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Linking Sequence to Function in Soil Bacteria: Sequence-Directed Isolation of Novel Bacteria Contributing to Soilborne Plant Disease Suppression[▽]†

María-Soledad Benítez and Brian B. McSpadden Gardener*

Department of Plant Pathology, The Ohio State University, OARDC, 1680 Madison Avenue, Wooster, Ohio 44691

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Microbial community profiling of samples differing in a specific ecological function, i.e., soilborne plant disease suppression, can be used to mark, recover, and ultimately identify the bacteria responsible for that specific function. Previously, several terminal restriction fragments (TRF) of 16S rRNA genes were statistically associated with damping-off disease suppression. This work presents the development of sequence-based TRF length polymorphism (T-RFLP)-derived molecular markers to direct the identification and isolation of novel bacteria involved in damping-off pathogen suppression. Multiple sequences matching TRF M139 and M141 were cloned and displayed identity to multiple database entries in the genera incertae sedis of the *Burkholderiales*. Sequences matching TRF M148, in contrast, displayed greater sequence diversity. A sequence-directed culturing strategy was developed using M139- and M141-derived markers and media reported to be selective for the genera identified within this group. Using this approach, we isolated and identified novel *Mitsuaria* and *Burkholderia* species with high levels of sequence similarity to the targeted M139 and M141 TRF, respectively. As predicted, these *Mitsuaria* and *Burkholderia* isolates displayed the targeted function by reducing fungal and oomycete plant pathogen growth in vitro and reducing disease severity in infected tomato and soybean seedlings. This work represents the first successful example of the use of T-RFLP-derived markers to direct the isolation of microbes with pathogen-suppressing activities, and it establishes the power of low-cost molecular screening to identify and direct the recovery of functionally important microbes, such as these novel biocontrol strains.

Understanding the associations between microbial population structure and ecosystem functions is important and relevant to the application and use of microorganisms for medical, agricultural, environmental, and industrial purposes (17, 34, 51, 53). Multiple approaches can be used to describe such associations, ranging from genomic comparative studies (e.g., see reference 19) to culture-based screens for specific activities (e.g., see references 1 and 39). The identification of microorganisms associated with plant pathogen suppression is important for the development of sustainable disease management strategies that employ natural or inoculated biocontrols (10, 36). The microbial basis for plant pathogen suppression has been well established (5), and the components of suppressiveness have been described for multiple pathosystems, especially for those involving a specific pathogen and microbial antagonist (10, 54). Uncovering microorganisms associated with general soilborne-disease suppression, i.e., that mediated by the activities of multiple microbial populations, is more challenging. Historically, the approach has been to recover bacteria and then screen them for activity. While this "recover and then identify" approach to finding biocontrol agents has been successful, most of the isolates recovered belong to a limited set of genera. To overcome this limitation and expand our understanding of the diversity of pathogen antagonists present in nature, we have developed and applied culture-independent molecular tools to "first identify and then recover" important biocontrol bacteria.

Broadly speaking, there are two distinct culture-independent approaches that can be followed to rapidly discover functionally important microbes, such as biocontrol agents. The first approach is to use genetic markers for a functionally important activity, such as antibiosis. The theory behind this approach is that natural variation in such marker genes will reveal concomitant natural variation in the functional activity. By isolating a diverse set of genetic variants, one can identify strains or subspecies with various capacities to colonize plant roots and/or suppress pathogens. This approach has been used to identify and recover novel genotypes of 2,4-diacetylphloroglucinol producers from the rhizosphere of field-grown crop plants (37). In the absence of knowledge about the mechanisms involved in biocontrol, PCR-based suppressive-subtractive hybridization can be used to identify new markers (26). The second approach is based on molecular profiling of microbial population structure, an approach sometimes referred to as microbial community profiling. In this approach, ribosomal gene sequences are targeted, amplified from the rhizosphere environment, and analyzed (9). Low-cost, low-resolution techniques, such as terminal restriction fragment (TRF) length polymorphism (T-RFLP) analyses, provide a cost-effective approach to finding generalist populations that consistently contribute to suppression across environments.

