Biological Control of Take-All by Fluorescent *Pseudomonas* spp. from Chinese Wheat Fields

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ABSTRACT

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Take-all disease of wheat caused by the soilborne fungus *Gaeumannomyces graminis* var. *tritici* is one of the most important root diseases of wheat worldwide. Bacteria were isolated from winter wheat from irrigated and rainfed fields in Hebei and Jiangsu provinces in China, respectively. Samples from rhizosphere soil, roots, stems, and leaves were plated onto King's medium B agar and 553 isolates were selected. On the basis of in vitro tests, 105 isolates (19% of the total) inhibited *G. graminis* var. *tritici* and all were identified as *Pseudomonas* spp. by amplified ribosomal DNA restriction analysis. Based on biocontrol assays, 13 strains were selected for further analysis. All of them aggressively colonized the rhizosphere of wheat and suppressed take-all. Of the 13 strains, 3 (HC9-

07, HC13-07, and JC14-07, all stem endophytes) had genes for the biosynthesis of phenazine-1-carboxylic acid (PCA) but none had genes for the production of 2,4-diacetylphloroglucinol, pyoluteorin, or pyrrolnitrin. High-pressure liquid chromatography (HPLC) analysis of 2-day-old cultures confirmed that HC9-07, HC13-07, and JC14-07 produced PCA but no other phenazines were detected. HPLC quantitative time-of-flight 2 mass-spectrometry analysis of extracts from roots of spring wheat colonized by HC9-07, HC13-07, or *Pseudomonas fluorescens* 2-79 demonstrated that all three strains produced PCA in the rhizosphere. Loss of PCA production by strain HC9-07 resulted in a loss of biocontrol activity. Analysis of DNA sequences within the key phenazine biosynthesis gene *phzF* and of 16S rDNA indicated that strains HC9-07, HC13-07, and JC14-07 were similar to the well-described PCA producer *P. fluorescens* 2-79. This is the first report of 2-79-like bacteria being isolated from Asia.

Additional keyword: antibiotic.

Take-all, one of the most important soilborne diseases of wheat worldwide, is caused by the fungus *Gaeumannomyces graminis* var. *tritici* and is highly sensitive to biological control by both indigenous and introduced microorganisms. Take-all has also been a model disease for studying both the process of biocontrol and the mechanisms by which antagonistic microorganisms suppress soilborne pathogens. For example, one of the best examples of indigenous microorganisms providing natural defense of wheat roots against take-all is take-all decline (TAD), a spontaneous remission of the incidence and severity of take-all during continuous wheat or barley monoculture and following a severe disease outbreak. Although many microorganisms have been reported to play a role in TAD (11,13,58), many TAD fields owe their suppressiveness to a build-up of *Pseudomonas fluorescens* producing the antibiotic 2,4-diacetylphloroglucinol (DAPG) (43). Farmers in nearly all major wheat-growing regions manage take-all through wheat monoculture (57).

In fields where the soil has not developed suppressiveness, take-all can be devastating, especially when wheat is grown under

irrigation or high precipitation. The lack of resistance in wheat to take-all and the availability of fungicides with only moderate effectiveness has prompted extensive studies of biological control with introduced microorganisms over the past 40 years. A wide spectrum of microorganisms, many from TAD fields, are known to have biocontrol activity against take-all (24,58). Examples include *Pseudomonas* spp., including *P. fluorescens* 2-79 (49,56), *P. fluorescens* Q8r1-96 (42), and *P. aureofaciens* 30-84 (41); *Bacillus* spp., including *Bacillus* spp. L324-92, *B. cereus* A47-2, *B. cereus* A47-3, and *B. subtilis* B908 (45); *Trichoderma* spp., including *Trichoderma koningii* and *T. pseudokoningii* (51); *Streptomyces* spp. (12); *Phialophora* spp. (13,19,29); *G. graminis* var. *graminis* (16); and avirulent *G. graminis* var. *tritici* (17,60). Among the several mechanisms of suppression shown to have a role in suppression of take-all, considerable emphasis has been focused on antibiosis by *Pseudomonas* spp. DAPG and phenazine-1-carboxylic acid (PCA) are two of the best-studied antibiotics by which introduced pseudomonads suppress take-all and other soilborne diseases (20).

China is the world's largest producer of wheat, and take-all is a serious problem in the provinces of Hebei, Shandong, and Gansu (6,7,21). Take-all-suppressive soils have been documented in China (31) but many fields remain highly conducive to the disease because wheat is grown in rotation with rice, which does not facilitate the induction of TAD. Several Chinese studies have focused on biocontrol of take-all (6,21,53) and growers are eager

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to adopt biocontrol technology. Chinese farmers have a history of using microbial biocontrol agents but their effectiveness and quality are not always known (53). The establishment of “cottage industries” that produce agents for local use is increasing worldwide as an alternative to registered chemical and biological fungicides but cottage industries need effective and reliable biocontrol agents (22).

This study is part of a broader biocontrol program being conducted in China with the goal of identifying new microbial agents for use in Chinese agriculture, especially on wheat and rice. Previously, as part of this program, an extensive analysis of the diversity, taxonomy, and antagonistic potential of rice-associated bacteria was conducted in order to identify new biocontrol agents for foliar pathogens of rice (61). Here, we focused on 13 antagonistic fluorescent *Pseudomonas* spp. that are part of a collection of epiphytic and endophytic bacteria isolated from fields of wheat in Hebei and Jiangsu provinces. These fields were selected as a source of microorganisms because they have had a long history of cereal production and have never been assayed for microbial biocontrol agents. Little is known about the taxonomic diversity of wheat-associated antagonistic bacteria from different agroecosystems in China and their potential role in biological control.

The aim of our work was to screen these isolates for the ability to produce known biocontrol antibiotics and to determine their ability to suppress take-all. We report that isolates producing PCA were among the most common biocontrol agents. They aggressively colonize wheat roots and produce PCA in the rhizosphere, and PCA production is an important component of their ability to suppress take-all.

MATERIALS AND METHODS

Bacteria, plasmids, and culture conditions. Bacteria and plasmids used in this work are described in Table 1. Strains of

Pseudomonas were routinely cultured on King’s medium B (KMB) agar or broth (27) at 27°C and stored in glycerol at –80°C. *Escherichia coli* strains were routinely grown at 37°C on Luria-Bertani (LB) agar or broth (1). Antibiotics were used at the following concentrations: rifampin, 100 µg/ml; cycloheximide, 100 µg/ml; chloramphenicol, 13 µg/ml; ampicillin, 25 µg/ml; tetracycline, 25 µg/ml; and gentamicin 15 µg/ml. For counterselection of *sacB*-containing plasmids, sucrose was added to a final concentration of 5%.

Sampling and isolation of bacteria. Bacteria were sampled from a rainfed field in Jiangsu province and an irrigated field in Hebei province. Fields were sampled several times beginning in late autumn 2007 and, at each sample time, plants were dug from five random locations and pooled. Plants were shaken vigorously to remove all but tightly adhering rhizosphere soil and transported to the laboratory in sterile plastic bags. Bacteria were isolated from the inside and surface of leaves and stems and from rhizosphere soil and the interior of roots by dilution plating using methods similar to those of Barraquio et al. (2). For each sample and at each sampling time, there were three independent replicates. To isolate from plant surfaces, 3 g of stems, leaves, or roots with rhizosphere soil were transferred into sterile Erlenmeyer flasks containing 27 ml of a 0.85% NaCl solution and the flasks were shaken at 200 rpm for 30 min. The washings were dilution-plated onto KMB agar and plates were incubated at 29°C for 48 h for the isolation of fluorescent *Pseudomonas* spp.

