AEM
Journals.ASM.org

Role of Bacterial Communities in the Natural Suppression of *Rhizoctonia solani* Bare Patch Disease of Wheat (*Triticum aestivum* L.)

Chuntao Yin,[a] Scot H. Hulbert,[a] Kurtis L. Schroeder,[a] Olga Mavrodi,[a] Dmitri Mavrodi,[a] Amit Dhingra,[b] William F. Schillinger,[c] Timothy C. Paulitz[d]

Department of Plant Pathology, Washington State University, Pullman, Washington, USA[a]; Department of Horticulture and Landscape Architecture, Washington State University, Pullman, Washington, USA[b]; Department of Crop and Soil Sciences, Washington State University, Pullman, Washington, USA[c]; USDA Agricultural Research Service, Root Disease and Biological Control Research Unit, Pullman, Washington, USA[d]

***Rhizoctonia* bare patch and root rot disease of wheat, caused by *Rhizoctonia solani* AG-8, develops as distinct patches of stunted plants and limits the yield of direct-seeded (no-till) wheat in the Pacific Northwest of the United States. At the site of a long-term cropping systems study near Ritzville, WA, a decline in *Rhizoctonia* patch disease was observed over an 11-year period. Bacterial communities from bulk and rhizosphere soil of plants from inside the patches, outside the patches, and recovered patches were analyzed by using pyrosequencing with primers designed for 16S rRNA. Taxa in the class *Acidobacteria* and the genus *Gemmatimonas* were found at higher frequencies in the rhizosphere of healthy plants outside the patches than in that of diseased plants from inside the patches. *Dyella* and *Acidobacteria* subgroup Gp7 were found at higher frequencies in recovered patches. *Chitinophaga*, *Pedobacter*, *Oxalobacteriaceae* (*Duganella* and *Massilia*), and *Chyseobacterium* were found at higher frequencies in the rhizosphere of diseased plants from inside the patches. For selected taxa, trends were validated by quantitative PCR (qPCR), and observed shifts of frequencies in the rhizosphere over time were duplicated in cycling experiments in the greenhouse that involved successive plantings of wheat in *Rhizoctonia*-inoculated soil. *Chryseobacterium soldanellicola* was isolated from the rhizosphere inside the patches and exhibited significant antagonism against *R. solani* AG-8 *in vitro* and in greenhouse tests. In conclusion, we identified novel bacterial taxa that respond to conditions affecting bare patch disease symptoms and that may be involved in suppression of *Rhizoctonia* root rot and bare batch disease.**

Rhizoctonia bare patch and root rot disease, caused by *Rhizoctonia solani* AG-8, results in major yield limitations in direct-seeded (no-till) cereal crops, as they develop circular or oval patches of stunted plants that produce little or no grain (1). It was discovered in Australia in the 1930s (2) and in the Pacific Northwest (PNW) in the mid-1980s (1, 3). To date, no wheat or barley (*Hordeum vulgare* L.) cultivars are resistant to *Rhizoctonia* (4). Furthermore, there are few management strategies for control of *Rhizoctonia* spp. except for tillage (5), and minimal control is obtained with fungicidal treatment (6–8). Seed treatments may improve seedling health in some cases (9), but a reduction of bare patch disease symptoms has not been observed (7). Growers can reduce damage by reducing greenbridge carryover of the pathogen from grassy weeds and crop volunteers. This carryover is most severe when herbicide treatment is applied right before the crop is planted (10).

Suppression of soilborne pathogens has been described for a number of diseases, including take-all disease of wheat (11), *Streptomyces* scab disease of potato (*Solanum tuberosum* L.) (12), *Fusarium* wilt disease of melons (13), and black rot disease of tobacco (*Nicotiana tabacum*) (14). In many cases, *Pseudomonas* species producing antifungal compounds, like phenazine and phloroglucinol, have been implicated in suppression of such pathogens. There are several lines of evidence that *Rhizoctonia* disease suppression may occur in agricultural fields. *Rhizoctonia* root rot disease declined to almost nil after 7 to 9 years of continuous wheat cropping in Australia (15). Roget (16) found that *Rhizoctonia* patch disease development decreased after 5 years of continuous direct-seeded wheat and reached negligible levels after 10 years. A similar *Rhizoctonia* decline or suppression was also observed in the PNW. *Rhizoctonia* patch disease sites started to appear 3 years after tillage was stopped at an 8-ha cropping systems rotation study site near Ritzville, WA, and reached a peak in the seventh year, based on annual global positioning system (GPS) mapping of patches. But since then, the areas of patches have declined significantly or even disappeared (17). Cores from the centers of patches were taken into a greenhouse and planted with cycles of monoculture barley over 9 months. Barley was stunted in all cores at the start of the experiment, but over half of the cores did not produce stunting at the end of the experiment (18). In the late 1990s, there was an intensive research effort to understand the basis of *Rhizoctonia* patch disease suppression in Australia (19–23). The findings suggested that disease suppression may involve mesofauna and macrofauna, or it may have been due to a long-term input of carbon in conservation tillage practices. However, the mechanisms influencing the dynamics of *Rhizoctonia* are still not well understood.

Soil microorganisms play a major role in soil health and suppression of root diseases (11, 24, 25). The diversity of soil microbial communities is important for the capacity of a soil to suppress soilborne plant diseases (26, 27). In disease-suppressive soils, some of the indigenous microorganisms protect susceptible crops

Received 24 May 2013 **Accepted** 17 September 2013
Published ahead of print 20 September 2013
Address correspondence to Timothy C. Paulitz, paulitz@wsu.edu.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.01610-13.

doi:10.1128/AEM.01610-13

from certain phytopathogens (28). Some isolates of *Mitsuaria* and *Burkholderia* inhibited fungal and oomycete growth *in vitro* and reduced disease severity in infected tomato (*Solanum lycopersicum* L.) and soybean (*Glycine max* L.) seedlings (29). Jung et al. (30) demonstrated that *Pseudomonas aureofaciens* 63-28 induced plant defense systems that led to improved resistance to *R. solani* AG-4 in soybean seedlings. Barnett et al. (31) suggested that *Rhizoctonia* patch disease suppression is an interaction among three bacteria, *Pantoea agglomerans*, *Exiguobacterium acetylicum*, and *Microbacteria* sp. Mendes et al. (32) reported that some other bacterial taxa (*Proteobacteria*, *Firmicutes*, and *Actinobacteria*) were consistently associated with suppression of root disease caused by *R. solani*. These studies indicated that soil disease suppression cannot simply be ascribed to a single microbial group and may involve more biocontrol microbes than previously reported or may even be the synergistic effect of many microbial groups. Therefore, the aim of this study was to compare the composition of the bacterial community inside *Rhizoctonia* bare patches, outside the patches, and in healthy areas that were previously patches (recovered patches) in wheat at a long-term cropping system study site near Ritzville, WA, that had demonstrated a *Rhizoctonia* decline. We used pyrosequencing to identify the key component(s) associated with this disease suppression. These trends were verified by using quantitative PCR (qPCR). We also mimicked the transition from conduciveness to disease suppression in greenhouse cycling experiments, and we isolated bacteria to test their biocontrol capabilities.

MATERIALS AND METHODS

Study site, sample collection, and preparation. This study was conducted at an 8-ha parcel on the Ron Jirava farm (47°8′34″N, 118°28′22″W) located near Ritzville, WA. The traditional cropping system practiced by growers throughout this region is a tillage-based winter wheat-summer fallow rotation by which only one crop is produced every other year. The study site had four replicate blocks, each with 14 different rotation strips, 9 m wide by 152 m long. Mean annual precipitation at the site is 290 mm, and the soil is a Ritzville silt loam (coarse-silty, mixed, superactive, mesic Calcidic Haploxerolls). The soil is >180 cm deep and has no restrictive layers or rocks. The slope at the site is <1%. *Rhizoctonia* disease bare patches appeared in the rotation strips 3 years after tillage was stopped. From 1999 to 2012, these patches were mapped with a high-resolution GPS device to record the dynamics of patch formation and recovery (33). This study only focused on the continuous annual spring wheat strips to minimize the effects of crop rotation. Samples were collected in April or May of 2008, 2009, and 2010. Both bulk and rhizosphere soil samples were collected from three locations: the center of an active *Rhizoctonia* disease patch (inside the patch), 1 m outside the same patch (outside the patch), and an adjacent site that did not have a noticeable patch but which had harbored a patch in the previous year (recovered patch). Approximately 1-kg samples of soil from between the rows was taken for the bulk soil samples. For rhizosphere soil samples, 5 to 10 plants were taken with intact roots. Roots with attached rhizosphere soil from wheat plants were cut from the shoot and placed in a 50-ml plastic tube containing 20 ml of sterile distilled water. The tubes were vortexed for 1 min, sonicated for 1 min, and stored at −20°C for further use. The factorial experiment for pyrosequencing of the field samples consisted of three locations (inside, outside, and recovered patches) for 2008 and two locations (inside and outside patches) for 2010 for each of two soil types (bulk and rhizosphere) in each of three replicates. Pyrosequencing of the 2009 samples was not conducted.