* Corresponding author. Mailing address: Department of Plant Pathology, The Ohio State University, OARDC, 1680 Madison Avenue, Wooster, OH 44691. Phone: (330) 202-3565. Fax: (330) 263-3841. E-mail: mcspadden-garden.1@osu.edu.

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Recently, we applied T-RFLP analyses to compare the bacterial community structure in soils differing in their disease-suppressive capacity. We first identified a series of soils that differed in their capacity to suppress endogenous and introduced oomycete pathogens that caused damping-off of tomatoes and soybeans (7). The damping-off suppressiveness had been induced by mixed-hay cropping and was durable, occurring over various greenhouse and field conditions. Following guidelines similar to those described by Borneman et al. (9), a population-based approach was used to correlate the abundance of microbial populations with the damping-off suppression differential. For this, T-RFLP was used to screen for microbial components that statistically associated with the observed damping-off suppression (8). Multiple bacterial populations (marked by different TRFs) revealed a positive association with disease suppression on both crops and across experiments. These TRFs were more abundant in disease-suppressive systems; however, they were not absent in the more-conducive soils. And, as a group, these TRFs showed a significantly greater proportion of negative correlations between TRF relative abundance and damping-off severity than TRFs associated with the more-conducive contexts. From that work, we hypothesized that the bacteria giving rise to the TRFs associated with damping-off suppression were generally able to contribute to the observed suppression and that general soilborne-disease suppression was at work in the system studied.

Based on our laboratory's previous work (7, 8), the primary aims of this study were to (i) identify bacterial sequences giving rise to TRFs previously associated with disease suppression (i.e., MspI-generated TRFs of 139, 141, and 148 nucleotides [nt]), (ii) obtain multiple isolates of those bacteria with corresponding TRF sizes and sequences, and (iii) characterize the pathogen-suppressive capacities of those isolates to further corroborate their association with general disease suppression in the system studied. The use of a culture-independent approach based on community profiling, such as T-RFLP and sequencing of individual TRFs, allowed for the development of a marker-assisted isolation strategy that targeted novel microorganisms associated with damping-off suppression. The success of that approach, detailed here, demonstrates the utility of microbial community profiling for rapidly identifying and recovering functionally important microbes, such as those that suppress soilborne pathogens, in a directed fashion.

MATERIALS AND METHODS

Cloning of MspI-generated 16S rRNA gene TRFs. The procedure for cloning and sequencing TRFs was modified from that of Widmer et al. (55). The 16S rRNA gene was amplified and digested with MspI (Promega) from multiple tomato and soybean soil and rhizosphere DNA samples. Individual samples were chosen for amplification from those described previously (8) based on high peak intensity (relative fluorescence) of the TRFs of interest, regardless of their origin, to maximize their recovery. As described previously, the target TRFs were on average less abundant but not absent under the more-disease-conducive treatment conditions. A double-stranded asymmetric adapter was ligated into the MspI site of the TRF. A concentration of 5 μM of MspI adapters 1 (5′-CGGT ACTCAGGACTCAT-3′) and 2 (5′-GACGATGAGTCCTGAGTAC-3′) was added to 1× buffer C (Promega) and incubated for 10 min at 65°C, 10 min at 37°C, 10 min at 25°C, and 10 min at 4°C. For ligation, 2 μl of the digested amplicon was mixed with 1 μl double-stranded adapter, 4.5 U T4 ligase (Promega), and 1× ligase buffer (Promega) in a 10-μl reaction mixture. The reaction mixture was incubated for 12 h at 16°C. Following ligation, TRFs were size selected from a portion of the agarose gel corresponding to 90 to 160 bp in length and purified by using an UltraClean GelSpin DNA purification kit (MoBio). The purified DNA was used to enrich the samples with 16S rRNA gene TRFs of the target sizes. PCR was performed using 16S primer 8F (5′-AGAGTTTGATCC TGGCTCAG-3′) in combination with MspI adapter primer (5′-GATGAGTCC TGAGTACCG-3′) (55). Amplification was carried out in 25-μl reaction mixture volumes containing 1× Mg-free buffer, 1.8 mM $MgCl_2$, 0.2 mM dideoxynucleoside triphosphates, 1 pmol μl^{-1} of each primer, 0.04 mg ml^{-1} RNase A (Novagen), 0.06 U μl^{-1} Go*Taq* Flexi DNA polymerase (Promega), and 2.5 μl template. The cycling program consisted of 5 min at 95°C, followed by 26 cycles of 94°C for 45 s, 54°C for 45 s, and 70°C for 45 s with an 8-min final extension at 70°C. The double-stranded adapter was removed by digestion with MspI, and the TRF-enriched samples were ligated into pGEM-T Easy vector (Promega) prior to introduction into *Escherichia coli* JM109 competent cells (Promega). A total of 56 transformants were selected for sequencing, based on insert size. Sequencing of this and other samples was performed at the Molecular and Cellular Imaging Center of the OARDC (Wooster, OH) in an ABI Prism 3100xl genetic analyzer system using 3′-BigDye dideoxynucleoside triphosphate-labeling chemistry.