To isolate endophytic bacteria, leaves, stems, and roots were first washed separately in running tap water for 10 min, then soaked in 1.5% sodium hypochlorite (NaClO) for 5 min, and finally rinsed five times (1 min each) in sterile water. Each sample was homogenized with a sterilized mortar and pestle. Macerated samples were serially diluted with sterile 0.85% NaCl solution, and resulting suspensions were plated onto KMB and incubated at 29°C for 48 h for the isolation of fluorescent *Pseudomonas* spp. Fluorescent colonies were picked randomly under UV light,

TABLE 1. Bacteria and plasmids used in this study

Strains or plasmids	Source, characteristics ^z	Reference
<i>Pseudomonas fluorescens</i>		
2-79	Phz ⁺ Rif ^r ; produces PCA	49
HC1-07	Hebei phyllosphere isolate; Phz [–]	This study
HC2-07	Hebei root endophyte; Phz [–]	This study
HC6-07	Hebei stem endophyte; Phz [–]	This study
HC8-07	Hebei phyllosphere isolate; Phz [–]	This study
HC9-07	Hebei stem endophyte; Phz ⁺ ; produces PCA	This study
HC10-07	Hebei stem endophyte; Phz [–]	This study
HC11-07	Hebei phyllosphere isolate; Phz [–]	This study
HC12-07	Hebei leaf endophyte; Phz [–]	This study
HC13-07	Hebei stem endophyte; Phz ⁺ ; produces PCA	This study
HC15-07	Hebei rhizosphere isolate; Phz [–]	This study
JC4-07	Jiangsu root endophyte; Phz [–]	This study
JC7-07	Jiangsu stem endophyte; Phz [–]	This study
JC14-07	Jiangsu stem endophyte; Phz ⁺ ; produces PCA	This study
HC9-07rif	Spontaneous Rif ^r derivative of HC9-07; Phz ⁺ ; produces PCA	This study
HC13-07rif	Spontaneous Rif ^r derivative of HC13-07; Phz ⁺ ; produces PCA	This study
JC14-07rif	Spontaneous Rif ^r derivative of JC14-07; Phz ⁺ ; produces PCA	This study
HC9M5 through HC9M10	Phz [–] derivatives of HC9-07; ΔphzCD::Tc ^r	This study
HC9M9comp	HC9M9 carrying pUCP22-phzCD; Phz ⁺ produces PCA	This study
<i>Escherichia coli</i>		
SM17-1 (λ-pir)	<i>thi pro hsdR hsdM recA rpsL</i> RP4-2 (Tc ^r ::Mu) (Km ^r ::Tn7) λ-pir	47
DH10B	F [–] <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) Δ <i>lacX74</i> φ80 <i>lacZ</i> ΔM15 <i>recA1 endA1 araD139</i> Δ(<i>ara, leu</i>)7697 <i>galU galK</i> λ [–] <i>rpsL nupG</i>	Invitrogen
Plasmids		
pT7-6ABCD	pT7-6 containing 4.5-kb <i>Bgl</i> II- <i>Pst</i> I fragment from strain 2-79 with <i>phz</i> ABCD genes	33
pEX18Gm	Gene replacement vector; Gm ^r <i>oriT sacB</i>	23
pUCP22	Broad-host-range vector; Gm ^r Ap ^r	59
pUCP22-phzCD	pUCP22 containing 3.15-kb <i>Eco</i> RI- <i>Pst</i> I fragment from pT7-6ABCD with <i>phz</i> CD genes	This study
pGEM-T Easy	SP6 and T7 promoters; Ap ^r <i>f1ori lacZα</i>	Promega
p34S-Tc	Donor of Tc ^r cassette; Ap ^r Tc ^r	15

^z Phz⁺, strain produces phenazine-1-carboxylic acid (PCA); Phz[–], strain does not produce PCA; Rif^r, rifampicin resistance; Tc^r, tetracycline resistance; Gm^r, gentamycin resistance; Km^r, kanamycin resistance; and Ap^r, ampicillin resistance.

replated to insure purity, and stored at -80°C in KMB broth amended with 40% glycerol.

In vitro inhibition of fungal pathogens. In Chinese studies, the ability of wheat-associated bacteria (553 isolates) to inhibit *G. graminis* var. *tritici*, *Phytophthora capsici*, *Rhizoctonia solani*, *Bipolaris sorokiniana*, *Fusarium graminearum*, and *Sclerotinia sclerotiorum* was tested on Waksman's Agar as described by Berg et al. (3). All isolates were tested in triplicate, plates were incubated at 25°C , and zones of inhibition were measured 5 days after inoculation. Isolates that showed antagonistic activity toward phytopathogenic fungi were selected for further assays.

Inhibition of Washington State isolates of *G. graminis* var. *tritici* (LD5, ARS-A1, and R3-111a-1) (28) by *Pseudomonas* spp. was tested in vitro on KMB and homemade potato dextrose agar (PDA) plates. For PDA, diced potato tubers (200 g) were cooked in water in a microwave for 15 min, the strained broth was brought to 1 liter, and dextrose (20 g) and agar (15 g) were added and then autoclaved (56). Aliquots (5 μl) from overnight cultures of each strain grown in KMB broth were spotted 1 cm from the edge of a petri dish and allowed to soak into the agar. A 0.5-cm plug from the leading edge of a culture of *G. graminis* var. *tritici* grown for 5 days at 24°C on PDA was placed in the center of the plate. Plates were incubated at 24°C and scored after 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. Each isolate was replicated three times. Inhibition was expressed relative to a noninoculated control.

Amplified ribosomal DNA restriction analysis and BOX-polymerase chain reaction. 16S ribosomal DNA (rDNA) was amplified from cell suspensions using oligonucleotide primers 8-27 (5'-AGAGTTTGATC(AC)TGGCTCAG-3') and L1494-1514 (5'-CTACGG(AG)TACCTGTTCAGAC-3') (61). Amplification products were purified using a Mini BEST Bacterial Genomic DNA Extraction kit (Takara Biotechnology Co., Dalian, China) and digested with restriction endonuclease *MspI*. The resultant fragments were separated on a mixed gel (1.5% agarose + 1.5% Synergel) in 1.0 $\times$ Tris-borate-EDTA (TBE) buffer at 80 V for ≈ 2 h, stained with ethidium bromide, and then photographed under UV light. The experiment was repeated three times to verify the reproducibility of the results.