DNA extraction, purification, and sequencing. For bulk soil, DNA was extracted as described previously (34). For rhizosphere soil, 2 ml of the root wash was used for DNA extraction by using an UltraClean soil DNA kit (MO BIO Laboratories, CA) with the alternative protocol for wet soil samples. For bacterial cultures, genomic DNA was extracted from bacteria by using a cetyltrimethylammonium bromide (CTAB) miniprep procedure (35). The DNA was quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and stored in a −20°C freezer for subsequent procedures.

The 16S rRNA fragments were amplified from bulk or rhizosphere soil DNA with universal primers. For the 2008 samples, primers were designed to amplify the V3 hypervariable region of bacterial 16S rRNA as described previously (34). The primers were as follows: U342-FC-B, 5′-GCCTTGCCAGCCCGCTCAG**CCTACGGGRSGCAGCAG**-3′, and U529R-FC, 5′-GCCTCCCTCGCGCCATCAG*NNNNN***ACCGCGGCKGCTGGC**-3′ (the underlined sequences are the 454 Life Science sequencing primers B or A, and the bold letters denote the universal 16S rRNA primers U342-FC-B and U529R-FC). The 5-bp barcode within primer U529R-FC is denoted by 5 italic Ns, and this applied to each 2008 sample. Because technological advances allowed longer sequence reads for the 2010 field samples and cycling experiment samples in the greenhouse, universal primers 27F and 533RAmpTagR were designed to amplify the V1-to-V3 hypervariable regions of 16S rRNA. The primers were as follows: 27F, 5′-CCTATCCCCTGTGTGCCTTGGCAGTCTCAGTC**AGAGTTTGATYMTGGCTCAG**-3′, and 533RAmpTagR, 5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG*NNNNNNNNN*TC**ATTACCGCGGCTGCTGGCA**-3′, where the underlined sequences are the 454 Life Science sequencing primers B or A, the bold regions are primers targeted to the conserved region of 16S rRNA, the barcode within the reverse primer is denoted by 8 italic Ns, which applied to each soil sample, and the TC after the 8 italic Ns is a linker sequence. A total of 39 barcoded reverse primers are listed in Table S1 of the supplemental material. PCR mixtures consisted of 2 ng of soil DNA, 1× reaction buffer, 0.2 mM deoxynucleotides, 0.25 μM each primer, 5 μl dimethyl sulfoxide, and 0.125 U of *Taq* DNA polymerase (Invitrogen Corp., CA) in a total volume of 25 μl. PCR was performed using a hot start program, as follows: 80°C for 3 min; 94°C for 5 min; 30 cycles of 94°C for 30 s, 55 to 60°C for 30 s, and 72°C for 30 s; final extension at 72°C for 7 min. Each soil DNA template was amplified in 5 or 6 separate individual reactions. The presence of amplicons was confirmed by gel electrophoresis on a 1.5% (wt/vol) agarose gel, and only specific amplification product was pooled. PCR products were quantified by using a GelDoc quantification system (Bio-Rad). Equimolar amounts (100 ng) of the amplicons of each sample were mixed in a single tube. PCR products were cleaned using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions for pyrosequencing.

To characterize bacterial isolates with antagonistic activity against *R. solani* AG-8, near-full-length 16S rRNA genes were amplified by PCR from bacterial genomic DNA with conserved primers 8F and 1492R, as described previously (36). The PCR mixtures consisted of 100 ng of purified bacterial DNA, 1× reaction buffer, 0.2 mM deoxynucleotides, 0.4 μM each primer, and 1.25 U of GoTaq DNA polymerase (Promega, Inc., Madison, WI) in a total volume of 25 μl. The amplification was performed using a cycling program that included a 2-min initial denaturation at 94°C followed by 30 cycles of 94°C for 20 s, 55°C for 15 s, and 72°C for 1.5 min, with a final extension at 72°C for 5 min. Amplicons were cleaned with QIAquick PCR purification spin columns (Qiagen, Valencia, CA) and end-sequenced by using the 8F primer (Applied Biosystems, Foster City, CA). The sequences were analyzed by using the Classifier and Seqmatch tools available via the Ribosomal Database Project server (http://rdp.cme.msu.edu).

Barcoded sample pyrosequencing and sequence analysis. DNA amplicons of the 2008 samples were sequenced as previously described (34). The DNA amplicons of the 2010 samples were sequenced on the genome sequencer (GS) FLX 454 pyrosequencer (Roche) at the University of Idaho. Pyrosequencing reads were processed to remove adapters, barcodes, and primers by using Cross Match (version 1.080806). Base quality clipping was performed using the application Lucy (version 1.20p), and then sequences were aligned by using the SILVA bacterial sequence data-

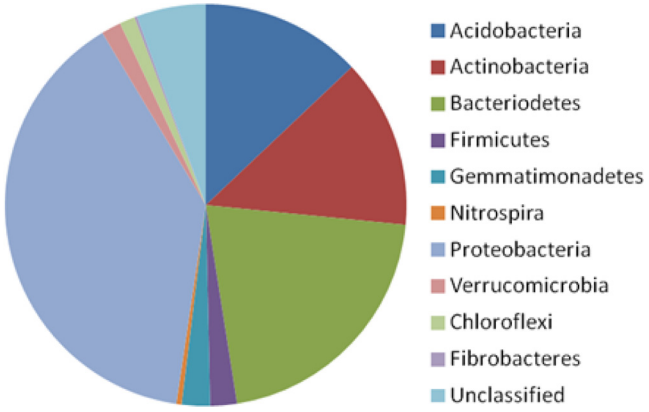

FIG 1 Frequencies of bacterial phyla in three locations (inside or outside the patches and in recovered patches) in 2008 (with a 3% dissimilarity cutoff).

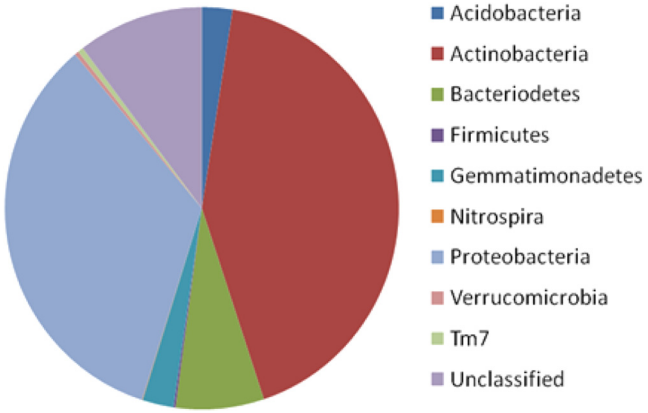

FIG 2 Frequencies of bacterial phyla in two locations (inside or outside the patches) in 2010 (with a 3% dissimilarity cutoff).

base and MOTHUR (version 1.12.1). Sequence reads were filtered according to the following criteria: (i) sequences were at least 100 bp in length; (ii) maximum hamming distance of barcode of 1; (iii) maximum number of matching errors to forward primer sequences of 2; (iv) presence of <2 ambiguous bases; (v) a homopolymer run of <7 bp in the sequence; (v) alignment to the SILVA bacterial database within 75 bp of the expected alignment start position as identified by the trimmed mean of all read alignments (trim, 10%); (vii) the read alignment started within the first 5 bp and extended through the read to within the final 5 bp. The RDP Bayesian classifier (Ribosomal Database Project, version 10 [http://rdp.cme.msu.edu/]) was used to assign sequences to phylotypes. Sequences were clustered into operational taxonomic units (OTUs) at a 3% dissimilarity cutoff. To avoid the bias created by analyzing data with different numbers of sequencing reads from each sample, all reported OTU abundances were converted to a relative frequency by dividing the number of reads for any given OTU by the total number of reads obtained for that sample.

Quantitative PCR analysis. Quantitative PCR was conducted for two bacterial species to validate the results of the sequence analysis and the trends in bacterial populations. These were tested on soil samples from 2008 and 2010 that were subjected to pyrosequencing and also on soil samples from 2009 that were not pyrosequenced. The primers were designed to amplify the specific regions in the 16S rRNA of two species (*Acidobacteria* subgroup Gp7 and *Flavobacterium* sp.), and the qPCR methods were described previously (34). The primers were as follows: GP7-F3, 5′-GCTGACAGGAGTTTACAATCCA-3′, GP7-R2, 5′-GCAGCAGTGGGGAATTTTGCG-3′, 859-RT-F2, 5′-ACAATCCATAGGACCGTCATCCTG-3′, and 859-RT-R2, 5′-TGAGACACGGACCAGACTCCTAC-3′. To normalize for differences in DNA extraction and PCR efficiencies for each sample, the universal primers U1406F and U1501R of 16S rRNA were designed to quantify amplification of total bacterial DNA from each sample. The primers for this were as follows: U1406F, 5′-TTGTACACACCGCCCGTCA-3′, and U1501R, 5′-ACGGTTACCTTGTTACGACTT-3′. The fraction of target bacterial DNA was calculated by dividing the amount of target bacterial DNA by the amount of total bacterial DNA in each sample. All reactions were carried out in triplicate.