Extension of target 16S rRNA gene TRFs. The cloned TRF sequences overlap with the first variable loop region between *E. coli* base pair positions 69 to 101 (13) (www.rna.ccbb.utexas.edu). Sequence alignments were used for designing variable loop-specific primers M139F (5′-TAACGCGGGGCAAC CTGGCGA-3′) and M141F (5′-CAGCACGGGAGCAATCCTGGTGG-3′) to target the *Burkholderiales*-like clones (see Fig. S4 in the supplemental material). The sequence diversity within the M148 clones precluded the design of a primer pair that could target a set of these clones. These primers were used independently, in combination with universal primer 518R (primer 2 from reference 42; 5′-ATTACCGCGGCTGCTGG-3′), to generate extended amplicons from multiple DNA samples from samples obtained during our previous work (8; see above), with the following variations in the cycling program: 30 cycles of 94°C for 1 min, 65°C for 45 s, and 70°C for 45 s. Amplicons from two independent samples were cloned as described above, and 16 transformants were selected for sequencing.

Culture-based screening for M139- and M141-positive isolates. A bacterial collection was generated from the rhizosphere of hay grown in soils previously described as suppressive (7). The hay mix contained Festulolium duo (36% [vol/vol]), alfalfa (14%), Starfire red clover (11%), Jumbo white clover (9%), Tekapo orchard grass (9%), Tuukka timothy (9%), Lancelot plantain (6%), and chicory (6%). The hay was grown in the greenhouse during the spring of 2007, with temperatures for the period ranging from 23°C to 31°C. Roots and soils were thoroughly mixed, and 5 g of the mixture was sampled and diluted in 50 ml of sterile water (SW). The suspension was vortexed (1 min), sonicated (1 min), vortexed again (15 s), serially diluted in SW, and spread plated in *Leptothrix* strain medium (LM; per liter, 5g peptone, 0.2 g magnesium sulfate heptahydrate, 0.15 g ferric ammonium citrate, 0.05 g calcium chloride, 0.01 g anhydrous ferric chloride, 0.01 g manganese sulfate monohydrate, 15 g agar [3]), yeast agar van Niel's (YAN; per liter, 10 g yeast extract, 1 g dipotassium phosphate, 0.5 g magnesium sulfate heptahydrate, 15 g agar [3]), buffered nutrient agar (NB; per liter, 4 g peptone, 4 g sodium chloride, 2 g yeast extract, 1 g beef extract, 0.45 g monopotassium phosphate, 1.78 g disodium hydrogen phosphate heptahydrate, 15 g agar [3]), and R2A (medium for growth of heterotrophic organisms; Difco BD). These culture media were previously reported to support the growth of various *Burkholderiales* species, including members of the *Comamonadaceae* and genera incertae sedis (R2A, NB, LM, and YAN) (3, 29, 35, 49). Plates were incubated for 48 h at room temperature (RT) in the dark. From each plate, eight colonies were picked and transferred into a 96-well plate prefilled with 200-μl $well^{-1}$ of corresponding liquid medium. A total of 11 mixed-hay pots were sampled, resulting in a collection of 704 isolates. Liquid cultures were pooled (eight per well) prior to DNA isolation performed with a Wizard genomic DNA purification kit (Promega). DNA pools (1:100 dilution) were PCR screened for the presence of M139- and M141-like sequences as described above, with a 25-cycle amplification program. The primer and amplification protocol for M141 was modified (M141F2 primer, 5′-GGAGCAATCCTGG TGGCGA-3′; amplification reaction mixture with final 1.0 mM $MgCl_2$) to maximize the recovery of isolates matching the targeted variable loop sequence. Individual amplifications were performed from individual cultures present in PCR-positive pools only. Colony PCR was performed with the 8F and 1492R primer combination using previously described conditions (8). 16S amplicons were purified with ExoSAP-IT (USB) and sequenced. Consensus sequences for each isolate were constructed by using Sequencher 4.7 (Gene Codes Corporation).