For BOX-polymerase chain reaction (PCR) fingerprint analysis, bacterial DNA was prepared using the Mini BEST Bacterial Genomic DNA Extraction kit (Takara Biotechnology Co.). BOX-PCR was carried out using the BOXA1R primer as described by Rademaker and De Bruijn (44). Amplification was performed with a DNA Engine PTC-200 thermal cycler (Bio-Rad, Hercules, CA) using the following cycling conditions: initial denaturation at 95°C for 6 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, and extension at 65°C for 8 min; and a final extension at 65°C for 16 min. Amplifications were carried out in 25- μl reaction mixtures, each containing 0.15 U of DNA polymerase (Takara Biotechnology Co.), 1.75 mM MgCl_2 , 1 $\times$ polymerase buffer, 0.1 mM dNTPs, 0.2 μM BOXA1R primer, and 1 μl of DNA template. A 5- μl aliquot of amplified PCR product was separated by gel electrophoresis in a mixed gel (0.5% agarose + 0.75% Synergel) in 1.0 $\times$ TBE buffer at 120 V for 6 h. The gel was stained with ethidium bromide and photographed under UV light. The reproducibility of the banding patterns was verified in three independent experiments. Computer-assisted evaluation of BOX-PCR-generated fingerprints was carried out using GelComparII software (version 4.5; Applied Maths, Kortrijk, Belgium). The analysis of the banding patterns was performed using Pearson correlation applied to the densitometric curves formed by the fingerprints (44). Clustering analysis was performed using the unweighted pair-grouping method based on arithmetic averages (UPGMA).

Detection of extracellular metabolites and cell-wall-degrading enzymes. All antagonistic bacterial isolates were assessed for

their ability to produce cell-wall-degrading enzymes and siderophores. Chitinase activity was tested in minimal medium as described by Chernin et al. (8), and cellulase activity was determined according to Ghose (18). Protease activity, indicated by casein degradation, was determined by the appearances of zones of clearing around colonies grown on skim milk agar (50 ml of sterilized skimmed milk mixed at 55°C with 2% agar) after incubation at 30°C for 3 days. Siderophores were detected as described by Shin et al. (46).

Detection of genes involved in antibiotic biosynthesis by PCR. A screen for key genes involved in the biosynthesis of different classes of antibiotics was conducted by PCR with previously reported oligonucleotide primer sets. Briefly, the DAPG biosynthesis gene *phlD* was amplified with primers B2BF1 and BPR4 (38). A key gene in the biosynthesis of pyrrol-nitrin, *prnC*, was targeted with primers prnC1 and prnC3 (35). The pyoluteorin biosynthesis pathway was detected by PCR with *phlB*-specific primers plt1 and plt2 (35). Genes for the biosynthesis of PCA were targeted by the *phzF*-specific primer set Ps_up1 and Ps_low1 (34). The amplifications were carried out in 25- μl reactions that contained: 2 μl of bacterial cell suspension template; 1 $\times$ GoTaq DNA polymerase buffer, 200 μM dNTPs, 1.5 mM MgCl_2 , 25 pmol of each primer, and 1.5 U of GoTaq DNA polymerase (Promega Corp., Madison, WI). The annealing temperatures and cycling conditions for each primer pair were adjusted as reported elsewhere (34,35,38). After amplification, an aliquot of each reaction was separated by agarose gel electrophoresis and processed as described above.

Sequencing of 16S rDNA and *phzF* and sequence analyses. Genomic DNA of selected strains was extracted using a GenElute Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis) and 16S rDNA and *phzF* genes were amplified using primer sets 8F-1492R (54) and Ps_up1-Ps_low1 (34), respectively. Amplifications were carried essentially as described above using 50 ng of purified DNA as a template. The annealing temperature and cycling conditions were as reported elsewhere (34). The amplified fragments were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA) and sequenced by using the BigDye Terminator Cycle Sequencing kit (v. 3.1; Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. In addition to primers 8F and 1492R, internal primers 16Sps3, r16Sps3, 16Sps4, and r16Sps4 (34) were used to obtain a full-length sequence of the $\sim 1,500$ -bp amplified fragment of 16S rDNA. Sequence data were assembled by using Vector NTI Advance software (v. 10; Invitrogen Corp., Carlsbad, CA).

The 16S rDNA database similarity searches were carried out using the nucleotide BLAST function implemented in the Ribosomal Database Project 10 (10). For *phzF* sequences, database searches for similar DNA and proteins sequences were carried out with the National Center for Biotechnology Information's BLAST network service (<http://www.ncbi.nlm.nih.gov/BLAST>). Sequences were aligned using Clustal X (30) and neighbor-joining phylogenies were inferred using the Molecular Evolutionary Genetics Analysis (MEGA) software, v. 4.0.2 (48), with DNA distances corrected by using the Kimura two-parameter model of evolution.

Construction and genetic complementation of PCA-deficient mutants of strain HC9-07. A spontaneous rifampin-resistant derivative of strain HC9-07 (HC9-07rif) was used for gene-replacement mutagenesis. DNA cloning was performed using standard methods (1). To inactivate the phenazine biosynthesis pathway, a 1.4-kb DNA fragment containing *phzC* and *phzD* genes was amplified by PCR with primers PHZ1 and PHZ2 (14). The amplified fragment was cleaned with a QIAquick PCR purification kit (Qiagen) and cloned into a pGEM-T Easy vector (Promega Corp.). *phzD* was inactivated by insertion of an 1.39-kb *tet* cassette from p34S-Tc into a unique *ClaI* site. The resulting plasmid was digested with *EcoRI* and the 2.8-kb interrupted insert

was subcloned into pEX18Gm. The pEX18Gm-based plasmid was mobilized in *P. fluorescens* HC9-07 from *E. coli* S17-1 (λ -pir) and double-crossover events were selected and verified by PCR with oligonucleotide primers targeting plasmid-borne *sacB*, tetracycline resistance, and gentamycin resistance genes as described earlier (36). The presence of the mutated *phzD* allele was confirmed by PCR performed with oligonucleotide primers PHZ1 and PHZ2. The cycling program included an initial denaturation for 2 min at 94°C followed by 25 cycles of 94°C for 1 min, 56°C for 45 s, 72°C for 1.75 min, and a final extension at 75°C for 1 min.

For complementation of the PCA-deficient mutant, we used phenazine biosynthesis genes *phzC* and *phzD* of *P. fluorescens* 2-79. The genes were excised from plasmid pT7-6ABCD (33) by using restriction endonucleases *EcoRI* and *PstI* and the 3.15-kb fragment was subcloned into broad-host-range plasmid vector pUCP22. The resulting plasmid with intact *phzC* and *phzD* under the control of a P_{lac} promoter was transformed in a PCA-deficient mutant of strain HC9-07, and transformants were selected on LB amended with gentamycin and confirmed by PCR with oligonucleotide primers PHZ1 and PHZ2.

Isolation and detection of phenazines in vitro. *Pseudomonas fluorescens* 2-79 and isolates HC9-07, HC13-07, and JC14-07 were grown with shaking in test tubes containing 5 ml of LB broth supplemented with 0.2% glucose or KMB broth at 28°C for 48 h. Cells were removed by centrifugation (5,000 rpm for 5 min) and the culture supernatants were acidified with 45 μ l of 10% trifluoroacetic acid and extracted twice with 10 ml of ethyl acetate. Organic phases were pooled and evaporated to dryness, and residues were redissolved in 99% acetonitrile–0.1% trifluoroacetic acid. Extracts were fractionated using an Alliance 2695/996 PDA high-performance liquid chromatography (HPLC) system and a C18 reverse-phase Symmetry column (5 μ m, 150 by 3.5 mm) (all from Waters Corp., Milford, MA). The photodiode array detection range was 180 to 470 nm, with screening for PCA at 248 nm. The HPLC gradient profile was a 2-min initiation at 10% acetonitrile–0.1% trifluoroacetic acid followed by a 20-min linear gradient to 100% acetonitrile–0.1% trifluoroacetic acid (5).