Cycling experiments in the greenhouse. To validate the observed shifts of bacterial frequencies in the rhizosphere in the field over time, a cycling experiment was conducted in the greenhouse. This experiment mimicked the planting cycles in the field but compressed the rotation cycle to approximately 1 month instead of 1 year, because of the optimum temperatures in the greenhouse. The soil was collected from the upper 25 cm of the soil profile by using a shovel, from a field adjacent to the study site. The field was managed with conventional tillage with a traditional wheat-fallow rotation. Plastic pots (16 cm in diameter and 18 cm in height) were filled with the soil amended with 1.0% (wt/wt) ground oat kernel inoculum of *R. solani* AG-8 isolate C1 as described by Paulitz et al. (18). Nine pots filled with natural soil without AG-8 inoculum served as controls. Three seeds of *Triticum aestivum* L. cv. Alpowa wheat were sown in each pot. A total of 18 pots were incubated in the greenhouse at 15 to 18°C in a dark/light cycle of 12 h and watered two times per week and also with diluted (1:3, vol/vol) Hoagland's solution once a week. After 4 weeks, the plants with intact roots from three pots (with AG-8 inoculum) were harvested, rhizosphere soils were collected as described above, and DNA was extracted for cycle 1 samples. The rhizosphere soils of three plants from each pot served as replicates. The above-ground parts of plants in the remaining 12 pots were cut without disturbing the soils, and the seeds of the same wheat cultivar were replanted in the pots. After another 4 weeks, the rhizosphere soils were collected from three pots (with AG-8 inoculum), and DNA was extracted for cycle 2 samples. Similarly, cycle 3 samples were obtained from the last three pots (with AG-8 inoculum) after four more weeks. 16S rRNA fragments were amplified from rhizosphere soil DNA with the primers 27F and 533RAmpTagR (described above and listed in Table S1 of the supplemental material). DNA amplicons were used for pyrosequencing.

Isolation of bacteria from rhizosphere soil and culture conditions. To identify bacteria antagonistic to AG-8, bacteria were isolated from rhizosphere soil collected from inside the patches of the Ron Jirava farm. Five plants with intact roots from inside patches were collected and placed into large plastic bags, brought back to the laboratory, and immediately processed. The wheat roots with adhering rhizosphere soil were added to a 50-ml tube containing sterile distilled water, and the tube was vortexed for 1 min and then sonicated in an ultrasonic cleaner for 1 min. The root wash was serially diluted in a 96-well microtiter plate, and the resulting dilutions were plated on ½ TSA medium (1 liter double-distilled water [ddH_2O], 5 g tryptone, 5 g sucrose, and 0.5 g glutamic acid) at 28°C. Representative colony types were picked from the most dilute plate and restreaked to obtain pure colonies. Bacterial isolates were stored in nutrient broth plus glycerol at −80°C.

***In vitro* antagonistic activities of bacteria against *Rhizoctonia solani*.** The antagonistic activities of bacterial strains against AG-8 were tested via a dual culture method (37). Paired cultures were incubated at 25°C and scored after 7 days by measuring the diameters of radial growth of AG-8 in the control and in dual cultures. The percent inhibition of radial growth was calculated as follows: $100 \times [(R1 - R2)/R1]$, where R1 was the radial growth of the pathogen in the control and R2 was the radial growth of the pathogen in the dual culture with antagonist. The experiment was repeated three times with three replicates of each treatment. Isolates that showed antagonistic activity against AG-8 were selected for further assays.

Greenhouse biocontrol assays. To verify antagonistic activity against *R. solani* AG-8, bacterial isolates that showed antagonistic activity in dual

TABLE 1 Diversity, richness, abundance, and evenness indices for 2008 and 2010 field samples[a]

Soil type	Sample source	Shannon diversity index[b]		Richness index[c]		Abundance[d]		Evenness[e]	
		2008	2010	2008	2010	2008	2010	2008	2010
Bulk soil	Inside the patch	4.20 a	4.84 a	207	340	2,149 ab	1,451 b	0.79 ab	0.83
	Outside the patch	4.07 a	4.96 a	186	365	1,744 b	1,570 ab	0.78 ab	0.84
	Recovered patch	4.32 a		178		1,152 c		0.83 a	
Rhizosphere soil	Inside the patch	3.24 b	4.83 a	170	322	2,779 a	1,763 a	0.63 c	0.83
	Outside the patch	3.64 ab	4.84 a	183	330	2,216 a	1,593 ab	0.70 b	0.83
	Recovered patch	3.83 ab		201		2,917 a		0.72 b	

[a] The values followed by different letters in the same column were significantly different according to Fischer's LSD test ($P \leq 0.05$). Each sample included three replicates.
[b] The Shannon diversity index (H) was calculated as the follows: $-\Sigma(P_i \times \log[P_i])$, where P_i is the proportion of the total population for each individual species or OTU.
[c] The richness index, S, is the total number of OTUs/sample.
[d] The abundance represents the total number of individual sequences in each sample.
[e] The evenness, E, is calculated as follows: H/ln(S).

culture assays were tested in the greenhouse. The experiment was performed in two separate assays, with soil collected from the healthy areas, which was sieved through a 0.5-cm-mesh screen. Part of the soil was pasteurized at 60°C for 30 min to reduce interference from other nonsporulating soilborne microorganisms. Both soils were air dried at room temperature and were amended with 1.0% (wt/wt) ground oat kernel inoculum of AG-8. Fresh bacterial cultures grown on ½ TSA plates were scraped, suspended in double-distilled water, and centrifuged for 3 min at 13,000 rpm. The pellet was resuspended in sterile ddH_2O and adjusted to 1×10^8 CFU/ml. Plastic cones (2.5 cm in diameter and 16.5 cm long) were filled with 70 g of *Rhizoctonia*-inoculated soil and 20 ml of the bacterial cell suspension. Soil samples amended or not amended with oat kernel inoculum served as controls. Three wheat seeds (cultivar Alpowa) were sown in each cone. Cones were arranged in a randomized complete block design in plastic racks and incubated in the controlled-environment greenhouse as described above. After 3 weeks, the seedlings were removed from the cones, the roots were washed, the plants were evaluated for *Rhizoctonia* patch disease root rot severity on a scale of 0 to 8, and root length was measured as described previously (10, 38). Each treatment had 10 replicates, and the experiment was conducted twice.

Statistical analysis. Data analyses were performed using the general linear models (GLM) procedures within the SAS statistical software package (SAS Institute, Inc., Cary, NC). The numbers of individual OTUs per sample were normalized by dividing the number of OTUs by the total number of sequences in each sample. These frequency data were used for statistical analysis. Multiple comparisons were performed using Tukey's test. Significance was accepted at an α level of ≤ 0.05. The Shannon diversity index, richness, abundance, and evenness of OTUs were calculated as described previously (34). Greenhouse biocontrol assay data were analyzed with STATISTIX 8.0 software (Analytical Software, St. Paul, MN). Differences in root disease or root lengths among treatments were determined by standard analyses of variance, and mean comparisons among treatments were performed by using Fisher's protected least significant difference (LSD) test ($P \leq 0.05$).

Nucleotide sequence accession numbers. Sequences from four antagonistic species (*Pseudomonas* sp. CY25, *Chryseobacterium* sp. CY31, *Pedobacter* sp. CY44, and *Chryseobacterium* sp. CY43) are available from NCBI (accession numbers JX987480 to JX987483). All sequencing reads are accessible under NCBI BioProject number PRJNA218469.

RESULTS

Bacterial community structure and composition. A total of 72,360 high-quality reads were generated in 2008, providing 2,070 to 5,746 sequences (150 to 220 bp in length) for each soil sample, and these were assembled into 345 OTUs by using a 3% dissimilarity cutoff. A total of 35,922 high-quality reads were generated in 2010, providing 2,566 to 3,441 sequences (350 to 400 bp in length)

TABLE 2 Bacterial taxa with higher abundance in rhizosphere soils than bulk soil in the 2008 and/or 2010 field samples

Taxa with a significantly[a] higher abundance in rhizosphere soil than bulk soil in[b]:			
2008		2010	
Family	Genus	Family	Genus
Pseudomonadaceae	*Pseudomonas**	*Pseudomonadaceae*	*Pseudomonas**
Flavobacteriaceae	*Flavobacterium**	*Flavobacteriaceae*	*Flavobacterium** (OTU258, OTU367)
Comamonadaceae	*Variovorax**	*Comamonadaceae*	*Variovorax**
Burkholderiaceae	*Burkholderia**	*Burkholderiaceae*	*Burkholderia**
Sphingobacteriaceae	*Pedobacter**	*Sphingobacteriaceae*	*Pedobacter** (OTU26, OTU52, OTU380)
Microbacteriaceae	*Microbacterium*	*Oxalobacteraceae*	*Duganella* (OTU4)
			Duganella (OTU200)
			Herminiimonas
			Herbaspirillum
Phyllobacteriaceae	*Phyllobacterium*	*Phyllobacteriaceae*	*Mesorhizobium*
Sphingomonadaceae	*Sphingomonas*	*Burkholderiaceae*	*Ralstonia*
		Chitinophagaceae	*Chitinophaga*
		Gemmatimonadaceae	*Gemmatimonas* (OTU13, OTU1597)

[a] Statistical significance was defined as $P \leq 0.05$.
[b] An asterisk indicates that the sequences from the 2008 and 2010 samples showed 100% identity. Sequences were clustered into OTUs at a 3% dissimilarity level.

