antibiotic production by bacterial biocontrol agents. *Antonie van Leeuwenhoek* **81**:537–547.
41. Ramey, B. E., M. Koutsoudis, S. B. von Bodman, and C. Fuqua. 2004. Biofilm formation in plant-microbe associations. *Curr. Opin. Microbiol.* **7**:602–609.
42. Rich, J. J., and D. K. Willis. 1990. A single oligonucleotide can be used to rapidly isolate DNA sequences flanking a transposon Tn5 insertion by the polymerase chain reaction. *Nucleic Acids Res.* **18**:6673–6676.
43. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
44. Sauer, K., A. H. Rickard, and D. G. Davies. 2007. Biofilms and biocomplexity. *Microbe* **2**:347–353.
45. Staskawicz, B. J., D. Dahlbeck, N. Keen, and C. Napoli. 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. *J. Bacteriol.* **169**:5789–5794.
46. Thomashow, L. S., and D. M. Weller. 1988. Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var. *tritici*. *J. Bacteriol.* **170**:3499–3508.
47. Thomashow, L. S. 1996. Biological control of plant root pathogens. *Curr. Opin. Biotechnol.* **7**:343–347.
48. Timms-Wilson, T. M., R. J. Ellis, A. Renwick, D. J. Rhodes, D. V. Mavrodi, D. M. Weller, L. S. Thomashow, and M. J. Bailey. 2000. Chromosomal insertion of phenazine-1-carboxylic acid biosynthetic pathway enhances efficacy of damping-off disease control by *Pseudomonas fluorescens*. *Mol. Plant-Microbe Interact.* **13**:1293–1300.
49. Toohey, J. I., C. D. Nelson, and G. Krotkov. 1965. Toxicity of phenazine carboxylic acid to some bacteria, algae, higher plants, and animals. *Can. J. Bot.* **43**:1151–1155.
50. Turner, J. M., and A. J. Messenger. 1986. Occurrence, biochemistry and physiology of phenazine pigment production. *Adv. Microb. Physiol.* **27**:211–275.
51. Walsh, U. F., J. P. Morrissey, and F. O'Gara. 2001. *Pseudomonas* for biocontrol of phytopathogens: from functional genomics to commercial exploitation. *Curr. Opin. Biotechnol.* **12**:289–295.
52. Weller, D. M. 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Annu. Rev. Phytopathol.* **26**:379–407.
53. Weller, D. M. 2007. *Pseudomonas* biocontrol agents of soilborne pathogens: looking back over 30 years. *Phytopathology* **97**:250–256.
54. Whipps, J. M. 1997. Developments in the biological control of soilborne plant pathogens. *Adv. Bot. Res.* **26**:1–133.
55. Whistler, C. A., and L. S. Pierson III. 2003. Repression of phenazine antibiotic production in *Pseudomonas aureofaciens* strain 30-84 by RpeA. *J. Bacteriol.* **185**:3718–3725.
56. Wink, M. 2003. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry* **64**:3–19.
57. Wood, D. W., and L. S. Pierson III. 1996. The *phzI* gene of *Pseudomonas aureofaciens* 30-84 is responsible for the production of a diffusible signal required for phenazine antibiotic production. *Gene* **168**:49–53.
58. Zhang, Z., and L. S. Pierson III. 2001. A second quorum-sensing system regulates cell surface properties but not phenazine antibiotic production in *Pseudomonas aureofaciens*. *Appl. Environ. Microbiol.* **67**:4305–4315.