Phenazines were also detected by using thin-layer chromatography (TLC). PCA was extracted as described above; 20 μ l of methanol was added to the residue and shaken to dissolve it, and 2 μ l of each sample was loaded onto the thin-layer silica plate. The plate was then placed in a mix of benzene/acetic acid (95:5 by volume) and allowed to develop for 1 h. PCA was visualized by UV irradiation at 256 nm.

Preparation of oat-kernel inoculum. Inoculum of *G. graminis* var. *tritici* was prepared essentially as described by Ownley et al. (39). 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* strain Ng07 (from China) or LD5 (from Washington State). After 3 to 4 weeks of growth at room temperature, the contents of each jar were air dried and stored at 4°C.

Bacterial treatment of wheat seed. For biocontrol studies in China, bacterial isolates were streaked onto LB plates and incubated at 30°C overnight. A single colony of each isolate was transferred into a flask containing 500 ml of LB broth, and the flask was incubated at 30°C with agitation at 200 rpm overnight. Bacterial cells were pelleted by centrifugation and resuspended in sterile 0.85% NaCl, and the concentration was adjusted to 10^8 CFU ml⁻¹. Seed of wheat ('Mianyang 8545') were treated by soaking them in the bacterial suspension for 1 h.

For biocontrol studies in Washington State, wheat seed ('Pena-wawa') were treated using methods similar to those described previously (40). KMB plates were spread inoculated with the test bacterium and incubated for 48 h at room temperature. A 1%

solution of methyl cellulose (M-6385; 4 ml) (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 $\approx 10^7$ CFU seed⁻¹.

Biocontrol assays. For biocontrol assays in China, pasteurized soil (300 g) was amended with 1.2% ground oat-kernel inoculum and added to each pot (12 cm in diameter and 15 cm in height). Seven wheat seeds treated with a bacterial isolate were sown in each pot and covered with 1.5 cm of soil. Controls consisted of soil amended or not amended with oat kernel inoculum and sown to nontreated seed. Each pot was then given 30 ml of water. All pots were incubated in a greenhouse at 22°C with a cycle of 16 h of light and 8 h of darkness and watered three times per week. Each treatment was replicated three times and each pot served as a replicate. Three weeks after planting, the plants were removed from the pots and roots were washed free of soil under a stream of water. The amount of disease was assessed using a 0 to 5 scale, where 0 = no visible disease symptoms and 1 = 1 to 10, 2 = 11 to 30, 3 = 31 to 60, and 4 = 61 to 100% of entire the root system diseased. Disease severity and biocontrol efficacy were calculated as follows: disease severity = $[\Sigma(\text{number of diseased plants in this index} \times \text{disease index}) / (\text{total number of plants investigated} \times \text{highest disease index})] \times 100\%$ and biocontrol efficacy = $[(\text{disease severity of the control} - \text{disease severity of a treatment}) / \text{disease severity of control}] \times 100\%$.

To determine the biocontrol activity of the bacteria to take-all in a Washington soil, a modification of the tube assay previously described by Ownley et al. (39) was used. Plastic tapered tubes (2.5 cm in diameter and 16.5 cm long), each with a hole at the bottom (Stuewe and Sons, Inc. Tangent, OR), were supported in a hanging position in plastic racks, 200 tubes per rack. Each tube had a cotton ball placed in the bottom and then was filled with a 6.5-cm-thick column of sterile vermiculite followed by 10 g of air-dried and sieved Quincy Virgin soil (Shano sandy loam). For some experiments, the soil was pasteurized (60°C for 30 min to reduce interference from other soilborne pathogens) and then air dried. *G. graminis* var. *tritici* was blended into the soil as colonized oat kernels that had been pulverized in a Waring Blender and sieved into fractions of known particle sizes. The fraction used was 0.25 to 0.5 mm and was added to the soil at 0.7% (wt/wt). Three bacteria-treated wheat seeds were placed on the soil and covered with a 1.5-cm-thick topping of vermiculite. Each tube then received 10 ml of water. The rack of tubes was covered with plastic for 4 days and incubated at 15 to 18°C in a dark/light cycle of 12 h. 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. After 3 to 4 weeks, the seedlings were removed from the tubes, the roots were washed, and the plants were evaluated for disease severity on a scale of 0 to 8 as previously described (40), where 0 = no disease evident and 8 = plants dead or nearly so.

Root colonization. The population dynamics of the *Pseudomonas* isolates were determined on spring wheat ('Louise') grown without irrigation at the Washington State University (WSU) Plant Pathology Research Farm, Pullman. 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. Treatments included *P. fluorescens* 2-79, HC9-07rif, HC13-07rif, and JC14-07rif. Nontreated and methyl-cellulose-treated seed served as controls. All bacteria used were spontaneous rifampicin-resistant derivatives of wild-type isolates (Table 1). Bacteria were applied to the seed as described above using the Washington protocol. Treatments were 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 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 an introduced strain were determined by the dilution end-point method (38) in one-third-strength KMB broth supplemented with rifampicin, cycloheximide, ampicillin, and chloramphenicol (1/3 $\times$ KMB⁺⁺⁺Rif). Populations of total culturable aerobic bacteria were determined by transferring dilutions into 1/10 strength tryptic soy broth supplemented with cycloheximide (1/10 $\times$ TSB⁺). Bacterial growth was assessed after 72 h for 1/3 $\times$ KMB⁺⁺⁺Rif and 48 h for 1/10 $\times$ TSB⁺ using a Model 680 microplate reader (Bio-Rad). An optical density at 600 nm > 0.1 was scored as positive for growth. When necessary, PCR was used to verify that cells of the target isolate were in the terminal dilution.

Extraction of PCA from the rhizosphere and quantification by mass spectrometry. Roots of extra plants collected from each replicate of treatments 2-79, HC9-07rif, and HC13-07rif of the root colonization study described above were excised, placed in plastic bags, and stored at -20°C . PCA was extracted from 15 g of roots that were cut into small pieces as previously described (5), with 2 μg of phenazine added to each sample as an internal standard. Each sample was brought up to 1 ml with 98% acetonitrile–2% acetic acid. The analysis used 30- μl injections on a C18 reverse-phase symmetry column (5 μm , 150 by 3.5 mm, Waters Corp.). HPLC quantitative time-of-flight (Q-TOF) 2 mass spectrometer conditions consisted of a corona current of 2.7 μA , cone voltage of 25 V, collision energy of 12, source temperature of 120°C , and probe temperature of 650°C for exact (TOF) mass measurements. For tandem mass spectrometry (MS-MS) profiles, the cone voltage was 35 V and the collision energy was 25.

Gradient elution profiles (2-min initiation at 10% acetonitrile–2% acetic acid, followed by a 20-min linear gradient to 100% acetonitrile–2% acetic acid) were monitored using a photodiode array detection range of 180 to 470 nm with screening for PCA at 248 nm. Q-TOF 2 profiles were monitored at 225.0664 (PCA) exact mass (TOF) positive-ion mode, atmospheric chemical ionization (APCI) with an Ion Saber probe (Waters Corp.). Resolution was $\geq 8,000$, with an average sensitivity of 0.16 mDa (0.70 ppm) exact mass. For MS-MS profiles, library matching was used with a forward matching range of 833 to 957 and a reverse matching range of 918 to 977, where a perfect match of 100% = 1,000 for both forward and reverse library matching (5).