TABLE 3 Relative abundances of bacterial taxa that differed based on patch sampling area (inside, outside, or recovered) in rhizosphere soil from field samples

Family[a]	Genus[b]	Year	Frequency ($\times 10^{-3}$)[c]		
			Inside the patch	Outside the patch	Recovered patch
Chitinophagaceae	*Chitinophaga** (OTU38)	2008	59.07 a	39.53 b	
		2010	4.88 a	1.17 b	
Comamonadaceae	*Variovorax**	2008	26.59 a	17.67 b	
		2010	51.41	42.9	
Sphingobacteriaceae	*Pedobacter* (OTU380)*	2008	31.67 a	17.43 b	
		2010	3.02 a	1.88 b	
	Pedobacter (OTU52)*	2010	3.21 a	1.26 b	
Cytophagaceae	*Dyadobacter*	2008	7.81 a	1.62 b	
Flavobacteriaceae	***Flavobacterium***	**2008**	**26.6 a**	**2.81 b**	
Microbacteriaceae	*Microbacterium*	2008	2.88 a	0.92 b	
Moraxellaceae	*Acinetobacter*	2008	27.92 a	1.98 b	
Phyllobacteriaceae	*Phyllobacterium*	2008	5.16 a	0.29 b	
Flavobacteriaceae	*Chryseobacterium* (OTU437)	2010	3.64 a	0.23 b	
Oxalobacteraceae	*Duganella* (OTU4)	2010	12.32 a	4.49 b	
	Massilia (OTU1244)	2010	1.83 a	0 b	
Pseudomonadaceae	*Pseudomonas* (OTU9)	2010	12.07 a	2.88 b	
Sphingobacteriaceae	*Mucilaginibacter*	2010	1.71 a	0.2 b	
Acidobacteria	*Acidobacteria* subgroup Gp3	2008	4.43 b	10.67 a	7.44 a
		2010	0.57 b	2.51 a	
Acidobacteria	***Acidobacteria* subgroup Gp7***	**2008**	**1.8 c**	**2.45 b**	**6.85 a**
		2010	**0 b**	**1.67 a**	
Burkholderiaceae	*Burkholderia**	2008	4.12 b	24.98 a	17.95 a
		2010	13.8	18.41	
Phyllobacteriaceae	*Mesorhizobium**	2008	3.7	4.11	5.14
		2010	7.56 b	13.81 a	
Xanthomonadaceae	*Dyella*	2008	0.37 b	0.48 b	1.54 a
		2010	3.02 b	6.7 a	
Acidobacteria	*Acidobacteria* subgroup Gp1	2008	9.38 b	21.1 a	
Actinomycetaceae	*Actinobacteria*	2008	4.78 b	12.96 a	
Acidobacteria	*Acidobacteria* subgroup Gp4 (OTU170)	2010	1.29 b	2.69 a	
	Acidobacteria subgroup Gp4 (OTU788)	2010	0 b	0.83 a	
Flavobacteriaceae	*Flavobacterium* (OTU258)	2010	6.05 b	18 a	
	***Flavobacterium* (OTU367)**	**2010**	**5.1 b**	**15.48 a**	
Gemmatimonadaceae	*Gemmatimonas* (OTU107)	2010	0.36 b	1.69 a	
	Gemmatimonas (OTU1105)	2010	1.02 b	4.06 a	

[a] Except for the order *Sphingobacteriales*.
[b] An asterisk indicates that the sequences from the 2008 and 2010 samples shared 100% identity. Sequences were clustered into OTUs at the 3% dissimilarity level. For genera shown in boldface, the abundance was confirmed by qPCR.
[c] Values followed by different letters in the same row were significantly different, according to Tukey's test ($P \leq 0.05$). The values are average frequencies, from three replicates, of the sequence per 1,000 reads.

for each sample, and these were assembled into 697 OTUs. Among all the phyla in 2008, *Proteobacteria* was the most abundant taxonomic group, followed by *Bacteroidetes*, *Actinobacteria*, and *Acidobacteria* (Fig. 1). In 2010, the most abundant taxonomic group was *Actinobacteria*, followed by *Proteobacteria* and *Bacteroidetes* (Fig. 2).

The diversity of bacterial communities was determined based on the Shannon diversity index, along with richness, evenness, and abundance (Table 1). In 2008, the diversity was higher in the bulk soil than in the rhizosphere soil from inside the patches, but the abundance was higher in the rhizosphere soil. Within the bulk soil of 2008, the abundance was significantly lower in the recovered patches than in the other two locations. In 2010, there was no effect of location on bacterial diversity, evenness, or richness, but inside the patches, the abundance was higher in the rhizosphere than in the bulk soil.

The higher abundance levels of bacteria in the rhizosphere soil or in soil from different locations (inside the patch, outside the patch, and the recovered patch) were explored by comparing relative frequencies of all OTUs (Tables 2 and 3). We noted that a few taxa (*Flavobacterium*, *Pedobacter*, *Duganella*, *Acidobacteria* subgroup Gp4, and *Gemmatimonas*) included multiple OTUs. Each OTU may represent different species or strains, but they cannot be differentiated in the RDP databases. Table 2 lists bacterial taxa with higher abundances of bacterial OTUs in the rhizosphere soil than in bulk soil ($P \leq 0.05$) in 2008 and 2010 field samples (inside of patch, outside of patch, and recovered patch). *Pseudomonas*, *Flavobacterium*, *Variovorax*, *Burkholderia*, and *Pedobacter* showed

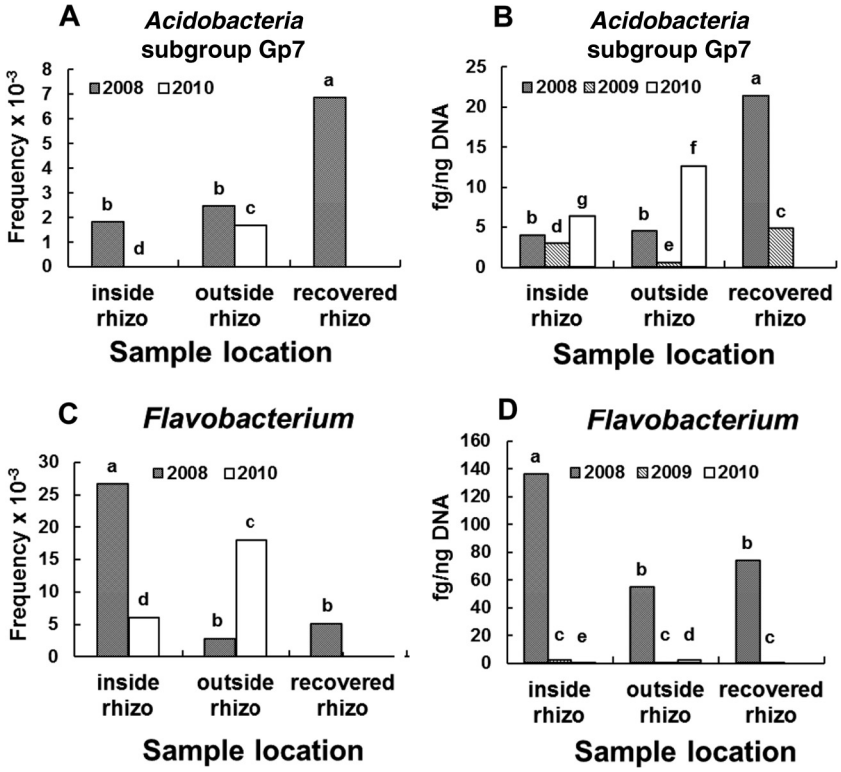

FIG 3 Frequencies of bacterial species in the rhizosphere soil of three locations (inside and outside the patches and in recovered patches) in 2 or 3 years (2008 and 2010, and also 2009 for some species). (A) Frequency of *Acidobacteria* subgroup Gp7, measured by pyrosequencing; (B) amount of *Acidobacteria* subgroup Gp7, measured by qPCR; (C) frequency of *Flavobacterium* sp., measured by pyroseqencing; (D) amount of *Flavobacterium* sp., measured by qPCR. Bars with different letters in the same year indicate statistically significant differences according to Tukey's test ($P \leq 0.05$). The values are means of three replicates.

a similar trend of a significantly higher frequency in the rhizosphere soil than in the bulk soil in both years. However, a number of taxa, including *Microbacterium*, *Phyllobacterium*, and one in the *Sphingobacteriales* order, were significantly more abundant in the rhizosphere soil in 2008 only, and other taxa, such as a few genera in the *Oxalobacteraceae* family (*Duganella*, *Herminiimonas*, and *Herbaspirillum*), *Mesorhizobium*, *Ralstonia*, *Chitinophaga*, and *Gemmatimonas*, were more abundant in the rhizosphere soil in 2010 only (Table 2).

Table 3 lists the bacterial distributions in the rhizosphere soil that were affected by sampling location. Taxa within *Chitinophaga*, *Variovorax*, and *Pedobacter* were detected at greater abundance inside the patches in both years. Other bacteria, such as from taxa within *Dyadobacter*, *Flavobacterium*, *Microbacterium*, *Acinetobacter*, and *Phyllobacterium*, were more abundant inside the patches in 2008 only. *Chryseobacterium*, *Oxalobacteraceae* (*Dunganella* and *Massilia*), *Pseudomonas*, and *Mucilaginibacter* were more abundant inside the patches in 2010. On the other hand, *Acidobacteria* subgroup Gp3, *Acidobacteria* subgroup Gp7, *Burkholderia*, *Mesorhizobium*, and *Dyella* were observed at higher frequencies outside the patches in both years and in recovered patches in 2008 (Table 3). Some bacteria, such as *Acidobacteria* subgroup Gp1 and *Actinobacteria*, were more abundant outside the patches in 2008. *Acidobacteria* subgroup Gp4, *Flavobacterium*, and *Gemmatimonas* were abundant outside the patches in 2010 (Table 3).