In vitro inhibition of pathogen growth. Pathogen growth inhibition was tested in multiple contexts. For *Mitsuaria* isolates, assays were performed on R2A, LM, and 1/10 trypticase soy agar (TSA). For *Burkholderia* isolates, R2A, LM, and 1/3 King's medium B (3) were used. Bacteria from 48-h-old culture plates were resuspended in SW, and a 10-μl drop was placed on a plate with a test pathogen in the center. Plates were incubated at RT, and growth inhibition was scored after between 4 and 10 days, depending on the pathogen. In vitro inhibition was scored as positive or negative, though phenotypes scored as positive varied somewhat depending on the pathogen and medium combination used. Positive scores reflected the formation of clear inhibition zones between the pathogen and the bacteria, diminished total growth of the pathogen in comparison to that of the control, melanization or morphology change in the pathogen colony, and/or bacterial swarming over the pathogen culture. In vitro inhibition tests were performed against *Pythium aphanidermatum* isolate 349 and *Phytophthora capscici* provided by S. Miller (OARDC); *Pythium sylvaticum* 134, *Phytophthora sojae* race 25, and *Rhizoctonia solani* AG4 provided by A. Dorrance (OARDC); *Fusarium graminearum* provided by P. Paul (OARDC); and *Alternaria solani* Mg23 and *Fusarium oxysporum* Ft25 (22).

Seedling lesion bioassays. Soybean and tomato seeds were surface sterilized and germinated on water agar (WA; 7.5 g agar liter^{-1}) at RT in the dark. After 4 days, three seedlings were transferred to petri plates containing WA (for tomatoes, 100- × 15-mm diameter, and for soybeans, 150- × 15-mm diameter). A 5-mm pathogen plug was placed in the center of the plate, and seedlings were treated with ~10^7 cells ml^{-1} seedlings^{-1} in a ≤100-μl volume. Inoculum was prepared from 24-h cultures in 1/10× TS broth, collected by centrifugation, and washed twice with SW. Control plates with water-treated seedlings with and without pathogen inoculum were also prepared. Each plate was prepared in triplicate. Seedling disease was scored after 4 and 5 days for soybeans and tomatoes, respectively. For each seedling ($n \geq 9$ per assay), total seedling length and lesion length were measured, and disease severity was expressed as the percentage of the seedling that showed a lesion. Seedlings from noninoculated control plates occasionally developed symptoms, due to a low percentage of seedborne pathogens/endophytes that colonized the plate or inefficient surface sterilization. Three bacterial isolates of each genus recovered were selected for analysis based on their independent isolation from different hay-containing pots. For *Mitsuaria* isolates, soybean assays were run against *P. aphanidermatum*, *P. sojae*, and *R. solani*, and for tomatoes against *P. aphanidermatum* and *R. solani*. For *Burkholderia* isolates, soybean and tomato assays were run against *R. solani* only. All experiments were run at least twice.