Standard curves consisting of six data points (in triplicate) for phenazine were generated by spiking 15 g of roots plus rhizosphere soil with 200 ng to 10 μg of phenazine standard. The relationship between peak area (y) and spiked standards (x) was

linear ($y = mx + b$), with $r^2 = 0.9813$ to 0.9913 (mean 0.9884). In our hands, the detection limit by Q-TOF 2 mass spectrometry of PCA produced in the rhizosphere of wheat was 800 pg.

Statistical analysis. MEGA version 4.0.2 (48) was used to infer trees based on neighbor joining (NJ) of genetic distances and maximum parsimony (MP). The NJ method was used with DNA and protein distances corrected by using the Kimura two-parameter (26) and Jones-Taylor-Thornton (25) models of evolution, respectively.

Colonization experiments were arranged in a randomized complete block design, and data were analyzed with STATISTIX 8.0 software (Analytical Software, St. Paul, MN). Population data were converted to log CFU g⁻¹ fresh root weight. Differences in population densities, root disease, or plant height 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 by the Kruskal-Wallis test ($P = 0.05$).

RESULTS

Population densities of bacteria associated with wheat. At both Hebei and Jiangsu, population sizes of bacteria that were culturable on KMB were consistently lower inside of plant tissues than on the surface of tissues, and these populations differed by several orders of magnitude (Table 2). For example, throughout the growing season at Hebei, densities ranged from 5.1×10^6 to 3.7×10^7 CFU g⁻¹ fresh weight compared with 3.8×10^3 to 8.7×10^5 CFU g⁻¹ fresh weight. Isolates with the ability to inhibit *G. graminis* var. *tritici* comprised 17.7 and 20.7% of all of the isolates selected at Hebei and Jiangsu, respectively. There was no significant relationship between ability to inhibit *G. graminis* var. *tritici* in vitro and isolate source (Hebei or Jiangsu) or plant part. However, it is notable that, at Jiangsu, no antagonistic isolates were collected from the stem surface or leaf interior (Table 2).

Amplified rDNA restriction and BOX-PCR analysis of antagonistic isolates. Of the 553 isolates collected from wheat in Hebei and Jiangsu provinces, 105 isolates inhibited *G. graminis* var. *tritici* in vitro and all were identified as *Pseudomonas* spp. based on amplified rDNA restriction (ARDRA) analysis and comparison with known *Pseudomonas* strains (data not shown). BOX-PCR analysis was used to sort these isolates and it identified 30 distinct groups among the 105 antagonistic isolates (data not shown). Of these, 27 representative isolates were selected for further study as part of a larger biocontrol project in China, and 13 isolates that showed consistent biocontrol activity were selected for this study.

Biological properties and biocontrol activity. Of the 13 isolates (10 from Hebei and 3 from Jiangsu) selected for this study, 9 were from the inside of the root (endophytic), 3 were from the phyllosphere, and only 1 was from the rhizosphere. All but strains JC4-07, JC7-07, and JC14-07 produced protease; only strains HC10-07 and HC15-07 produced chitinase; all but isolate

TABLE 2. Population densities of cultural bacteria on wheat and ability to inhibit *Gaeumannomyces graminis* var. *tritici*^w

Plant part	Hebei			Jiangsu		
	CFU g ⁻¹ fresh weight ^x	Number ^y	Inhibitory (%) ^z	CFU g ⁻¹ fresh weight ^x	Number ^y	Inhibitory (%) ^z
Rhizosphere soil	5.1×10^6 to 3.7×10^7	72	9.7	5.4×10^5 to 6.6×10^6	50	18.0
Interior of roots	3.8×10^3 to 8.7×10^5	51	21.6	2.3×10^3 to 8.4×10^4	25	28.0
Surface of stems	2.4×10^5 to 7.0×10^7	34	26.5	1.1×10^5 to 6.2×10^6	27	0
Interior of stems	1.9×10^3 to 6.7×10^4	51	15.7	1.0×10^3 to 3.5×10^4	40	20.0
Surface of leaves	5.3×10^5 to 3.8×10^7	55	18.2	1.4×10^5 to 7.6×10^6	65	40.0
Interior of leaves	7.2×10^3 to 2.0×10^5	48	20.8	3.0×10^2 to 1.6×10^4	35	0

^w Fields sampled were located in Hebei and Jiangsu provinces in China.

^x Range of population densities in samples collected throughout the growing season.

^y Number of isolates collected.

^z Inhibitory to *G. graminis* var. *tritici*.

HC15-07 produced a siderophore; and only isolates HC1-07, HC9-07, and HC11-07 produced cellulase (Table 3). Analysis of the partial 16S rDNA sequence confirmed that all 13 isolates were *Pseudomonas* spp. (data not shown).

All 13 isolates consistently inhibited *G. graminis* var. *tritici* isolates Ngf07 (Table 3), LD5, ARS-A1, and R3-111a-1 (Table 4) in vitro. However as a group, the bacteria did not consistently inhibit other common soilborne pathogens from Chinese soils (i.e., *F. graminearum*, *R. solani*, *B. sorokiniana*, *Phytophthora capsici*, and *S. sclerotiorum*) (Table 3). All 13 isolates applied as seed treatments suppressed take-all in greenhouse trials conducted in China (Table 3) and in Washington State (data not shown).

Detection of antibiotic biosynthesis genes and PCA. PCR with primers specific for sequences within *phlD*, *prnC*, and *pltB* did not amplify any fragments from the 13 Chinese strains that suppressed take-all (data not shown). Fragments were amplified only from the control strains *Pseudomonas fluorescens* Pf-5 (containing *phlD*, *prnC*, and *pltB*) and Q8r1-96 (containing *phlD*). PCR with primers Ps_up1 and Ps_low1 specific for sequences in *phzF* amplified the predicted 427-bp *phzF* fragment in 3 of the 13 Chinese isolates (HC9-07, HC13-07, and JC14-07) and the

control strain *P. fluorescens* 2-79 (data not shown). *phzF* is required for phenazine biosynthesis and is highly conserved in all known phenazine producers (34). Results of PCR screening were in agreement with results of HPLC analyses, which demonstrated that extracts of 2-day-old bacterial culture supernatants of HC9-07, HC13-07, and JC14-07 contained a metabolite with the same retention time (15.3 min) and peak identity (absorption maxima of 203.2, 247.9, and 363.1 nm) as PCA (data not shown). On the basis of results described above, we focused on determining the mechanisms that are responsible for suppression of take-all by HC9-07, HC13-07, and JC14-07.

Phylogenetic analysis of *phzF* and sequence analysis of 16S rDNA. The phylogenetic relationship among isolates HC9-07, HC13-07, JC14-07, and other known phenazine-producing strains was determined by analysis of sequences within *phzF* in 56 reference strains found in the GenBank database using the same approach as Mavrodi et al. (34). The analysis divided strains into four groups, with HC9-07, HC13-07, and JC14-07 clustering closely with *P. fluorescens* 2-79 (Fig. 1). These findings were consistent with comparisons of partial 16S rDNA sequences of strains HC9-07, HC13-07, and JC14-07 by BLAST with se-

TABLE 3. Characteristics, in vitro antagonism, and biocontrol efficacy of Chinese *Pseudomonas* spp.