Quantitative PCR. In order to validate the results of the pyrosequencing, the abundance of two bacterial species (*Flavobacterium* and *Acidobacteria* subgroup Gp7) were determined with qPCR in rhizosphere soil DNA extracts from 2008, 2009, and 2010 field samples. *Flavobacterium* and *Acidobacteria* subgroup Gp7 were selected because *Flavobacterium* was abundant in the rhizosphere soil and *Acidobacteria* subgroup Gp7 was abundant outside the patches and in the recovered patches. The results showed that the DNA concentration of *Acidobacteria* subgroup Gp7 was higher in recovered patches in 2008 and 2009 and outside the patches in 2010 (Fig. 3B), confirming the trend found with the pyrosequencing data (Fig. 3A), but the DNA concentration was lower outside the patches in 2009. For *Flavobacterium*, the highest concentration of DNA was found inside the patches in 2008, while the highest concentration of DNA of *Flavobacterium* shifted to outside the patches in 2010 (Fig. 3D), which was also consistent with the pyrosequencing frequency results (Fig. 3C). The DNA concentration of *Flavobacterium* in 2009 was not significantly different among samples from inside the patch, outside the patch, and the recovered patch (Fig. 3D).

Greenhouse cycling experiments. To duplicate bacterial shifts in the rhizosphere soil in field samples, cycling experiments were conducted in the greenhouse to mimic field treatments, as described in Materials and Methods. After the first cycle, severe wheat stunting was observed in pots with AG-8 inoculum compared with the control pots without AG-8 inoculum. After the second cycle, mild wheat stunting was still observed in pots with AG-8 inoculum. However, after the third cycle, wheat in the pots with AG-8 inoculum no longer showed stunting, similar to wheat growth in the control pots (data not shown). Similar disease suppression phenomena were observed in previous greenhouse tests

TABLE 4 Frequencies of bacterial taxa in rhizospheres of field samples versus greenhouse cycling experiment samples

Family	Genus	Frequency ($\times 10^{-3}$) of genus in[a]:				
		Greenhouse samples			Field samples (in 2010)	
		Cycle1	Cycle2	Cycle3	Inside the patch	Outside the patch
Oxalobacteraceae	*Massilia* (OTU288)	1.21 c	5.50 b	14.89 a	2.40 a	0.20 b
	Duganella (OTU4)	1.14 c	22.89 b	32.36 a	12.32 a	4.49 b
	Herbaspirillum (OTU94)	1.14 b	5.88 b	72.82 a	7.27 a	0 b
Flavobacteriaceae	*Chryseobacterium* (OTU437)	0 c	65.54 a	15.21 b	3.64 a	0.23 b
Chitinophagaceae	*Chitinophaga* (OTU38)	2.50 b	7.50 a	5.60 a	4.88 a	1.17 b
Sphingobacteriaceae	*Pedobacter* (OTU380)	3.85 b	11.86 a	3.27 b	3.02 a	1.88 b
Pseudomonadaceae	*Pseudomonas* (OTU9)	1.71 b	6.16 a	5.64 a	12.07 a	2.88 b

[a] Values followed by different letters in the same row were significantly different for greenhouse samples versus field samples according to Tukey's test ($P \leq 0.05$). The values are average frequencies, from three replicates, of the sequence per 1,000 reads.

(18). This suggested that soils in the pots with AG-8 inocula had become suppressive after 3 cycles of wheat growth in the greenhouse. To monitor the bacterial community changes, pyrosequencing was conducted to characterize bacterial organisms in the rhizosphere soil after each cycle. A few genera (*Massilia* [OTU288], *Duganella*, and *Herbaspirillum* [OTU94] in the family *Oxalobacteraceae*) became more abundant in the rhizosphere soil with increasing cycles of wheat seedling growth (Table 4). A number of other taxa, including *Chryseobacterium* (OTU437), *Chitinophaga*, *Pedobacter*, and *Pseudomonas* (OTU9), increased dramatically in the second cycle, and then in the third cycle they decreased a little or to the level of the first cycle. Interestingly, all bacteria mentioned above showed significantly higher abundance in the rhizosphere soils inside the patches than outside the patches (Table 4). Furthermore, a strong correlation was observed between qPCR and pyrosequencing results for *Flavobacterium* (Fig. 4A and B).

Antifungal capabilities of bacteria *in vitro*. To identify bacterial taxa that may be involved in soils naturally suppressive to *R. solani*, 48 bacterial isolates were obtained from the rhizosphere soil from inside the patches that represented the most abundant colony types. In dual culture tests, six isolates significantly inhibited the radial mycelial growth of AG-8 (Table 5). The percent inhibition of radial growth (PIRG) values ranged from 34.4% to 59.3%. The highest PIRG value (59.3%) was observed with isolate 38, and the lowest (34.4%) was recorded with isolate 44 (Fig. 5B to G and Table 5). These six isolates' identities were determined from the 16S rRNA sequences (Table 5). Three isolates (isolates 31, 37, and 38) were considered the same species (*Chryseobacterium soldanellicola*) because their 16S rRNA sequences showed 100% identity, similar antagonistic activity on petri dishes, and similar gliding motility on the ¼ PDA medium (Fig. 5C, D, and E). These isolates displayed 100% sequence identity to OTU437 (*Chryseobacterium*) from the field samples and cycling experiments. Isolate 43 was also identified as *C. soldanellicola* according to the 16S rRNA sequence, but the gliding motility on the ¼ PDA medium was different from the above three (Fig. 5F). The other two were *Pseudomonas* (isolate 25) and *Pedobacter* (isolate 44), which displayed 100% sequence identities to OTU9 (*Pseudomonas*) and OTU26 (*Pedobacter*), respectively (Table 5). The remaining 42 bacterial isolates did not exhibit significant inhibition of the radial growth of AG-8.

Biocontrol capabilities of bacterial isolates in greenhouse assays. All six antagonists (isolates 25, 31, 37, 38, 43, and 44) were tested to determine their biological control capability against AG-8 in the greenhouse. In the natural soil (nonpasteurized), treatments with three *C. soldanellicola* isolates (isolates 31, 37, and 38) significantly reduced disease caused by AG-8 (Table 6). The root rot rating decreased and root length increased in treated soils compared with the nonpasteurized AG-8-inoculated control. But *Pseudomonas* sp. (isolate 25), *Chryseobacterium* (isolate 43), and *Pedobacter* sp. (isolate 44) did not show significant disease reductions in the greenhouse in natural soil. Treatment with *Pseudomonas* sp. slightly increased disease severity (Table 6). In pasteurized soil, the root rot rating for treatments of *C. soldanellicola* (isolate 43) and *Pseudomonas* sp. isolates decreased slightly compared to the AG-8-inoculated control. *C. soldanellicola* (isolates 38 and 43) increased the root

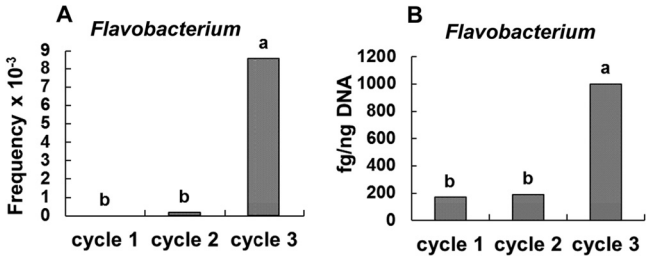

FIG 4 Bacterial frequencies in the rhizosphere soil in the cycling experiment in the greenhouse. (A) Frequency of *Flavobacterium* sp., measured by pyrosequencing; (B) amount of *Flavobacterium* sp., measured by qPCR. Bars with different letters indicate statistically significant differences according to Tukey's test ($P \leq 0.05$). The values are means of three replicates.

TABLE 5 Inhibition of radial growth of *R. solani* in dual culture by selected bacteria

Isolate ID no.	Genus[a]	% inhibition of radial growth[b]
Control	*R. solani* AG-8	0 c
25	*Pseudomonas* sp. (OTU9)	39.9 ± 7.7 ab
31	*Chryseobacterium soldanellicola* (OTU437)	53.1 ± 13.6 ab
37	*Chryseobacterium soldanellicola* (OTU437)	56.0 ± 20.3 ab
38	*Chryseobacterium soldanellicola* (OTU437)	59.3 ± 15.0 a
43	*Chryseobacterium soldanellicola* (OTU18)	54.7 ± 22.7 ab
44	*Pedobacter* sp. (OTU26)	34.4 ± 9.7 b

[a] Identified by 16S rRNA sequencing.

[b] The values followed by different letters within a row were significantly different according to Tukey's test ($P \leq 0.05$). The values are means of three replicates. The experiments were repeated three times with similar results.

