Biological Control of Wheat Root Diseases by the CLP-Producing Strain *Pseudomonas fluorescens* HC1-07

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ABSTRACT

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Pseudomonas fluorescens HC1-07, previously isolated from the phyllosphere of wheat grown in Hebei province, China, suppresses the soilborne disease of wheat take-all, caused by *Gaeumannomyces graminis* var. *tritici*. We report here that strain HC1-07 also suppresses Rhizoctonia root rot of wheat caused by *Rhizoctonia solani* AG-8. Strain HC1-07 produced a cyclic lipopeptide (CLP) with a molecular weight of 1,126.42 based on analysis by electrospray ionization mass spectrometry. Extracted CLP inhibited the growth of *G. graminis* var. *tritici* and *R. solani* in vitro. To determine the role of this CLP in biological control,

plasmid mutagenesis was used to generate two nonproducing mutants, HC1-07viscB and HC1-07prtR2. Analysis of regions flanking plasmid insertions in HC1-07prtR2 and HC1-07viscB revealed that the inactivated genes were similar to *prtR* and *viscB*, respectively, of the well-described biocontrol strain *P. fluorescens* SBW25 that produces the CLP viscosin. Both genes in HC1-07 were required for the production of the viscosin-like CLP. The two mutants were less inhibitory to *G. graminis* var. *tritici* and *R. solani* in vitro and reduced in ability to suppress take-all. HC1-07viscB but not HC1-07prtR2 was reduced in ability to suppress Rhizoctonia root rot. In addition to CLP production, *prtR* also played a role in protease production.

Additional keyword: biosurfactant.

Take-all and Rhizoctonia root rot, caused by the soilborne necrotrophic fungal pathogens *Gaeumannomyces graminis* var. *tritici* and *Rhizoctonia solani* AG-8, respectively, cause severe damage to irrigated and dryland wheat and barley (6,48,49) in the Inland Pacific Northwest (PNW) of the United States. Take-all is considered one of the most important root diseases of wheat in the United States and worldwide and Rhizoctonia root rot is a severe problem in Australian cereal production; however, surprisingly, in the United States, root rot caused by *R. solani* AG-8 seems to be confined to the PNW. The incidence and severity of Rhizoctonia root rot is greatly exacerbated by reduced tillage, and this disease is the primary cause for the slow adoption of no-till (direct seeding), which is needed to reduce soil erosion in the PNW. Because of the lack of genetic resistance and the fact that fungicides are effective only during the seedling phase of these diseases, biological control by either introduced or indigenous agents and sustainable management practices represent the best options for long-term control (6,47,48,61,62). A disease decline phenomenon

resulting in soils suppressive to either take-all (62,63) or Rhizoctonia root rot (36,54) has been described and many growers have utilized this highly sustainable natural form of biocontrol to manage these diseases. For example, Cook (6) reported that 0.8 million ha of PNW farmland are now cropped continuously or nearly so to rotations of spring wheat, spring barley, and winter wheat, and these crops suffer little damage from take-all owing to take-all decline (TAD) (6). Several promising biocontrol agents capable of suppressing take-all and Rhizoctonia root rot have been described and tested in the PNW and elsewhere. In particular, antibiotic-producing strains of gram-positive *Bacillus* spp. and gram-negative *Pseudomonas* spp. have proved to be effective as seed treatments in controlling *G. graminis* var. *tritici* or *R. solani* AG-8 under field conditions in the PNW (7,28,51,61).

Several groups of antibiotics are known to mediate the suppression of fungal phytopathogens by fluorescent *Pseudomonas* spp. (20,21,35), and antibiotics of special interest have included phenazines (37,38,58), pyoluteorin (20,25), pyrrolnitrin (20,24), and 2,4-diacetylphloroglucinol (40,62), as well as biosurfactants like cyclic lipopeptides (CLPs) and rhamnolipids (27,45,52, 53,57). CLPs are versatile molecules with antimicrobial, cytotoxic, and surfactant properties produced by many different bacterial genera, including plant-associated *Pseudomonas* spp. (8,52). The potential of CLPs produced by *Pseudomonas* spp. to suppress diverse phytopathogens is documented in several different studies. For example, Tran et al. (59) demonstrated that the CLP massetolide A from *Pseudomonas fluorescens* SS101

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played an important role in suppression of *Phytophthora infestans*, whereas viscosin contributed to the control of broccoli head rot by *Pseudomonas fluorescens* PfA7B (4). The CLP viscosinamide produced by *Pseudomonas* sp. DR54 triggered the encystment of *Pythium* zoospores and reduced the mycelial growth of *R. solani* and *Pythium ultimum* (43). Amphisin played an important role in the surface motility of *Pseudomonas* sp. strain DSS73, allowing efficient containment of root-infecting plant-pathogenic fungi (44,45). Lipodepsipeptides produced by the pathogen *Pseudomonas syringae* pv. *syringae* mediate the antagonism of this organism towards several phytopathogenic fungi (19). van den Broek et al. (60) reported that a lipopeptide was likely involved in the suppression of take-all of wheat by *Pseudomonas* sp. PCL1171. Finally, CLPs produced by *Pseudomonas* sp. CMR12a acted synergistically with phenazines in the control of *Pythium* and *Rhizoctonia* spp. on bean (9,50).

The biosynthesis and regulation of CLPs in *Pseudomonas* spp. has been well studied (8,52), with biosynthesis governed by nonribosomal peptide synthetases (NRPSs) encoded by large gene clusters and composed of modules, one for each amino acid incorporated in the oligopeptide. Each module consists of several conserved domains, responsible for recognition, activation, transport, and binding of the amino acid to the peptide chain. A special thioesterase domain in the last module coordinates cyclization and release of the peptide product (8,18,52). In addition, many studies have focused on the genetic regulation of CLP production in pseudomonads. The well-known GacS/GacA two-component regulatory system appears to serve as a master switch for CLP production as it does for other secondary metabolites (10,16). Furthermore, acyl-homoserine lactone quorum sensing is involved in the regulation of some of the CLPs produced by *Pseudomonas* spp. (10,17). LuxR type transcriptional regulator genes, such as *salA* and *syrG*, were also found to play a role in CLP biosynthesis regulation (3,12,15). DnaK, a member of the Hsp70 heat-shock protein family, is involved in regulation of CLP synthesis in *P. putida* (16).