Isolate	Inhibition of pathogens ^w					Extracellular metabolites ^x					Disease severity (%) ^y	Biocontrol efficacy ^z
	Fg	Ggt	Rsl	Bs	Pc	Ss	Pro	Chi	Sid	Cell		
HC1-07	–	++	–	–	–	+	++	–	++	++	41.3 cde	62.4 ± 6.1 def
HC2-07	–	+	+	–	–	–	++	–	+	–	32.0 efgh	68.8 ± 2.6 abcd
JC4-07	–	+	–	+	–	+	–	–	++	–	39.8 cde	61.0 ± 7.9 def
HC6-07	–	+++	+	+	+	–	++	–	+	–	24.4 h	76.4 ± 3.0 a
JC7-07	–	+	–	+	–	++	–	–	++	–	41.7 cd	59.11 ± 4.4 ef
HC8-07	–	++	–	+++	–	–	++	–	+	–	36.8 defg	66.9 ± 6.3 bcde
HC9-07	–	+	–	–	–	+	++	–	+	++	28.7 gh	75.1 ± 2.4 ab
HC10-07	–	+	–	+	+	–	++	+	+	–	57.1 b	46.4 ± 7.1 g
HC11-07	–	+	–	+	–	+	++	–	+	++	45.7 c	55.1 ± 5.6 f
HC12-07	–	+	–	–	+	+	++	–	+	–	29.0 gh	71.8 ± 4.4 ab
HC13-07	–	+++	–	+	+	–	++	–	+	–	45.4 cd	58.3 ± 6.2 ef
JC14-07	–	+	–	+	–	+	–	–	++	–	38.9 cdef	64.7 ± 5.6 cdef
HC15-07	–	+	–	–	+	–	+++	+	–	–	35.9 defg	64.9 ± 10.3 bcde
Check	–	–	–	–	–	–	–	–	–	–	100 a	...

^w Isolates were tested in vitro for inhibition of *Fusarium graminearum* (Fg), *Gaeumannomyces graminis* var. *tritici* (Ggt), *Rhizoctonia solani* (Rsl), *Bipolaris sorokiniana* (Bs), *Phytophthora capsici* (Pc), and *Sclerotinia sclerotiorum* (Ss); – represents no inhibition zone and +, ++, and +++ represent zones of 0 to 5, 5 to 10, and >10 mm in width, respectively.

^x Isolates were tested for ability to produce extracellular metabolites protease (Pro), chitinase (Chi), siderophore (Sid), and cellulase (Cell) as described by Yang et al. (61).

^y Percent disease severity = $[\Sigma(\text{number of diseased plants in this index} \times \text{disease index}) / (\text{total number of plants investigated} \times \text{highest disease index})] \times 100\%$.

^z Biocontrol efficacy (%) = $[(\text{disease severity of the control} - \text{disease severity of a treatment}) / \text{disease severity of control}] \times 100\%$.

TABLE 4. In vitro inhibition by Chinese isolates of Washington strains of *Gaeumannomyces graminis* var. *tritici* on King's medium B (KMB) and potato dextrose agar (PDA)

Treatment	<i>G. graminis</i> var. <i>tritici</i> strain ^z					
	LD5		ARS-A1		R3-111a-1	
	KMB	PDA	KMB	PDA	KMB	PDA
HC1-07	+	+	+	+	+	++
HC2-07	+	+	+	+	+	+
P32	++	++	++	++	+	++
HC4-07	++	+	++	+	++	+
2-79	+	+	++	+	+	+
HC6-07	+	+	+	+	+	+
JC7-07	+	+	+	+	+	+
HC8-07	++	+	+	+	+	++
HC9-07	++	+	++	+	++	+
HC10-07	+	+	+	+	+	+
HC11-07	+	+	+	++	+	+
HC12-07	+	–	+	+	+	–
HC9-13	++	++	+	+	+	+
JC14-07	++	++	+	++	+	+
HC15-07	+	+	+	+	+	+

^z Symbols: + and ++ indicated zones of inhibition of 0 to 5 and 5 to 10 mm, respectively; – indicates no inhibition.

quences in the database that showed 99 to 100% sequence similarity with *P. fluorescens* 2-79 (data not shown). Isolates HC9-07, HC13-07, and JC14-07 were designated as *P. fluorescens*.

PCA-deficient mutant derivatives of strain HC9-07 are reduced in ability to suppress *G. graminis* var. *tritici*. Using gene-replacement mutagenesis, we interrupted *phzC* and *phzD* genes of isolate HC9-07rif with insertion of the tetracycline resistance cassette. As a result, six mutants were recovered and selected for further testing. In all six mutant strains, the PCR primers PHZ1 and PHZ2 that flank the cassette insertion site amplified the expected 2.8-kb fragment, which is larger than the 1.4-kb fragment amplified from the intact *phzC* and *phzD* genes. The six mutants were also screened by TLC analysis and failed to produce PCA, which coincided with the loss of inhibitory activity against *G. graminis* var. *tritici* under in vitro conditions (data not shown).

Isolates HC9-07, HC13-07, and JC14-07 and *P. fluorescens* 2-79 showed significant ($P = 0.05$) suppression of take-all (Table 5) in the tube bioassay based upon lower disease ratings and greater plant height compared with the nontreated controls (CK and CK+MC). Isolates HC9-07 and HC9-07rif did not differ in ability to suppress take-all. Wheat treated with the PCA-deficient mutants of isolate HC9-07rif had significantly higher root disease ratings than wheat treated with the parental isolate when tests

were conducted in either pasteurized or raw Quincy virgin soil. Mutants had disease ratings that were no different than the CK and CK+MC or the disease rating was intermediate between that of isolate HC9-07rif and that of the controls. Complementation of the mutant restored PCA production as determined by TLC. The biocontrol activity of HC9-07rif and the complemented mutant were equivalent, and both were significantly different than the nontreated check or the check with methyl cellulose (Table 5).

Root colonization. The root-colonizing ability of *P. fluorescens* isolates HC9-07rif, HC13-07rif, JC14-07rif, and 2-79 were tested in a field on the WSU Plant Pathology Farm in Pullman. Introduction of these bacteria on the seed at $\approx 10^7$ CFU seed⁻¹ resulted in rhizosphere populations of between 10^8 and 10^9 CFU g⁻¹ fresh root weight at 3 weeks after planting, and population sizes of these strains gradually declined over the growing season. The population dynamics of these four strains were typical of what has been seen in past studies of strain 2-79 (55). All three Chinese strains showed similar population dynamics and, at 19 weeks after planting, their population sizes were greater than that of strain 2-79. The population size of HC13-07rif was greater than that of 2-79 at all samplings except week six (Fig. 2).

Isolation of PCA from the rhizosphere. Roots of wheat treated with strains HC9-07rif, HC13-07rif, and 2-79 were tested

Fig. 1. Phylogenetic relationship of *phzF* sequences from HC9-07, HC13-07, and JC14-07 and known phenazine-producing *Pseudomonas* spp. The neighbor-joining tree with bootstrap support was constructed and visualized by using Molecular Evolutionary Genetics Analysis 4.0.2 programs. Thick lines indicate nodes with bootstrap support values $\geq 60\%$. The scale bar indicates 0.02 substitutions per site.

for the presence of PCA. Because of limitations in the number of root samples that could be effectively processed, we selected roots only from replicates 2, 4, and 6 of wheat treated with 2-79, HC9-07rif, and HC13-07rif in the field experiment. PCA was isolated from the rhizosphere of all three treatments and at all four sampling dates tested: 3, 6, 9, and 12 weeks after planting (roots from week 19 were not assayed). For strains 2-79, HC9-07rif, and HC13-07rif, the amounts of PCA were 21 to 48, 75 to 589, and 98 to 1,333 ng g⁻¹ of root, respectively, across the entire growing season (Table 6). HC9-07rif and HC13-07rif produced significantly more PCA than 2-79. Representative MS-MS chromatograms and MS-MS spectra of PCA produced by 2-79 and HC9-07rif, respectively, in the wheat rhizosphere are shown in Figure 3.