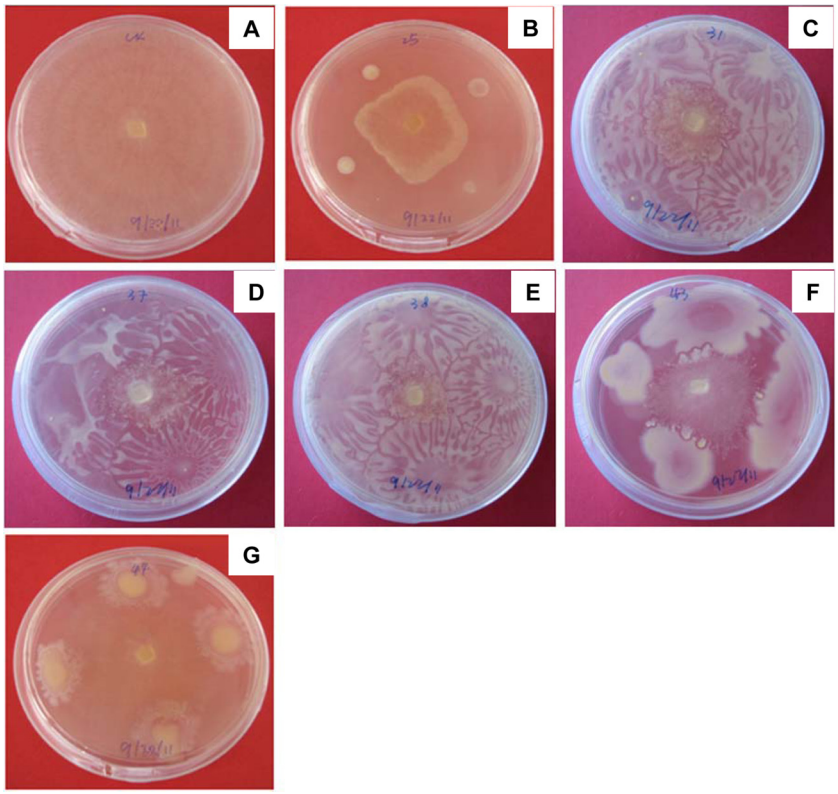

FIG 5 Dual culture assays for *in vitro* inhibition of growth of *R. solani* AG-8 by bacterial isolates on ¼ PDA medium 7 days after incubation. (A) Control (only AG-8); (B) *Pseudomonas* sp. (isolate 25) and *R. solani* AG-8; (C) *C. soldanellicola* (isolate 31) and *R. solani* AG-8; (D) *C. soldanellicola* (isolate 37) and *R. solani* AG-8; (E) *C. soldanellicola* (isolate 38) and *R. solani* AG-8; (F) *C. soldanellicola* (isolate 43) and *R. solani* AG-8; (G) *Pedobacter* sp. (isolate 44) and *R. solani* AG-8.

length (Table 6). However, treatments with two *C. soldanellicola* isolates (isolate 31 or 37) in pasteurized soil did not result in significant suppression of disease. The plants in the soil treated with only bacteria showed a similar phenotype as the control soil (without the AG-8 inoculum) (Table 6).

DISCUSSION

We have reported here the first detailed study of the bacterial communities associated with *Rhizoctonia* patch disease-induced decline and the first example of this phenomenon outside Australia (16). We found a number of taxonomic groups that were associated with the rhizosphere of diseased plants, with two predominating: the phylum *Bacteroidetes* (families *Sphingobacteriaceae* and *Flavobacteriaceae*) and the class *Betaproteobacteria* (family *Oxalobacteraceae*). In 2008, the most dominant genus was *Chitinophaga*, a member of the class *Sphingobacteria*, a gliding chitin-decomposing myxobacterium. It has been isolated from a number of soils in South Korea (39) and was the most dominant taxa in four soils from North and South America (40). Within the *Bacteroidetes*, we found the gliding bacteria *Flavobacterium* and *Chryseobacterium* were dominant in the rhizospheres of diseased plants. Previous work with soil from western Washington State reported that the abundance of *Chryseobacterium* and *Flavobacterium* increased significantly in the rhizosphere of take-all disease-affected plants (41). This suggests that both root pathogens (*Gaeumannomyces graminis* var. *tritici* and *Rhizoctonia solani*) promote similar bacteria, possibly from exudates leaking from diseased roots. However, *Chryseobacterium* isolates displayed no ability to inhibit *Gaeumannomyces graminis* var. *tritici in vitro* and did not interfere with the antagonism caused by an isolate of *Pseudomonas* antagonistic to take-all disease (42). In this study, we were able to isolate a number of strains of *Flavobacterium* and *Chryseobacterium* from the rhizosphere soils of diseased plants and demonstrated that three isolates (isolate 31, 37, and 38) of *Chryseobacterium* could inhibit *R. solani in vitro* and reduce *Rhizoctonia* patch disease in inoculated natural soil but not in pasteurized soil in greenhouse experiments (Tables 5 and 6). It is possible that these isolates may interact with other microorganisms in the natural soil and suppress *Rhizoctonia* patch disease. Benítez and Gardener (29) successfully used sequence-based molecular markers (terminal restriction fragment length polymorphisms) to isolate novel bacterial genera, *Mitsuaria* and *Burkholderia*, which were involved in the suppression of damping-off disease pathogens. Our current results further supported that identification and isolation of functionally important microbes from diverse microbial communities by use of sequence-based molecular screening is a low-cost and powerful approach to develop sustainable disease management. Additionally, taxa as dominant communities in the rhizosphere of wheat, in association with phloroglucinol-producing *Pseudomonas fluorescens* genotypes, have been described (41, 42). In both studies, *Flavobacterium* isolates were identified by molecular techniques. In the mid-1990s, J. Raaijmakers and D. M. Weller (43) also found that *Flavobacterium* became a dominant component of the wheat rhizosphere after successive cycling or monocropping experiments in the greenhouse. *Flavobacterium* has been identified as a

TABLE 6 Effects of different treatments of bacterial cultures in greenhouse assays on the suppression of root rot of wheat plants caused by *R. solani* AG-8

Presence of *R. solani*	Treatment: Nonpasteurized soil	Treatment: Pasteurized soil	Bacterium (isolate no.)	Root rot rating[a]	Root length (mm)[a]
−	+		None	0.6 e	14.9 a
−	+		*Pseudomonas* sp. (25)	0.6 e	14.4 ab
−	+		*Chryseobacterium soldanellicola* (31)	1.0 e	14.4 ab
−	+		*Chryseobacterium soldanellicola* (37)	0.5 e	14.6 a
−	+		*Chryseobacterium soldanellicola* (38)	1.1 e	13.6 abc
+	+		None	6.4 ab	9.1 e
+	+		*Pseudomonas* sp. (25)	6.7 a	7.1 f
+	+		*Chryseobacterium soldanellicola* (31)	4.2 d	12.6 bcd
+	+		*Chryseobacterium soldanellicola* (37)	4.9 c	11.5 d
+	+		*Chryseobacterium soldanellicola* (38)	4.0 d	12.4 cd
+	+		*Chryseobacterium soldanellicola* (43)	5.8 b	11.5 d
+	+		*Pedobacter* sp. (44)	5.9 b	11.2 d
−		+	None	0.3 d	13.4 a
−		+	*Pseudomonas* sp. (25)	0.5 d	14.3 a
−		+	*Chryseobacterium soldanellicola* (31)	0.5 d	14.3 a
−		+	*Chryseobacterium soldanellicola* (37)	0.3 d	14.5 a
−		+	*Chryseobacterium soldanellicola* (38)	0.3 d	14.0 a
+		+	None	6.8 ab	8.7 cd
+		+	*Pseudomonas* sp. (25)	6.2 c	8.8 cd
+		+	*Chryseobacterium soldanellicola* (31)	6.9 a	7.7 d
+		+	*Chryseobacterium soldanellicola* (37)	6.5 bc	9.9 bc
+		+	*Chryseobacterium soldanellicola* (38)	6.4 bc	10.9 b
+		+	*Chryseobacterium soldanellicola* (43)	6.2 c	11.5 b
+		+	*Pedobacter* sp. (44)	6.7 abc	10.3 bc

[a] For each treatment, each datum is an average of results from 10 replicates. The experiments were repeated twice with similar results. Means with different letters were significantly different according to Fisher's LSD test ($P \leq 0.05$).

common component in the rhizospheres of barley (44), turfgrass (45), and tomatoes (46). There is extensive literature on this group in terms of bioremediation and biodegradation and for breakdown of mycotoxins. These bacteria are found in aquatic habitats, and some are fish pathogens. They have served as models for study of the molecular basis of gliding. There have also been some reports of *Flavobacterium* as a biocontrol agent for *Verticillium dahliae* (47), *Colletotrichum* (48), sunflower (*Helianthus annuus* L.) pathogens (49), and *Botrytis* (50).

In 2008, the total diversity was higher in the bulk soil than in the rhizosphere soil from inside the patches; this trend was not seen in 2010. However, inside the patches, bacterial abundance was higher in the rhizosphere than in the bulk soil for both years (Table 1). This suggested that there is a higher diversity of niches in the bulk soil but that the rhizosphere selects for a narrow diversity of taxa, with higher populations supported by the large amounts of exudates. In a study correlating biomass productivity with rhizosphere communities on wheat grown in raw soil, OTUs of *Duganella* and *Janthinobacterium*, two genera of the *Oxalobacteriaceae*, were positively correlated with plant biomass, along with *Pseudomonas* (51). OTUs of *Chryseobacterium* and *Enterobacterium* were negatively associated. However, those investigators did not look at pathogens in the system, and it is possible that pathogens caused the reduction in biomass and that these genera were associated with diseased roots, as we found in our study.