Sequence data analysis. Vector sequence and bad quality sequence data were trimmed either manually or by using Sequencher 4.7 (Gene Codes Corporation). Sequences were aligned, and pairwise comparisons calculated with ClustalW2 (EMBL-EBI Tools). Graphic alignments were prepared by using the Jalview (version 2.3) alignment editor. Shorter sequences were compared to the Ribosomal Database Project (release 10.0 beta) using SeqMatch to determine the best match to isolate data only. Full-length sequences were compared also to the nonredundant nucleotide collection NCBI database (nr/nt) using BLAST (blastn program). Database searches include data as of 10 June 2008. Phylogenetic analyses were performed by using MEGA 4 (50). Trees were generated by using the neighbor-joining algorithm from distances calculated using the maximum composite likelihood method with the complete deletion option (all positions containing gaps and missing data were eliminated from the data set). The bootstrap test was performed with 1,000 replicates to determine the percentage of replicate trees in which data cluster together. For comparison of topologies between different algorithms, trees were also generated with the maximum parsimony method using the close-neighbor-interchange algorithm. Both sets of tree topologies were equivalent (data not shown).

Statistical analyses. All analyses were performed by using JMP version 7.0 (SAS Institute, Inc.). Seedling lesion bioassays were analyzed as a completely randomized design, following the nonparametric Kruskall-Wallis test, to determine differences in lesion severity (expressed as percentage of root length marked by a lesion) in response to treatment. Five treatment levels were considered: three bacterial isolates in the presence of pathogen and water-treated seedlings with or without pathogen with 9 to 16 replicates per treatment. In addition, pairwise comparisons with the Wilcoxon two-sample test were performed between individual isolate treatments and water-treated controls (plus pathogen). Contrast analysis (one-tailed Wilcoxon two-sample test) was performed to determine the overall effect of bacterial treatment compared to the results for water-treated seedlings (plus pathogen).

Nucleotide sequence accession numbers. Sequences generated within this study have been deposited in GenBank under accession numbers EU714905 to EU714956.

RESULTS

Classification of 16S eubacterial sequences corresponding in size to a target TRF. The identities of bacteria giving rise to MspI-generated TRFs previously associated with damping-off suppression in the microbial community profiles studied (8) were first assessed by cloning TRFs of the selected size range. Of 56 clones sequenced, 20 were confirmed as targeted TRFs (seven targeted to M139, eight to M141, and five to M148). These sequences were compared to isolate sequences only, available at the Ribosomal Database Project (release 10.0). Table 1 summarizes the best match data for each individual TRF clone. Five M139 clones shared >90% sequence identity with one another and likely arose from *Betaproteobacteria*, and of these, four, recovered from three independent samples, shared sequence identity (>0.75 S_ab SeqMatch score) with database sequences belonging to members of the order *Burkholderiales* not assigned to a named family (i.e., genera incertae sedis) and to a member of the *Comamonadaceae*. Similarly, four M141 clones derived from independent samples showed a high degree of similarity to one another and were classified as *Burkholderiales*. These clones, however, had a higher number of sequence matches to members from multiple families and individual genera (Table 1). These four M141 clones were recovered from two independent samples yet still displayed 98 to 100% sequence identity. Other M141 clones differed substantially from this group (68 to 82% sequence identity) and among themselves (66 to 78% sequence identity) and might belong to the divisions *Firmicute*, *Actinobacteria*, *Proteobacteria*, and/or *Spirochaete*. The greatest sequence variation was observed within the sampled population of M148 clones, which shared only 46 to 71% sequence identity with each other. Two clones matched *Proteobacteria* sequences, one matched *Planctomycete* sequences, and the other three matched with sequences of members from multiple divisions. Within each cloned TRF subset, at least one did not show any significant match, as reflected by matches to multiple divisions. These data further support our initial hypothesis that multiple novel bacterial populations are associated with the suppressive activity that develops from the hay-based transition strategy.

Over half of the M139 and M141 TRF clones likely arose from novel bacterial species not previously associated with plant disease suppression (i.e., *Burkholderiales* genera incertae sedis). Hence, the sequence information from these was used to recover longer and more phylogenetically informative sequences from the samples studied. For this, the sequences of known *Burkholderiales* species and M139 and M141 clones were aligned to determine sequence variation within the first variable loop of the 16S rRNA (13). These data were then used to design M139- and M141-specific primers (see Fig. S4 in the supplemental material). These primers were used in combination with eubacterial primer 518R to generate extended amplicons from two independent DNA samples with high peak intensity for the TRF of interest, to maximize chances of recovery. Four of the M139 extended sequences showed similarity to bacteria of the genera incertae sedis (up to three genera), and three M141 sequences matched the *Comamonadaceae* (up to two genera) (see Table S4 in the supplemental material). The high sequence diversity observed among the M148 clones, as well as the lowest level of taxonomic resolution observed