This study focused on the biocontrol agent *P. fluorescens* strain HC1-07, isolated from the phyllosphere of wheat grown in a field in Hebei province. In this region of China, there is a long history of cereal production; take-all is a chronic problem and little is known about the communities of wheat-associated, disease-suppressive bacteria (64). Strain HC1-07 was selected for further study because it was representative of one of the morphotypes of fluorescent pseudomonads isolated from wheat. This strain suppressed take-all of wheat caused by isolates of *G. graminis* var. *tritici* from both China and the PNW of the United States (64).

This study was part of an ongoing collaboration to select and develop biocontrol agents for use in Chinese agriculture, identify mechanisms of action, and determine the relatedness of the Chinese agents to others worldwide. Our specific aim was to determine the role of CLPs in the suppression *G. graminis* var. *tritici* and *R. solani* AG-8 by strain HC1-07. A CLP produced by

strain HC1-07 was isolated and identified as a viscosin-like compound and two genes, *viscB* and *prtR*, involved in the CLP production were identified by random plasposon mutagenesis. We characterized the two CLP-deficient mutants and showed them to be less effective at controlling take-all compared with the wild-type strain; however, only the mutant disrupted in *viscB* was less suppressive of *Rhizoctonia* root rot.

MATERIALS AND METHODS

Microorganisms, media, and culture conditions. Bacteria and fungi used in this study are listed in Table 1. A spontaneous rifampicin-resistant derivative of *P. fluorescens* HC1-07 (64) with growth characteristics similar to those of the wild-type strain was used throughout this study. *P. fluorescens* and *Escherichia coli* were routinely cultured in King's medium B (KMB) agar and broth (29) and Luria-Bertani (LB) broth (1), respectively. When needed, antibiotics were supplemented in the following concentrations: chloramphenicol (13 µg ml⁻¹), tetracycline (25 µg ml⁻¹), ampicillin (40 µg ml⁻¹), cycloheximide (100 µg ml⁻¹), and rifampicin (100 µg ml⁻¹) (Table 1). Bacteria were stored at -80°C in LB broth supplemented with 40% (vol/vol) glycerol.

G. graminis var. *tritici* isolates LD5, ARS-A1, and R3-111a-1 (32) and *R. solani* AG-8 isolate C-1 (26) were grown on one-fifth-strength potato dextrose agar (1/5 PDA) prepared from either fresh potato and adjusted to pH 6.5 as described by Yang et al. (64) or commercial potato dextrose broth (PDB) (Becton Dickinson, Sparks, MD). To prepare homemade PDA, diced potato tubers (200 g) were cooked in water in a microwave for 15 min and the resultant broth was strained and brought to 1 liter, supplemented with dextrose (20 g) and agar (15 g), and then autoclaved (64). All fungi were stored at 4°C on 1/5 PDA made from fresh potato and amended with rifampicin (100 µg ml⁻¹) (31).

Generation of CLP-deficient mutants by transposon mutagenesis. CLP-deficient mutants of HC1-07rif were generated by subjecting the strain to mutagenesis with plasposon TnMod-OTc' (13). The plasposon was delivered into HC1-07rif using biparental matings with *E. coli* S17-1 (λpir). The transposants were selected on LB amended with tetracycline and rifampicin, then screened for the loss of biosurfactant production by the drop-collapse assay (14), which measures the ability of a bacterial metabolite to reduce surface tension of a liquid spotted on a piece of Parafilm.

The number of TnMod-OTc' copies in genomes of the biosurfactant-deficient mutants was analyzed by Southern blot hybridization. Briefly, genomic DNA was isolated using a GenElute Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis) following the manufacturer's instructions. The extracted DNA was dissolved in 100 µl of sterile deionized water and stored at -20°C. Genomic DNA of the mutants was digested to completion with endonucleases *EcoRV* and *PstI*. The DNA fragments were

TABLE 1. Bacteria and plasmids used in this study

Organism, strain, or plasmid	Genotype, characteristics ^z	Source/reference
<i>Pseudomonas fluorescens</i> HC1-07	CLP ⁺	64
HC1-07rif	Rif ^r CLP ⁺	This study
HC1-07viscB	Rif ^r viscB::TnMod CLP ⁻	This study
HC1-07viscB-1	Rif ^r CLP ⁺ complemented mutant; viscB ⁺	This study
HC1-07prtR2	Rif ^r prtR::TnMod CLP ⁻	This study
HC1-07prtR2-1	Rif ^r CLP ⁺ complemented mutant; prtR2 ⁺	This study
<i>Escherichia coli</i>		
S17-1(λpir)	thi pro hsdR hsdM recA rpsL RP4-2 Tet ^r ::Mu Kan ^r ::Tn7 λpir	Laboratory collection
DH5α	F ⁻ endA1 hsdR17 supE44 thi-1 gyrA96 relA1 ΔargF-lacZYA U169 Φ80d lacZΔM15 λ-	Laboratory collection
Plasmids		
p TnMod-OTc'	TnMod; oriR; Tc ^r	13
pEX18Gm	Gene replacement vector; Gm ^r oriT sacB	Laboratory collection

^z CLP = cyclic lipopeptide; Rif^r, Tet^r, Kan^r, Tc^r, and Gm^r = resistant to rifampicin, tetracycline, kanamycin, tetracycline, and gentamicin, respectively.

separated on a 1% agarose gel and transferred onto a Hybond N nylon membrane (GE Healthcare, Piscataway, NJ) using standard techniques (1). The hybridization probe contained a 515-bp fragment of the tetracycline resistance gene and was obtained by amplification of p TnMod-OTc' with primers Tet_UP and Tet_LOW (38). The probe was labeled with digoxigenin (DIG) using DIG High Prime DNA Labeling and Detection Starter Kit (Roche Diagnostics GmbH, Germany).