The rhizosphere of recovered or healthy patches may represent a more stable community with a higher diversity. We found *Acidobacteria* subgroup Gp7 and *Dyella* at higher frequencies in the recovered patches in 2008 (Table 3). *Dyella* was first described as a genus within the family *Xanthomonadaceae* when it was isolated from soil in 2005 (52) and subsequently from the rhizosphere of bamboo (53), from the nodules of *Lespedeza* sp. (54), and also as a seed-borne endophyte of rice (55). *Dyella japonica* was shown to comigrate with *Burkholderia* on the hyphae of *Lyophyllum*, a genus of edible mycorrhizum-forming mushrooms, in soil microcosms (56) and was selected in the mycosphere of the basidiomycetes. On the contrary, the rhizosphere of diseased plants had less diversity, as it was dominated by an abundance of copiotrophic, fast-growing bacteria that utilize the exudates leaking from infected roots. This concept was demonstrated in our cycling experiments in the greenhouse. The soils inoculated with AG-8 became suppressive after three cycles of wheat growth under direct seed planting conditions. Interestingly, we found that a few taxa of *Chryseobacterium* and also *Pseudomonas* became more prevalent in the rhizosphere soil with increasing cycles (Table 4). Compared to the microorganism community in the field samples, these taxa showed higher abundances in the rhizospheres of diseased plants. This indicated that our cycling experiments in the greenhouse could duplicate some taxonomic shifts in bacteria that operate at the field level. Kyselková et al. (28) found that taxa of *Pseudomonas* and *Bacteroidetes* were higher in soil suppressive to tobacco black rot caused by *Thielaviopsis basicola* when they used a microarray

based on 16S RNA, similar to our results. We further demonstrated that *Chryseobacterium* and *Pseudomonas* exhibited inhibitory activities against *R. solani in vitro* or reduced *Rhizoctonia* disease in inoculated natural soils (Table 5 and 6). These findings indicate that these organisms may play important roles in the transition of soil from conducive to suppressive conditions. Suppression of soilborne disease has been well documented for declines in take-all disease, in which *Pseudomonas* colonizes microsites on diseased roots and produces antibiotics that lead to disease suppression (41, 57, 58). *Pseudomonas* comprised 2 to 5% of sequences from the rhizosphere in our study. Phenazine-producing *Pseudomonas* has been found at high frequencies in samples taken at this location, as reported elsewhere (59). There are at least four groups of *Pseudomonas* that have been identified from phylogenetic studies of a number of genes (60), but the species cannot be distinguished by the pyrosequencing method used in our study. A few *Oxalobacteraceae* (*Massilia*, *Duganella*, and *Herbaspirillum*) and *Chitinophaga* also increased significantly in the rhizospheres of diseased plants in the field and with increasing cycles. Whether these taxa have similar inhibition abilities is unclear. It is possible that some of them may interact with *Chryseobacterium* to suppress *Rhizoctonia* patch disease. However, much more work needs to be done to address their functions. Additionally, *Pedobacter* and *Variovorax* were also common in the rhizosphere of wheat plants inside the patches in our study (Table 3), the latter comprising 1 to 5% of the sequences.

In conclusion, we have identified members of a highly complex bacterial community that may be involved in the suppression of *Rhizoctonia* bare patch and root rot disease of wheat, as documented in a 14-year study (17). If early shifts of community structure are indicative of the later development of suppression, such shifts can be used as a tool to test crop rotations and cultural techniques that may enhance and hasten the development of disease suppression. Can this suppression develop faster than in 5 to 7 years? What are the implications for wheat growers? Better knowledge of this phenomenon may result in recommendations for cultural practices and crop rotations that will enhance this natural disease suppression. Once it is established, growers will not need to provide any chemical inputs, or they can rely on resistant cultivars, which have eluded plant breeders. This may provide an impetus for more growers to adapt direct seed or no-till conservation agriculture methods, reducing the loss of the valuable soil resource.

ACKNOWLEDGMENTS

This work was supported by Washington State University, the USDA Agricultural Research Service, the Columbia Plateau PM_{10} Project, and the Solutions to Economic and Environmental Problems (STEEP) Project.

We thank Suresh Iyer and Matt Settles at the University of Idaho and Qingsheng Zhang for pyrosequence and data analysis. We gratefully acknowledge wheat grower Ron Jirava for providing the field site and Washington State University agricultural research technicians Harry Schafer, Timothy Smith, and Steven Schofstoll for their excellent technical assistance.

REFERENCES

1. **Pumphrey FV, Wilkins DE, Hane DC, Smiley RW.** 1987. Influence of tillage and nitrogen fertilizer on Rhizoctonia root rot (bare patch) of winter wheat. Plant Dis. **71:**125–127.
2. **Samuel G, Garrett SD.** 1932. *Rhizoctonia solani* on cereals in South Australia. Phytopathology **22:**827–836.
3. **Weller DM, Cook RJ, MacNish G, Bassett EN, Powelson RL, Petersen RR.** 1986. Rhizoctonia root rot of small grains favored by reduced tillage in the Pacific Northwest. Plant Dis. **70:**70–73.
4. **Smith JD, Kidwell KK, Evans MA, Cook RJ, Smiley RW.** 2003. Evaluation of spring cereal grains and wild *Triticum* germplasm for resistance to *Rhizoctonia solani* AG-8. Crop Sci. **43:**701–709.
5. **Mac Nish GC.** 1985. Methods of reducing rhizoctonia patch of cereals in Western Australia. Plant Pathol. (Lond.) **34:**175–181.
6. **Cook RJ, Weller DM, Youssef El-Banna A, Vakoch D, Zhang H.** 2002. Yield responses of direct-seeded wheat to rhizobacteria and fungicide seed treatments. Plant Dis. **86:**780–784.
7. **Paulitz TC, Smiley R, Cook RJ.** 2002. Insights into the prevalence and management of soilborne cereal pathogens under direct seeding in the Pacific Northwest, USA. Can. J. Plant Pathol. **24:**416–428.
8. **Bolton MD, Panella L, Campbell L, Khan MF.** 2010. Temperature, moisture, and fungicide effects in managing Rhizoctonia root and crown rot of sugar beet. Phytopathology **100:**689–697.
9. **Paulitz TC, Scott R.** 2006. Effect of seed treatments for control of Rhizoctonia root rot in spring wheat, 2005. Fungicide Nematicide Tests **61:** ST014.
10. **Babiker EM, Hulbert SH, Schroeder KL, Paulitz TC.** 2011. Optimum timing of preplant applications of glyphosate to manage Rhizoctonia root rot in barley. Plant Dis. **95:**304–310.
11. **Weller DM, Raaijmakers JM, McSpadden Gardener BB, Thomashow LS.** 2002. Microbial populations responsible for specific soil suppressiveness to plant pathogens. Annu. Rev. Phytopathol. **40:**309–348.
12. **Lorang JM, Liu D, Anderson NA, Schottel JL.** 1995. Identification of potato scab inducing and suppressive species of *Streptomyces*. Phytopathology **85:**261–268.
13. **Alabouvette C.** 1990. Biological control of Fusarium wilt pathogens in suppressive soils, p 27–43. *In* Hornby D (ed), Biological control of soilborne plant pathogens. CAB International, Wallingford, United Kingdom.
14. **Stutz EW, Defago G, Kern H.** 1986. Natural occurring fluorescent pseudomonads involved in suppression of black root rot of tobacco. Phytopathology **76:**181–185.
15. **MacNish GC.** 1988. Changes in take-all (*Gaeumannomyces graminis* var. *tritici*), rhizoctonia root rot (*Rhizoctonia solani*) and soil pH in continuous wheat with annual applications of nitrogenous fertilizer in Western Australia. Aust. J. Exp. Agric. **28:**333–341.
16. **Roget DK.** 1995. Decline in root rot (*Rhizoctonia solani* AG-8) in wheat in a tillage and rotation experiment at Avon, South Australia. Aust. J. Exp. Agric. **35:**1009–1013.
17. **Schillinger WF, Paulitz TC.** Natural suppression of Rhizoctonia bare patch in a long-term no-till cropping systems experiment. Plant Dis., in press.
18. **Paulitz TC, Schillinger WF, Cook RJ.** 2003. Greenhouse studies of Rhizoctonia bare patch disease in soil cores from direct seeded fields. Am. Soc. Agron. Annu. Meet., ASA, CSSA, and SSSA Abstr., Denver, CO. American Society of Agronomy, Madison, WI.
19. **Gupta VVSR, Neate SM.** 1999. Root disease incidence: a simple phenomenon or a product of diverse microbial/biological interactions, p 3–4. *In* Magarey RC (ed), Proceedings of the First Australasian Soilborne Disease Symposium. BSES, Brisbane, Australia.
20. **Gupta VVSR, Neate SM, Dumitrescu I.** 1999. Effects of microfauna and mesofauna on *Rhizoctonia solani* in a South Australian soil, p 134–136. *In* Magarey RC (ed), Proceedings of the First Australasian Soilborne Disease Symposium. BSES, Brisbane, Australia.
21. **Neate SM, Gupta VVSR.** 1999. Assessment of differences in soil biota and microbiological processes in diseases suppressive and non-suppressive soils: an experimental approach, p 131–133. *In* Magarey RC (ed), Proceedings of the First Australasian Soilborne Disease Symposium. BSES, Brisbane, Australia.
22. **Roget DK, Coppi JA, Herdina, Gupta VVSR.** 1999. Assessment of suppression to *Rhizoctonia solani* in a range of soils across SE Australia, p 129–130. *In* Magarey RC (ed), Proceedings of the First Australasian Soilborne Disease Symposium. BSES, Brisbane, Australia.
23. **Roget DK, Gupta VVSR.** 2006. Rhizoctonia control through management of disease suppressive activity in soils, p 172. Proc. 18th World Congr. Soil Sci., Philadelphia, PA. International Union of Soil Sciences, Wageningen, Netherlands.
24. **van Elsas JD, Garbeva P, Salles J.** 2002. Effects of agronomical measures