DISCUSSION

Chinese farmers, like those throughout the world, are being challenged to grow more food using sustainable practices and fewer chemical pesticides because of concerns about the toxicity and residues of pesticides. This study was part of an ongoing collaboration to both select biocontrol agents for use in China and identify their mechanisms of action. Take-all of wheat was selected as a target of this study because China is the world's largest producer of wheat and take-all remains a chronic and acute problem in both the United States and China. In addition, the wheat-take-all pathosystem has been a model for studying the biocontrol process by both introduced and indigenous microorganisms (57).

We selected a nonirrigated field in Jaingsu province and an irrigated field in Hebei province as sources of biocontrol agents because putative agents had never been selected from or tested there, and the fields differed in the type of traditional management practices used. In the Hebei and Jiangsu fields, annual precipitation is ≈500 and 1,000 mm per annum and wheat is rotated with corn and rice, respectively. Both of these rotations promote take-all and inhibit the development of TAD. Bacteria were selected from all parts of the wheat plant and from both the surface and inside of tissues in order to sample the widest range of putative antagonists possible. Little is known about the wheat-associated antagonistic bacteria in these regions, especially bacterial endophytes. As has been seen in other studies (4), including an earlier study on rice in China (61), population sizes were consistently greater on the surface of tissues compared with the inside of tissues. In addition, population sizes on all sampled tissues generally were greater on wheat from Hebei province

compared with Jiangsu province, perhaps as a result of the extra water from irrigation.

In this study, we focused on isolates that inhibited *G. graminis* var. *tritici* in vitro because introduced and indigenous antibiotic producers have consistently been shown to suppress take-all (58) and other soilborne diseases (37). One example of this is the involvement of DAPG-producing *P. fluorescens* isolates in the suppression of take-all in TAD soils (57). Of the 105 antagonistic isolates from both fields, ARDRA analysis and comparisons to known *Pseudomonas* strains revealed that all of these isolates were *Pseudomonas* spp. This is not surprising because the initial selection was done on KMB, and pseudomonads are well-known to colonize the rhizosphere and plant surfaces and produce a broad spectrum of antibiotics. Further analysis of this collection of isolates by BOX-PCR identified 30 distinct groups. From these BOX-PCR groups, 27 isolates were selected for further biocontrol studies without having any information about the types of antibiotics they produced. Finally, 13 isolates, 10 from Hebei and 3 from Jiangsu, were shown to consistently suppress take-all caused by Chinese and Washington isolates of *G. graminis* var. *tritici*. Surprisingly, none of these isolates produced any of the common biocontrol antibiotics: DAPG, pyrrolnitrin, or pyoluteorin. Pseudomonads producing one or more of these antibiotics (i.e.,

Fig. 2. Population dynamics of 2-79, HC9-07rif, HC13-07rif, and JC14-07rif in the rhizosphere of spring wheat ('Louise') planted on 7 May 2010 at the Washington State University Plant Pathology Research Farm.

TABLE 5. Suppression of take-all by phenazine-1-carboxylic acid (PCA)-producing isolates and PCA-deficient mutants^y

Treatment ^z	Experiment 1		Experiment 2		Experiment 3		Experiment 4		Experiment 5		Experiment 6	
	Rating	Height	Rating	Height	Rating	Height	Rating	Height	Rating	Height	Rating	Height
CK	6.8 a	14.3 c	6.6 a	13.1 b	6.4 a	14.1 b	6.4 a	13.9 d	5.1 a	16.6 c	6.1 ab	14.0 bc
CK+MC	—	—	6.5 a	13.1 b	6.2 a	14.3 b	6.0 a	14.2 cd	5.1 a	16.8 c	6.3 a	13.6 c
2-79	5.5 c	15.9 ab	6.3 ab	14.0 ab	5.0 b	15.4 a	3.8 cd	15.7 b	—	—	—	—
JC14-07	6.0 b	16.6 a	5.5 b	14.4 a	5.3 b	15.1 ab	—	—	—	—	—	—
HC13-07	5.6 bc	16.5 a	5.4 b	14.3 a	4.6 b	15.9 a	—	—	—	—	—	—
HC9-07	5.7 bc	15.6 b	5.5 b	14.0 ab	5.3 b	15.0 ab	—	—	—	—	—	—
HC9-07rif	—	—	5.6 b	14.1 ab	—	—	3.4 d	15.8 a	3.2 c	19.9 a	4.8 c	15.2 ab
HC9M7	—	—	—	—	—	—	4.1 bc	15.4 b	4.6 ab	18.4 bc	—	—
HC9M6	—	—	—	—	—	—	—	—	4.5 ab	18.6 ab	—	—
HC9M8	—	—	—	—	—	—	4.4 bc	14.9 bcd	4.5 ab	18.9 ab	—	—
HC9M9	—	—	—	—	—	—	4.1 bc	15.1 bc	4.4 b	19.0 ab	5.3 bc	15.0 ab
HC9M5	—	—	—	—	—	—	—	—	4.3 b	18.9 ab	—	—
HC9M10	—	—	—	—	—	—	4.8 b	14.8 bcd	4.3 b	18.5 abc	—	—
HC9M9 comp	—	—	—	—	—	—	—	—	—	—	4.7 c	15.7 a

^y Experiments 1 to 4 and 6 were conducted in pasteurized Quincy virgin soil (60°C, 30 min) and experiment 5 was conducted in raw soil. 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 or Kruskal-Wallis all-pairwise comparison; — indicates not tested in this experiment.

^z CK = nontreated check; CK + MC = seed treated with methyl cellulose; — bacteria were applied to the seed with methyl cellulose at a dose of 10⁷ CFU seed⁻¹; HC9-07rif is a spontaneous mutant of HC9-07 that is resistant to rifampicin; HC9M5 to HC9M10 are mutants deficient in production of PCA; HC9M9comp was genetically complemented to restore PCA production.

isolates similar to well-studied *P. fluorescens* strains Pf-5, CHA0, and Q8r1-96) are widespread in agroecosystems (57). However, 3 of the 13 isolates (HC9-07, HC13-07, and JC14-07) produced PCA based upon the detection of the key biosynthesis gene *phzF* using the specific primer set Ps_up1–Ps_low1 and detection of the antibiotic in vitro by HPLC. Most unexpected, however, was that these three isolates were nearly identical to the well-studied biocontrol agent *P. fluorescens* 2-79, based upon phylogenetic analysis of *phzF* (Fig. 1) and sequence analysis of 16S rDNA. Equally unexpected was that the three PCA producers were isolated from the inside of the stem. Special attention was given to insure that the surface of the stems was sterilized and, thus, we are confident that these strains are indeed endophytes and not surface contaminants. This is the first report that 2-79-like pseudomonads can have an endophytic phase.