The hybridization conditions included prehybridization (1.5 h at 65°C) and hybridization (12 h at 65°C) steps followed by stringency washes (twice each for 5 min with 2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] and 0.1% sodium dodecyl sulfate [SDS] at room temperature, and twice each for 30 min with 0.1× SSC and 0.1% SDS at 65°C). The hybridized DIG-labeled probe was immunodetected according to the protocol provided by Roche.

To characterize regions flanking plasposon insertion sites, 1 µg of total genomic DNA was digested with *EcoRI* (does not cut within TnMod-OTc') and the resultant fragments were self-ligated under conditions favorable for intramolecular reactions. The ligation mixture (2 µl) was transformed into *E. coli* DH5α by electroporation and clones containing the resultant TnMod-OTc'-based plasmids were selected by plating on LB amended with tetracycline. Regions of the HC1-07 genome flanking the TnMod-OTc' insertion sites were sequenced using the plasposon-based oligonucleotide primers Ori (5'-GGCCTTTTGCTCACATGT-3') and Tet (5'-TCAATTCTTGCGGAGAAC-3').

Genetic complementation of CLP-deficient mutants. The CLP-deficient mutants HC1-07viscB and HC1-07prtR2 were complemented as follows. The 1.0-kb fragment of viscB corresponding to the TnMod-OTc' integration site in HC1-07viscB was amplified by polymerase chain reaction (PCR) with primers viscB1 (5'-GGAGCAACTGGTACAGGCGT-3') and viscB2 (5'-GCACTTCGTAGGTAGTGGCGT-3') using DNA of HC1-07 as a template. Similarly, the 1.0-kb fragment of prtR that corresponds to the plasposon integration site in HC1-07prtR2 was amplified by PCR with primers PrtR1 (5'-AGCTCAACACCGAGCAGC-3') and PrtR2 (5'-TATCGGCAGCCAGGTGTT-3'), again using DNA of HC1-07 as a template. Both amplicons were cloned into pEX18Gm and the resultant plasmids were single-pass sequenced to ensure the absence of mutations. The plasmids were mobilized into HC1-07viscB or HC1-07prtR2, respectively, using biparental matings with *E. coli* S17-1 (λpir). Single crossover events were recovered on LB medium supplemented with gentamicin, and several gentamicin-resistant colonies were streaked on LB amended with 5% sucrose to select for double crossovers. The reversion to wild-type *viscB* and *prtR* alleles and the loss of the pEX18Gm backbone were confirmed by PCR with primer sets viscB1/viscB2, PrtR1/PrtR2, and GM_UP/GM_LOW (38). The complemented mutants were also assayed by the drop collapse assay for the gain of function to produce CLP.

Phenotypic profiling. Swimming and swarming motility were visualized by spot-inoculating bacteria on standard succinate medium (12) supplemented with 0.25% (wt/vol) (swimming motility assays) and 0.6% (wt/vol) (swarming motility assays) of agar. The inoculated plates were incubated overnight at 28°C. Biofilm formation was assessed in untreated polystyrene flat-bottom 96-well plates (Greiner Bio-One, Monroe, NC) prefilled with 200 µl of M63 medium, as described by O'Toole and Kolter (46) and Dubern et al. (17). Exoprotease production was assessed according to Lawrence et al. (34) by inoculating 20 µl of a 24-h-old bacterial culture in wells cut in 2.5% skim milk agar using a 4-mm cork borer. The inoculated plates were incubated for 48 h at 28°C and the diameter of the halo was measured. The production of siderophores was determined by measuring orange haloes after 2 days of growth at 28°C on CAS (chrome azurol S) agar (54). All assays were repeated twice, with three replicates per strain.

In vitro inhibition of fungal pathogens by strain HC1-07 and its isogenic derivatives. The capacity of HC1-07 and its isogenic mutant and recombinant derivatives to inhibit the growth of *G. graminis* var. *tritici* isolates LD5, ARS-A1, and R3-111a-1 and *R. solani* AG-8 isolate C-1 was tested on PDA made from fresh potato, as described by Yang et al. (64). Aliquots (5 µl) from overnight cultures of each strain grown in KMB broth were spotted 1 cm from the edge of a Petri dish containing homemade PDA and allowed to soak into the agar. A 0.5-cm plug from the leading edge of a 7-day-old 1/5 PDA culture of *G. graminis* var. *tritici* or 5-day-old culture of *R. solani* was placed in the center of the plate. The inoculated plates were incubated at 24°C and scored after 5 or 7 days by measuring the distance between the edges of the bacterial colony and the fungal mycelium. Each plate consisted of three isolates and a control that was not inoculated. All assays were repeated twice and each isolate was replicated three times. Inhibition was expressed relative to a noninoculated control.

Isolation and characterization of CLP. To extract the CLP, strain HC1-07 and its mutant derivatives were grown on KMB agar for 48 h at 28°C. The bacterial cells were scraped from the agar surface and washed twice in 20 ml of sterile deionized water. The supernatant was passed through a membrane filter (0.22-µm pore size), acidified to pH 2.0 with 9% HCl, and kept on ice for 1 h. The precipitate was collected by centrifugation (30 min at 6,000 rpm and 4°C) and washed twice with sterile deionized water (pH 2.0) (14). The precipitate was resuspended in 20 ml of sterile deionized water (pH 8.0) and lyophilized (Supermodul Yo-220; Thermo Savant, NY). The lyophilized extract was stored at -20°C. The lyophilized sample was analyzed by electrospray ionization mass spectrometry (ESI-MS) by the State Key Laboratory of Coordination Chemistry, Nanjing University, essentially as described by Hong et al. (23). Briefly, an aliquot of the biosurfactant sample was dissolved in methanol to obtain a stock solution of 1 mg ml⁻¹. Freshly prepared working solutions were made by diluting the stock solution with methanol to 10 or 15 µg ml⁻¹. ESI mass spectra were recorded using a Finnigan MAT LCQ ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA) in the positive ionization mode. Samples were injected at a flow rate of 2 µl min⁻¹. Source voltage and capillary voltage were 5.0 kV and 17 V in positive ion mode. Capillary temperature and sheath gas (N₂) flow were set at 290 and 12 arbitrary units, respectively, in both scan modes. Data were acquired in positive MS total ion scan mode (mass scan range of m/z 200 to 2,000).