on the microbial diversity of soils as related to the suppression of soilborne plant pathogens. Biodegradation **13:**29–40.
25. **Borneman J, Becker JO.** 2007. Identifying microorganisms involved in specific pathogen suppression in soil. Annu. Rev. Phytopathol. **45:**153–172.
26. **Raaijmakers JM, Paulitz TC, Steinberg C, Alabouvette C, Moënne-Loccoz Y.** 2009. The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. Plant Soil **321:**341–361.
27. **Adesina MF, Grosch R, Lembke A, Vatchev TD, Smalla K.** 2009. In vitro antagonists of *Rhizoctonia solani* tested on lettuce: rhizosphere competence, biocontrol efficiency and rhizosphere microbial community response. FEMS Microbiol. Ecol. **69:**62–74.
28. **Kyselková M, Kopecký J, Frapolli M, Défago G, Ságová-Marecková M, Grundmann GL, Moënne-Loccoz Y.** 2009. Comparison of rhizobacterial community composition in soil suppressive or conducive to tobacco black root rot disease. ISME J. **3:**1127–1138.
29. **Benítez MS, Gardener BB.** 2009. Linking sequence to function in soil bacteria: sequence-directed isolation of novel bacteria contributing to soilborne plant disease suppression. Appl. Environ. Microbiol. **75:**915–924.
30. **Jung WJ, Park RD, Mabood F, Souleimanov A, Smith D.** 2011. Effects of *Pseudomonas aureofaciens* 63-28 on defense responses in soybean plants infected by *Rhizoctonia solani*. J. Microbiol. Biotechnol. **21:**379–386.
31. **Barnett SJ, Roget DK, Ryder MH.** 2006. Suppression of *Rhizoctonia solani* AG-8 induced disease on wheat by the interaction between *Pantoea*, *Exiguobacterium*, and *Microbacteria*. Aust. J. Soil Res. **44:**331–342.
32. **Mendes R, Kruijt M, de Bruijn I, Dekkers E, van der Voort M, Schneider HM, Piceno YM, DeSantis TZ, Andersen GL, Bakker PA, Raaijmakers JM.** 2011. Deciphering the rhizosphere microbiome for disease-suppressive bacteria. Science **332:**1097–1100.
33. **Schillinger WF, Paulitz TC.** 2006. Reduction of Rhizoctonia bare patch in wheat with barley rotations. Plant Dis. **90:**302–306.
34. **Yin CT, Jones KL, Peterson DE, Garrett KA, Hulbert SH, Paulitz TC.** 2010. Members of soil bacterial communities sensitive to tillage and crop rotation. Soil Biol. Biochem. **42:**2111–2118.
35. **Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K.** 2002. Short protocols in molecular biology, 5th ed. John Wiley & Sons, New York, NY.
36. **Weisburg WG, Barns SM, Pelletier DA, Lane DJ.** 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. **173:**697–703.
37. **Fokkema NJ.** 1978. Fungal antagonism in the phylosphere. Ann. Appl. Biol. **89:**115–117.
38. **Kim DS, Cook RJ, Weller DM.** 1997. *Bacillus* sp. L324-92 for biological control of three root diseases of wheat grown with reduced tillage. Phytopathology **87:**551–558.
39. **Lee HG, An DS, Im WT, Liu QM, Na JR, Cho DH, Jin CW, Lee ST, Yang DC.** 2007. *Chitinophaga ginsengisegetis* sp. nov. and *Chitinophaga ginsengisoli* sp. nov., isolated from soil of a ginseng field in South Korea. Int. J. Syst. Evol. Microbiol. **57:**1396–1401.
40. **Fulthorpe RR, Roesch LFW, Riva A, Triplett EW.** 2008. Distantly sampled soils carry few species in common. ISME J. **2:**901–910.
41. **McSpadden Gardener BB, Weller DM.** 2001. Changes in populations of rhizosphere bacteria associated with take-all disease of wheat. Appl. Environ. Microbiol. **67:**4414–4425.
42. **Landa BB, Mavrodi DM, Thomashow LS, Weller DM.** 2003. Interactions between strains of 2,4-diacetylphloroglucinol-producing *Pseudomonas fluorescens* in the rhizosphere of wheat. Phytopathology **93:**682–994.
43. **Raaijmakers JM, Weller DM.** 1998. Natural plant protection by 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp. in take-all decline soils. Mol. Plant Microbe Interact. **11:**144–152.
44. **Johansen JE, Nieldsen P, Binnerup SJ.** 2009. Identification and potential enzyme capacity of flavobacteria isolated from the rhizosphere of barley (*Hordeum vulgare* L.). Can. J. Microbiol. **55:**234–241.
45. **Elliott ML, McInroy JA, Xiong K, Kim JH, Skipper HD, Guertal EA.** 2008. Taxonomic diversity of rhizosphere bacteria in golf course putting greens at representative sites in the southeastern United States. HortScience **43:**514–518.
46. **Kim JS, Dungan RS, Kwon SW, Weon HY.** 2006. The community composition of root-associated bacteria of the tomato plant. World J. Microbiol. Biotechnol. **22:**1267–1273.
47. **Berg G.** 1996. Rhizobacteria of oilseed rape antagonistic to*Verticillium dahliae* var. *longisporum* Stark. J. Plant Dis. Prot. **103:**20–30.
48. **Gunasinghe WKRN, Karunaratne AM.** 2009. Interactions of *Colletotrichum musae* and *Lasiodiplodia theobromae* and their biocontrol by *Pantoea agglomerans* and *Flavobacterium* sp. in expression of crown rot of "Embul" banana. Biocontrol **54:**587–596.
49. **Hebbar P, Berge O, Heulin T, Singh SP.** 1991. Bacterial antagonists of sunflower (*Helianthus annuus* L.) fungal pathogens. Plant Soil **133:**131–140.
50. **Moline H, Hubbard JE, Karns JS, Buyer JS, Cohen JD.** 1999. Selective isolation of bacterial antagonists of *Botrytis cinerea*. Eur. J. Plant Pathol. **105:**95–101.
51. **Anderson M, Habiger J.** 2012. Characterization and identification of productivity-associated rhizobacteria in wheat. Appl. Environ. Microbiol. **78:**4434–4446.
52. **Xie CH, Yokato A.** 2005. *Dyella japonica* gen. nov., sp. nov., a gammaproteobacterium isolated from soil. Int. J. Syst. Evol. Microbiol. **55:**753–756.
53. **An DS, Im WT, Yang HC, Yang DC, Lee ST.** 2005. *Dyella koreensis* sp. nov., a beta-glucosidase-producing bacterium. Int. J. Syst. Evol. Microbiol. **55:**1625–1628.
54. **Palaniappan P, Chauhan PS, Saravanan VS, Anandham R, Sa TM.** 2010. Isolation and characterization of plant growth promoting endophytic bacterial isolates from root nodule of *Lespedeza* sp. Biol. Fertil. Soils **46:**807–816.
55. **Hardoim PR, Hardoim CCP, van Overbeek LS, van Elsas JD.** 2012. Dynamics of seed-borne rice endophytes on early plant growth stages. PLoS One **7**(2)**:**e30438. doi:10.1371/journal.pone.0030438.
56. **Warmink JA, van Elsas JD.** 2009. Migratory response of soil bacteria to *Lycophyllum* sp. strain Karsten in soil microcosms. Appl. Environ. Microbiol. **7:**2820–2830.
57. **Weller DM.** 1983. Colonization of wheat roots by a fluorescent pseudomonad suppressive to take-all. Phytopathology **73:**1548–1553.
58. **Weller DM.** 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annu. Rev. Phytopathol. **26:**379–407.
59. **Mavrodi DV, Mavrodi OV, Parejko JA, Bonsall RF, Kwak YS, Paulitz TC, Thomashow LS, Weller DM.** 2012. Accumulation of the antibiotic phenazine-1-carboxylic acid in the rhizosphere of dryland cereals. Appl. Environ. Microbiol. **78:**804–812.
60. **Parejko JA, Mavrodi DV, Mavrodi OV, Weller DM, Thomashow LS.** 2012. Population structure and diversity of phenazine-1-carboxylic acid producing fluorescent *Pseudomonas* spp. from dryland cereal fields of central Washington State USA. Microb. Ecol. **64:**226–241.