Strain 2-79 was isolated in 1979 from the rhizosphere of the wheat growing in a TAD soil near Lind, WA (56) and it has been one of the most intensively studied biocontrol agents during the past three decades. It is considered to be the simplest of the

phenazine-producing *Pseudomonas* spp. (32,34), because it contains only the core biosynthesis locus *phzABCDEFG* and synthesizes only PCA. Other biocontrol *Pseudomonas* spp. such as *P. aureofaciens* (41) and *P. chlororaphis* (9) produce PCA and derivatives of PCA such as 2-hydroxyphenazine-1-carboxylic acid and phenazine-1-carboxamide, respectively, because they have additional genes that modify PCA (34). Whereas *P. aureofaciens* and *P. chlororaphis* strains have long been known to be common in soils worldwide, it had been thought that 2-79-like strains were endemic to eastern Washington State (D. M. Weller, *unpublished*). Recently, however, Mazurier et al. (37) reported that strains ChPhzLR105, ChPhzLR108, and ChPhzLR167 were genotypically similar to 2-79 and are part of a complex of phenazine producers that work in concert with nonpathogenic *F. oxysporum* to provide natural biocontrol of Fusarium wilts in suppressive soils of the Châteaurenard region of France. Our discovery of strains HC9-07, HC13-07, and JC14-07 in two fields in China indicates that 2-79-like strains are more cosmopolitan than previously thought and may be present in agroecosystems worldwide.

TABLE 6. Accumulation of phenazine-1-carboxylic acid (PCA) in the rhizosphere of wheat colonized by *Pseudomonas fluorescens* 2-79, HC9-07, and HC13-07

Weeks ^v	Bacterial treatment ^w	High mass accuracy ^x			Library match reverse ^y		PCA (ng/g) ^z
		Calculated MW	mDa	ppm	Forward	Reverse	
3	2-79	225.0666	0.2	0.9	950	972	34
	HC9-07rif	225.0664	0.0	0.0	955	977	589
	HC13-07rif	225.0666	0.2	0.9	950	958	1,333
9	2-79	225.0664	0.0	0.0	942	974	21
	HC9-07rif	225.0661	0.3	1.3	951	965	84
	HC13-07rif	225.0664	0.0	0.0	949	957	289
12	2-79	225.0662	0.2	0.9	873	919	48
	HC9-07rif	225.0666	0.2	0.9	957	965	187
	HC13-07rif	225.0663	0.1	0.4	949	962	98
15	2-79	225.0663	0.1	0.4	833	918	27
	HC9-07rif	225.0664	0.1	0.4	954	976	75
	HC13-07rif	225.0663	0.1	0.4	947	956	192

^v Weeks after planting.

^w Bacteria were applied as a seed treatment at 10⁷ CFU seed⁻¹.

^x Actual MW of PCA is 225.0664; calculated molecular weight (MW) is mass obtained from quantitative time-of-flight (Q-TOF) 2 analysis.

^y For tandem mass spectrometry (MS/MS) library matching, the forward match value, which applies to pure chromatographic peaks, is reduced when mass peaks are present in the spectrum of the unknown but not in that of the library, or present in the library spectrum but not in that of the unknown. In reverse library matching, which applies to peaks containing mixtures, the match value is reduced by mass peaks present in the library but not in the unknown spectrum. Contaminant peaks in the unknown spectrum but not present in the library spectrum will have no effect on the reverse hit (perfect fit equals 1,000). As a general rule, confirmation of high mass accuracy (MS-TOF), together with forward/reverse match values of ≥500, 600 to 700, and 800 to 1,000 correspond to compound identification at confidence levels of 70, 85, and 100%, respectively.

^z PCA in ng/g⁻¹ of root fresh weight.

Fig. 3. Examples of mass spectrometry time-of-flight (MS-TOF) and tandem mass spectrometry (MS-MS) chromatograms of phenazine-1-carboxylic acid (PCA) (left set of panels) and MS-MS spectra of PCA (right set of panels). PCA actual molecular weight (MW) is 225.0664 and the formula is C₁₃H₉N₂O₂ for positive-ion mode. **A**, MS time-of-flight (TOF) chromatogram of PCA standard; **B**, MS-MS chromatogram of PCA produced by 2-79 in the wheat rhizosphere (calculated MW is 225.0663); **C**, MS-TOF chromatogram of PCA produced by strain HC9-07 in the wheat rhizosphere (calculated MW is 225.0664); **D**, MS-MS spectrum of PCA standard; **E**, MS-MS spectrum of PCA produced by 2-79 in the wheat rhizosphere (forward 950, reverse 972); and **F**, MS-MS spectrum of PCA produced by HC9-07rif in the wheat rhizosphere (forward 951, reverse 965).

Although very similar to 2-79, the three Chinese strains in general had slightly greater rhizosphere competence than 2-79. In our field study, the population sizes of HC9-07rif, HC13-07rif, and JC14-07rif at week 19 were significantly greater than that of 2-79. In addition, HC9-07rif and HC13-07rif produced significantly more PCA in the rhizosphere than 2-79, which is probably reflective, in part, of higher population sizes of the Chinese strains. The amount of PCA produced over the season by 2-79, HC9-07rif, and HC13-07rif was 21 to 48, 75 to 589, and 98 to 1,333 ng g⁻¹ fresh weight, respectively, of root with rhizosphere soil. PCA produced by 2-79 in this study was in the range of concentrations previously reported in the rhizosphere of wheat grown in the greenhouse and field from seed treated with 2-79 (50). In addition, the levels of PCA produced by all three strains were comparable with amounts of the antibiotic detected on roots of wheat colonized by indigenous PCA-producing pseudomonads in commercial fields located in the low-precipitation zone of the Columbia Plateau of the Pacific Northwest (Mavrodi et al., unpublished). However, we think that the amounts of PCA we measured in root samples probably underestimate the actual concentrations of the antibiotic in and around microcolonies of 2-79, HC9-07rif, and HC13-07rif because our antibiotic extraction procedure uses a pooled sample of roots (15 g) from several plants. Furthermore, both indigenous and introduced bacteria inhabit only a small fraction of the rhizosphere volume (52) and introduced rhizobacteria generally compose only 1 to 10% of the culturable rhizosphere community. In this study, after the first sample, the introduced PCA producers composed <10% of the total aerobic bacteria detected in 1/10 tryptic soy agar (data not shown). We estimate that localized PCA concentrations could easily reach hundred nanomolar levels, which is close to the minimal inhibitory concentrations for PCA in vitro for some gram-positive bacteria and fungi.

In addition to using a direct approach to demonstrate the role of PCA in biocontrol activity of the Chinese strains against of take-all, we also generated mutants of HC9-07rif deficient specifically in biosynthesis of PCA. In tests in both pasteurized and raw soil, the six mutants lost all or part of their ability to suppress take-all in the greenhouse. These results are similar to those from previous studies demonstrating that PCA was responsible for the majority of the biocontrol activity of 2-79 against take-all (49).

In conclusion, our results indicate that 2-79-like PCA-producing *P. fluorescens* isolates are probably common in Chinese wheat fields. The fact that they can live as endophytes warrants further study of PCA production inside of wheat tissues and of the role of these bacteria in protecting wheat against not only soilborne disease but also foliar pathogens. Until now, emphasis has been placed on quantification of PCA in the rhizosphere and its role in inhibiting soilborne pathogens. Current topics of interest focus on the center of origin of 2-79-like strains of *P. fluorescens* and its role in natural suppression of soilborne and foliar diseases in different agroecosystems.

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