Effect of CLPs on the growth of *G. graminis* var. *tritici* and *R. solani* AG-8. The effect of the CLP on growth of the *G. graminis* var. *tritici* isolates and *R. solani* AG-8 C-1 was examined as follows. The CLP was extracted from strain HC1-07 as described above. A 5-mm-diameter plug was excised from the margin of a 7-day-old 1/5 PDA culture of *G. graminis* var. *tritici* or 5-day-old culture of *R. solani* AG-8 and placed in the center of a Petri plate containing 1/5 PDA amended with the CLP at 0, 10, or 100 µg ml⁻¹. The inoculated plates were incubated at room temperature and radial hyphal growth was measured at 5 days for *R. solani* AG-8 or 7 days for *G. graminis* var. *tritici*. Three replicate plates per concentration were included in the experiment and the entire experiment was repeated twice.

Treatment of wheat seed with bacteria. For greenhouse biocontrol or root colonization studies in the field, wheat seed ('Penawawa' or 'Louise') was treated with bacteria, as described by Yang et al. (64). Briefly, KMB plates were spread inoculated with the test bacterium and incubated for 48 h at room temperature. A 1% methyl cellulose solution (4 ml) (M-6385; Sigma-Aldrich) was added to the plate and the bacteria were scraped into a test tube, shaken for 30 s, and then mixed with 5.3 g of wheat seed. Treated seed were dried for 2 h under a stream of sterile air. The final density of bacteria was ≈10⁷ CFU seed⁻¹. For field studies, larger amounts of seed were treated using the same proportions.

Preparation of oat-kernel inoculum. Inoculum of *G. graminis* var. *tritici* and *R. solani* AG-8 was prepared essentially as described by Kwak et al. (31). Briefly, 250 ml of untreated whole oat grains plus 350 ml of water were combined in a 1-liter flask and autoclaved for 90 min on each of two successive days. Sterile oat grains were inoculated with agar disks of *G. graminis* var. *tritici* isolate LD5 or *R. solani* AG-8 isolate C-1. After 3 to 4 weeks of growth at room temperature, the contents of each jar were air dried and stored at 4°C.

Biological control assays. A tube assay previously described by Yang et al. (64) was used to determine the biocontrol activity of bacteria against take-all or Rhizoctonia root rot. Briefly, plastic tubes (2.5 cm in diameter and 16.5 cm long) were supported in a hanging position in racks (200 cones rack⁻¹). The hole in the bottom of each tube was plugged with a cotton ball and the tube was then filled with a 6.5-cm-thick column of sterile vermiculite followed by air-dried and sieved Quincy virgin soil (Shano sandy loam) (10 g). For some experiments, the soil was first pasteurized (60°C for 30 min) in order to reduce the interference from other soilborne pathogens. *G. graminis* var. *tritici* or *R. solani* colonized oat-kernel inoculum was pulverized in a Waring Blender and sieved into fractions of known particle sizes. The fraction of 0.25 to 0.5 mm was added to the soil at 0.7 and 1.0% (wt/wt) for *G. graminis* var. *tritici* and *R. solani*, respectively. Three bacteria-treated wheat seed were placed on the soil surface and covered with a 1.5-cm-thick topping of vermiculite. Each tube received 10 ml of water. The rack of tubes was incubated at 15 to 18°C in a 12-h cycle of light and darkness and, for the first 4 days, was covered with plastic. After the plastic was removed, each cone received 10 ml of water two times a week and diluted (1:3 [vol/vol]) Hoagland's solution (macroelements only) once a week. All treatments were replicated five times and arranged in a randomized complete block design. After 3 to 4 weeks, plants were removed from the tubes, the roots were washed free of soil, and plants were evaluated for disease severity on a scale of 0 to 8, as previously described (26,51), where 0 = no disease evident and 8 = plants dead or nearly so. All experiments were repeated at least twice with similar results.

Rhizosphere colonization assays. Bacterial strains were tested under both controlled and field conditions for ability to colonize roots of wheat. For assays under controlled conditions, bacteria (strains HC1-07rif, HC1-07viscB, HC1-07prtR2, HC1-07viscB-1, and HC1-07prtR2-1) were prepared and applied individually to Quincy virgin soil in a 1% methylcellulose suspension, as previously described (33,39), to obtain 10⁴ CFU g⁻¹ of soil. The actual density of each strain introduced into the soil was determined by assaying 0.5 g of inoculated soil, as described below. Control soils consisted of soil amended only with a 1% methylcellulose suspension. Each treatment was replicated six times with a single pot serving as a replicate. Spring wheat (Penawawa) seed were pregerminated in the dark on moistened sterile filter paper in petri dishes (24 h) and were sown (6 per pot) in square pots (height, 6.5 cm; width, 7 cm) containing 200 g of Quincy virgin soil (33) inoculated with one of the bacterial strains. Control treatments consisted of soil amended with a 1% methylcellulose suspension. Plants were grown for three successive cycles in a controlled environment chamber at 15 to 18°C with 12-h photoperiod. After 2 weeks of growth (one cycle), population densities of bacteria were determined essentially as described by Mavrodi et al. (39). Briefly, for each replicate, the roots plus adhering rhizosphere soil from three individual plants were excised and assayed by first vortexing in 10 or 20 ml of sterile water (depending on the amount of roots) followed by sonication in a water bath. Each plant was processed separately. Population densities of the introduced strains were monitored by the modified dilution end-point method (42,64). Briefly, to detect the introduced strains, the soil suspensions were serially diluted in wells of 96-well microtiter plates containing sterile water (200 µl) and these dilutions

were transferred to plates containing 1/3× KMB broth supplemented with rifampicin, cycloheximide, ampicillin, and chloramphenicol (1/3× KMB⁺⁺⁺Rif). Population densities of total culturable heterotrophic aerobic bacteria were determined by performing the same assay in 1/10× tryptic soy broth supplemented with only cycloheximide (1/10× TSB⁺). Plates with 1/3× KMB⁺⁺⁺Rif and 1/10× TSB⁺ were incubated for 72 and 48 h, respectively, at room temperature and an optical density at 600 nm ≥ 0.1, measured with a microplate reader, was scored as positive for growth.

A field test of the ability of strain HC1-07rif to colonize the rhizosphere of the spring wheat (Louise) grown without irrigation at the Washington State University Plant Pathology Research Farm, Pullman was conducted in 2010. As previously described (64), the plot was cultivated in the spring and fertilized with 89-25-20-10 (N-P-S-Cl), which was applied as a dry formulation and incorporated to a depth of 10 to 15 cm. Seed furrows (40.6 cm apart) were opened mechanically to a depth of 6 to 8 cm and seed were hand sown and covered with ≈3 cm of soil. Treatments included strain HC1-07rif, applied at ≈10⁷ CFU seed⁻¹ as described above, and nontreated seed as a control. Treatments were part of a larger experiment and arranged in a randomized complete block design.

Each treatment consisted of four 2.13-m-long rows and was replicated six times. Every 3 weeks, plants were sampled from three locations in a replicate by digging them with a shovel to a depth of ≈20 cm, and excess soil was removed from the roots by gentle shaking. Plants from each replicate were placed in a new plastic bag, transferred to the laboratory, and immediately processed. For each replicate, the roots plus adhering rhizosphere soil from three individual plants were processed separately. The population size of introduced strain HC1-07rif and the indigenous culturable heterotrophic aerobic bacteria in the rhizosphere was detected with the dilution end-point assay, as described above.

Data analysis. Statistical analyses were performed by using appropriate parametric and nonparametric procedures with the STATISTIX 8.0 software (Analytical Software, St. Paul, MN). All population data were converted to log CFU per gram (fresh weight) of root. Differences in population densities among treatments were determined by standard analysis of variance, and mean comparisons among treatments were performed by using Fisher's protected least-significant-difference test (*P* = 0.05) or the Kruskal-Wallis all-pairwise comparison test (*P* = 0.05). Data from phenotypic assays were compared by using a two-sample *t* test or the Wilcoxon rank sum test (*P* = 0.05).

Accession numbers. The nucleotide sequences of *viscB* and *prtR* genes of *P. fluorescens* HC1-07 were deposited in GenBank under accession numbers AFN27384.1 and AFN27386.1, respectively.

RESULTS

Isolation and characterization of biosurfactants produced by *P. fluorescens* HC1-07. Extraction of CLP from the culture supernatant of *P. fluorescens* HC1-07 yielded a white precipitate (≈90% pure) (data not shown) that was analyzed by ESI-MS. Results of the analysis revealed a major peak in the mass spectrum at *m/z* 1,125.42 [*M*+H]⁺, which closely matched the molecular weight of the biosurfactants viscosin, massetolide F, or massetolide L (produced by some strains of *P. fluorescens*) (Supplemental Figure 1) (10). Amino acid analysis of the CLP (conducted by the Washington State University Laboratory for Bioanalysis & Biotechnology, Pullman) narrowed the possibilities to massetolide F or viscosin (data not shown).

The CLP extracted from HC1-07 was tested for the capacity to inhibit hyphal growth of *G. graminis* var. *tritici* and *R. solani*. Addition of the CLP to growth media at concentrations of 10 and 100 µg ml⁻¹ resulted in strong inhibition of both pathogens (Table

2; Supplemental Figure 2) but *G. graminis* var. *tritici* was more sensitive to the CLP than *R. solani*. The hyphal growth of *G. graminis* var. *tritici* was completely inhibited by the viscosin-like CLP at 100 µg ml⁻¹, whereas the same amount of this CLP caused only 55% inhibition of *R. solani* (Table 2).

Plasposon mutagenesis and characterization of CLP-deficient mutants. To clone genes involved in the production of the CLP, *P. fluorescens* HC1-07 was subjected to mutagenesis with the TnMod-OTc⁺ plasposon. As a result, in total, 390 transformants were obtained. Two of these, HC1-07viscB and HC1-07prtR2, failed to produce CLP, which was evident from the absence of foam formation after vigorous shaking and inability to

lower the surface tension of water during the collapsed drop assay (Fig. 1). Southern hybridization showed that each of the two mutants contained single-transposon integration events. The mutants were further tested for the ability to swim and swarm on semisolid medium, and to produce siderophores, exoprotease, and biofilm. Results of the testing revealed that HC1-07viscB and HC1-07prtR2 were deficient in swimming and swarming motility (Table 3; Supplemental Figure 3A and B). In addition to that, HC1-07prtR2 lost the ability to secrete exoprotease at 23 and 29°C (Table 3; Supplemental Figure 3C), while HC1-07viscB retained the wild-type levels of exoprotease production. The production of siderophores in both mutants remained unaffected and similar to that in HC1-07 (Table 3; Supplemental Figure 3D). HC1-07viscB and HC1-07prtR2 were reduced in ability to form biofilms compared with the wild-type strain (Table 3; Fig. 1B). The genetic complementation of mutants with the corresponding wild-type DNA fragments restored the surface motility and production of exoprotease to the wild-type levels and partially restored biofilm formation (Table 3).

Identification of genes affected by the plasposon insertions. In order to identify genes interrupted by TnMod-OTc⁺ in strains HC1-07viscB and HC1-07prtR2, we recovered and sequenced DNA regions flanking the plasposon insertions. Comparison of the resultant sequences against the nonredundant GenBank dataset revealed that, in HC1-07viscB, the plasposon insertion disrupted a gene with strong similarity (90% identity at the amino acid level to NRPS; blastp E-value 0.0) to *viscB*, a gene involved

TABLE 2. Relative inhibition (%) of radial hyphal growth of *Gaeumannomyces graminis* var. *tritici* and *R. solani* pathogenic isolates cultured on one-fifth-strength potato dextrose agar (1/5 PDA) amended with cyclic lipopeptide (CLP) at the indicated concentration^z

Pathogens	CLP concentration (µg/ml)		
	0	10	100
<i>G. tritici</i> LD5	0	42.9	100.0
<i>G. tritici</i> ARS-A1	0	50.0	100.0
<i>G. tritici</i> R3-111a-1	0	35.0	100.0
<i>R. solani</i> AG-8	0	10.0	55.0

^z Data represent the mean from three replicate plates. Reduction of radial hyphal growth of the pathogens on 1/5 PDA amended with indicated concentration of CLP compared with that on the control plate (no CLP amended).

Fig. 1. Comparison of biosurfactant production and biofilm formation in *Pseudomonas fluorescens* HC1-07rif and its cyclic lipopeptide-deficient mutants and complemented mutant derivatives. **A**, Drop collapse assay with cultures of the wild-type strain HC1-07rif, two mutants (HC1-07viscB and HC1-07prtR2), and two complemented mutants (HC1-07viscB-1 and HC1-07prtR2-1). Bacterial cells grown for 2 days at 28°C were resuspended in sterile water, and 20-µl droplets were spotted on Parafilm. A flat droplet is indicative of viscosin production. **B**, Biofilm formation by strain HC1-07 and its mutants in polystyrene microtiter plates. Cells firmly attached to the walls of the wells were stained with 0.1% crystal violet.

TABLE 3. Phenotypes of HC1-07, cyclic lipopeptide (CLP)-deficient mutants, and complemented strains^x

Strain	CLP	Production of ^y		Biofilm formation ^z	Swarming motility	Swimming motility
		Exoprotease (cm)	Siderophores (cm)			
HC1-07rif	+	0.97 A	1.12 A	0.35 A	+	+
HC1-07 viscB	–	0.93 A	1.37 A	0.05 B	–	–
HC1-07 viscB-1	+	0.96 A	1.26 A	0.20 AB	+	+
HC1-07 prtR2	–	ND	1.13 A	0.06 B	–	–
HC1-07 prtR2-1	+	0.96 A	1.18 A	0.21 AB	+	+

^x Means in the same column followed by the same letter are not significantly different at *P* = 0.05 according to the Kruskal-Wallis all-pairwise comparison test. ND = no halo detected. HC1-07prtR2 was not included in the analysis of halo diameter.

^y Halo diameter.

^z Absorbance at 600 nm.

in the production of the biosurfactant viscosin in *P. fluorescens* SWB25, and closely related to NRPSs from other *Pseudomonas* spp. (Table 4). Collectively, this finding, along with data generated by MS and amino acid analyses, indicated that the CLP was similar to viscosin. In HC1-07prtR2, the plasposon insertion disrupted a gene encoding a transmembrane regulator with strong similarity (94% identity at the amino acid level; blastp E-value 5e-148) to PrtR of *P. fluorescens* SWB25. Results of blast searches revealed that homologous genes were also present in other fluorescent *Pseudomonas* spp. (Table 5).

Impact of *viscB* and *prtR* mutations on rhizosphere colonization of HC1-07. The impact of *viscB* and *prtR* mutations on the ability of HC1-07 to colonize and persist in the rhizosphere of wheat was tested under controlled conditions in a growth chamber. In the wheat cycling experiment, HC1-07rif, mutants, and complemented mutants were introduced individually into Quincy virgin soil and population sizes of each strain were determined in the wheat rhizosphere after each 2-week-long growth cycle of wheat. Of the two tested mutants, only HC1-07prtR2 was mildly but significantly impaired in the ability to persist in the wheat rhizosphere (Fig. 2A). In cycle 2, the population size of HC1-07prtR2 was ≈ 1 log unit lower than that of the wild-type parent and significantly less than the other strains. Complementation of the *prtR* mutation restored plant colonization to the wild-type levels. In cycle 3, the same trend was seen, and the population size of mutant HC1-07prtR2 was significantly lower than that of the wild type and the complemented strain HC1-07prtR2-1.

Because HC1-07 was originally isolated from the phyllosphere, we also determined its ability to colonize and survive in the rhizosphere of wheat in the field. Under field conditions, the population sizes of HC1-07 on the roots at the first sampling time (3 weeks after planting) were 10^8 CFU g⁻¹ fresh root weight and declined thereafter to $\approx 10^7$ CFU g⁻¹ fresh root weight over a period of 16 weeks (Fig. 2B).

Impact of *viscB* and *prtR* mutations on biocontrol of take-all and Rhizoctonia root rot. The capacity of *P. fluorescens* HC1-07 and its isogenic mutants and complemented mutant derivatives to inhibit hyphal growth of *G. graminis* var. *tritici* and *R. solani* was first assessed in vitro. The wild-type HC1-07 demonstrated strong inhibition of three isolates of *G. graminis* var. *tritici* and of *R. solani* AG-8 (Table 6; Supplemental Figure 4). In contrast, CLP-deficient mutants HC1-07viscB and HC1-07prtR2 were greatly reduced in ability to inhibit the growth of the pathogens, and complemented derivatives of both mutants regained the ability.

The wild-type HC1-07, its CLP-deficient mutants HC1-07viscB and HC1-07prtR2, and complemented mutants were also tested for the ability to suppress *G. graminis* var. *tritici* and *R.*

Fig. 2. A, Root colonization by HC1-07rif, cyclic lipopeptide-deficient mutants HC1-07viscB and HC1-07prtR2, and complemented mutants HC1-07viscB-1 and HC1-07prtR2-1 in a wheat cycling experiment conducted under controlled conditions in a growth chamber. Bacteria were introduced into the soil to give approximately 10^4 CFU g soil⁻¹. Cycle 0 shows population sizes of each strain in the soil at the time of planting. Each cycle of wheat growth lasted 2 weeks, at which time the wheat was harvested and another cycle of wheat was then planted. Bars indicate means and error bars indicate standard deviations. The same letter above bars for the same cycle indicates that the means are not significantly different ($P = 0.05$) according to the Fisher's protected least-significant-difference test (unless indicated otherwise). **B,** Root colonization of spring wheat ('Louise') in the field at Pullman, WA by strain HC1-07rif. Seed were treated with HC1-07rif at approximately 10^6 CFU/seed, population sizes were determined every 3 weeks after planting, and the last sample was taken at week 19. Error bars indicate standard deviations.

TABLE 4. Comparison of the putative nonribosomal peptide synthetase (NRPS) ViscB of *Pseudomonas fluorescens* HC1-07 to similar protein sequences from other *Pseudomonas* spp.

Organism	Gene function	NCBI accession number ^z	Blastp E-value	Identity (%)
<i>P. fluorescens</i> SBW25	Putative NRPS	YP002872142.1	0.0	90
<i>P. synxantha</i> BG33R	NRPS MassC	ZP10142895.1	0.0	81
<i>Pseudomonas</i> sp. Ag1	Putative NRPS	ZP10143315.1	0.0	79
<i>P. fluorescens</i> SS101	Putative NRPS	ABH06369.2	0.0	79

^z National Center for Biotechnology Information.

TABLE 5. Comparison of the regulatory protein PrtR of *Pseudomonas fluorescens* HC1-07 to similar protein sequences from other *Pseudomonas* spp.

Species	Strain	Gene function	NCBI accession number ^z	Blastp E-value	Identity (%)
<i>P. fluorescens</i>	SBW25	Putative transmembrane regulator	YP002873451.1	5e-148	94
<i>P. fluorescens</i>	LS107d2	Transmembrane regulator	AAF81075.1	2e-143	89
<i>P. extremaustralis</i>	14.3b	Transcriptional regulator	ZP101437425.1	2e-142	89
<i>P. fluorescens</i>	SS101	Transmembrane regulator	EIK58907.1	3e-136	85

^z National Center for Biotechnology Information.

solani AG-8 under controlled conditions. Strain HC1-07 applied as a seed treatment provided significant suppression of take-all and Rhizoctonia root rot based on root disease ratings when compared with the nontreated controls in both raw and pasteurized soil (Table 7). When compared with the wild-type parent, the two CLP-deficient mutants provided significantly less suppression ($P = 0.05$) of take-all based on root disease ratings. However, the mutants did not lose all of their biocontrol activity. Genetically complemented mutants regained the ability to control take-all (Table 7) to the same level as the wild type. The results were similar when tests were done in both raw and pasteurized soil. In contrast, these strains responded differently against Rhizoctonia root rot. Mutant HC1-07viscB but not HC1-07prtR2 suppressed Rhizoctonia root rot significantly less than the wild type in raw and pasteurized soil (Table 7). Only in pasteurized soil did the genetically complemented strain HC1-07viscB-1 show fully restored biocontrol activity (Table 7). Thus, the viscosin-like CLP appeared to be a more important determinant of disease suppression against take-all than Rhizoctonia root rot.

DISCUSSION

In this study, we demonstrated the involvement of a CLP from *P. fluorescens* strain HC1-07 in the biocontrol of take-all and Rhizoctonia root rot, two economically important diseases of wheat. Results of this work revealed that *P. fluorescens* HC1-07 produces a CLP with molecular structure similar to that of viscosin. Viscosin is a CLP that was first isolated from *P. viscosa* (30) and was later implicated as a key metabolite responsible for the ability of *Pseudomonas* spp. to control the brown blotch disease of mushrooms caused by *P. tolaasii* (56). Three lines of evidence suggested that the viscosin-like CLP is an important component of the biocontrol activity of strain HC1-07. First, the addition of CLP extracted from HC1-07 to the growth medium even at a concentration of $10 \mu\text{g ml}^{-1}$ inhibited the hyphal growth of both *G. graminis* var. *tritici* and *R. solani* AG-8. Second, both CLP-nonproducing mutants HC1-07viscB and HC1-07prtR2 were significantly less effective in biocontrol of take-all than HC1-07, and HC1-07viscB was reduced in activity against Rhizoctonia root rot. Finally, genetic complementation of the mutants HC1-07viscB and HC1-07prtR2 restored their biocontrol activity to wild-type levels. CLP production is only one mechanism of suppression in strain HC1-07 because the mutants still retained significant biocontrol activity compared with the nontreated controls. In addition, the viscosin-like CLP appears to be a much more important determinant of disease suppression against take-all than Rhizoctonia root rot because the mutant HC1-07prtR2 was not significantly different from the wild type in *Rhizoctonia* spp. control. This is not surprising given that both extracted CLP

and HC1-07 showed greater inhibition of *G. graminis* var. *tritici* than *R. solani* in vitro. In addition, these two organisms belong to different classes of fungi (Ascomycetes and Basidiomycetes, respectively). Furthermore, it is also known that *G. graminis* var. *tritici* is especially sensitive to many different mechanisms of biocontrol compared with other soilborne fungi (63).

In order to identify genes involved in the production of the viscosin-like CLP in HC1-07, we subjected this strain to plasmid mutagenesis and characterized genetic loci affected in the two resultant CLP-deficient mutants. In the first mutant, HC1-07viscB, the mutation site was located in the gene similar to *viscB* of *P. fluorescens* SBW25, which encodes for ViscB, a NRPS. In SBW25 (from the phyllosphere of sugar beet in the United Kingdom), *viscB* is one of the three key genes (*viscA*, *viscB*, and *viscC*) in the synthesis of viscosin (11). In the second mutant, the mutation site was found in the gene *prtR*, which encodes a transmembrane regulator PrtR. *prtR* is translationally coupled to an upstream gene, *prtI*, which encodes an ECF (extracytoplasmic function) σ factor. PrtR was reported to be required for protease expression at 29 but not 23°C in *Pseudomonas* LS107d2 (5). We found that mutating the *prtR* gene also resulted in the loss of protease activity but at both 23 and 29°C; thus, protease production in the mutant HC1-07prtR was not related to the temperature. Interestingly, *prtR* in HC1-07 not only plays a role in protease production but also functions in regulation of the production of the viscosin-like CLP. De Bruijn and Raaijmakers (12) reported a similar phenomenon in *P. fluorescens* SS101, where the serine protease ClpP regulates the biosynthesis of the CLP massetolides, which are involved in swarming motility, biofilm formation, and antimicrobial activity.

CLP production is known to play an important role in surface motility and biofilm formation (11,52). Our soft agar assays showed that the mutants HC1-07viscB and HC1-07prtR2 were completely impaired in surface motility, a result also reported for the viscosin-deficient mutants of *P. fluorescens* SBW25 (11). In addition, microtiter plate assays showed that the two mutants produced significantly less biofilm than the wild-type HC1-07 and the complemented mutants. Different CLPs, including arthrofactin and putisolvin, have been reported to influence biofilm formation; however, the way that this occurs is still unclear but their impact on cell surface hydrophobicity may be important in this process. Hydrophobic interactions and surface-active compounds, such as CLPs, are thought to play a role in the adherence of cells to surfaces (53).

The function of CLPs in colonization and dispersal of bacteria in natural habitats is still emerging but apparently varies by plant species and ecological niche. Tran et al. (59) showed that the

TABLE 6. In vitro inhibition of *Gaeumannomyces graminis* var. *tritici* and *Rhizoctonia solani* by the wild-type HC1-07, cyclic lipopeptide-deficient mutants, and complemented mutant derivatives

Treatment	Relative inhibition of radial hyphal growth (%) ^z			
	<i>G. graminis</i> var. <i>tritici</i>			<i>R. solani</i>
	LD5	ARS-A1	R3-111a-1	
HC1-07rif	56.1 A	56.3 A	56.8 A	46.1 B
HC1-07viscB	15.3 B	5.8 C	12.5 C	8.7 C
HC1-07viscB-1	50.1 A	47.2 B	49.9 AB	54.8 A
HC1-07prtR2	10.1 B	16.7 C	8.1 C	5.7 C
HC1-07prtR2-1	47.9 A	53.3 AB	44.9 B	50.8 AB

^z Data represent the mean from three replicate plates. Means in the same column followed by the same letter are not significantly different at $P = 0.05$ according to Fisher's protected least significant difference test. Distance between the bacterial colony and the edge of the mycelium on one-fifth-strength potato dextrose agar compared with that on the control plate (not inoculated with bacteria).

TABLE 7. Severity ratings of take-all and Rhizoctonia root rot disease in wheat inoculated with HC1-07 wild-type, cyclic lipopeptide-deficient mutants, and complemented mutants as seed treatments

Treatment ^y	Disease severity rating ^z			
	Take-all		Rhizoctonia root rot	
	Pasteurized	Raw	Pasteurized	Raw
HC1-07rif	2.8 D	3.2 C	2.5 C	2.4 C
HC1-07viscB	3.5 C	3.6 B	3.3 B	2.9 B
HC1-07viscB-1	3.1 D	3.4 C	2.6 C	2.6 BC
HC1-07prtR2	3.9 B	3.7 B	2.8 C	2.8 BC
HC1-07prtR2-1	3.0 D	3.3 C	2.6 C	2.5 BC
CK+pathogen	4.4 A	3.9 A	4.3 A	4.3 A
CK+MC+pathogen	4.1 AB	4.1 A	4.1 A	4.2 A

^y CK, a nontreated control; CK+MC, seed treated with methyl cellulose; bacteria were applied to the seed with methyl cellulose at a dose of 10^7 CFU seed⁻¹.

^z Ratings represent the mean disease severity on 90 replicate plants on a scale of 0 to 8, where 0 = no disease detected and 8 = dead plants. Means in the same column followed by the same letter are not significantly different at $P = 0.05$ according to the Kruskal-Wallis all-pairwise comparison test.

wild-type strain *P. fluorescens* SS101, when applied to tomato seed, was more effective in colonizing the root system of tomato seedlings than its massetolide-deficient mutant. Similarly, a viscosin-deficient mutant of *P. fluorescens* strain 5064 was unable to colonize the surface of intact broccoli florets (22). Surfactin and amphisin produced by *B. subtilis* strain 6031 and *Pseudomonas* sp. DSS73, respectively, were also shown to be important traits in the colonization of *Arabidopsis* roots and sugar beet seed (2,44). In contrast, the *massA*-minus mutant 10.24 was detected at higher populations in the wheat and apple rhizosphere and in soil compared with the wild-type *P. fluorescens* SS101 at 30 days after the strains were introduced into an orchard soil (41). We found that loss of the ability to produce the viscosin-like CLP had no effect on the ability of HC1-07 to colonize the wheat rhizosphere in the cycling experiment. The slight loss in rhizosphere competence in mutant HC1-07prtR2 may be related to loss of other traits that *prtR* impacts. The cycling experiment is an especially robust assay to test the role of a trait or gene in root colonization because bacteria introduced into the soil must survive, colonize the root, and then recolonize the root again after surviving in the soil.

In conclusion, our study specifically shows that the biocontrol strain *P. fluorescens* HC1-07 produces a viscosin-like CLP that plays a key role in the suppression of root diseases of wheat but the CLP does not contribute to the rhizosphere competence of the strain. In a broader context, our results add support to an emerging picture that certain biocontrol microorganisms, mechanisms, metabolites, or genes are cosmopolitan in agro-ecosystems worldwide, where they contribute to some level of natural suppression of soilborne and foliar plant diseases.

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