TABLE 1. Classification of 16S rRNA gene clones that match the size of targeted[a] MspI-generated TRFs

TRF size (bp)	Clone[b]	Sequence similarity[c] to:			
		Higher-rank taxon	Order	Family	No. of matching genera
139	S101D	*Betaproteobacteria*	*Burkholderiales*	Genera incertae sedis	2
				Comamonadaceae	1
	SB42G	*Betaproteobacteria*	*Burkholderiales*	Genera incertae sedis	3
				Comamonadaceae	1
	S102A	*Betaproteobacteria*	*Burkholderiales*	Genera incertae sedis	4
				Comamonadaceae	2
	SB81G	*Betaproteobacteria*	*Burkholderiales*	Genera incertae sedis	4
				Comamonadaceae	1
	SB11G	*Betaproteobacteria*	*Burkholderiales*		
			Rhodocyclales		
	SB12G	*Alphaproteobacteria*	*Rhizobiales*		
	SB41H	*Actinobacteria*			
		Firmicutes			
		Proteobacteria			
		Nitrospira			
141	SB41E	*Betaproteobacteria*	*Burkholderiales*	Genera incertae sedis	2
				Burkholderiaceae	2
				Comamonadaceae	3
	SB41G	*Betaproteobacteria*	*Burkholderiales*	Genera incertae sedis	2
				Burkholderiaceae	2
				Comamonadaceae	3
	SB42F	*Betaproteobacteria*	*Burkholderiales*	Genera incertae sedis	2
				Comamonadaceae	3
	SB71E	*Betaproteobacteria*	*Burkholderiales*	*Burkholderiaceae*	3
				Comamonadaceae	3
	SB31A	*Firmicutes*			
		Actinobacteria			
	S102G	*Proteobacteria*			
		Firmicutes			
		Actinobacteria			
	S101F	*Proteobacteria*			
		Actinobacteria			
	SB31C	*Spirochaetes*			
		Protoebacteria			
148	SB82F	*Alphaproteobacteria*	*Caulobacterales*		
			Rhizobiales		
	SB71D	*Sphingobacteria*			
		Bacteroidetes			
		Proteobacteria			
	SB42C	*Planctomycete*			
	SB21H	*Acidobacteria*			
		Plactomycete			
	SB32F	*Gammaproteobacteria*	*Xanthomonadales*		
			Methylococcales		

[a] Target MspI TRFs of the 16S rRNA gene were previously associated with general disease suppression (7).
[b] Identifiers for GenBank accession numbers for clones of 16S rRNA gene TRF sequences obtained in this study.
[c] Sequences were compared to the Ribosomal Database Project (release 10.0 beta) isolate data only, using SeqMatch. The number of genera per family are shown only for those samples matching sequences within the same order (correponding to SeqMatch hits with >0.75 S_ab scores).

within this set (Table 1), hindered the use of a similar approach for this third group of sequences.

Sequences from both cloning steps were aligned to determine identity at the region of overlap, including the first variable loop (see Fig. S5 in the supplemental material). For M139, three different extended sequences showed a 100% identity with the initial M139 TRF clones over a 76-nt overlap. Based on approximately 450 nt, these three M139 extended sequences exhibited similarity to database entries of genera incertae sedis, including the genera *Leptothrix*, *Ideonella*, *Methylibium*, and *Schlegelella* (see Table S4 in the supplemental material). In addition, sequence analysis revealed the presence of an MspI recognition site that will produce a TRF of 139 bp in these three genera incertae sedis-like sequences. For M141, one extended sequence, which matched to database entries of the *Comamonadaceae* (*Ramlibacter* and *Curvibacter*), exhibited a 97% sequence identity in a 78-nt overlap region (see Table S4 and Fig. S5 in the supplemental material). The *Comamonadaceae*-like sequence, however, lacked the MspI site to produce the expected 141-bp TRF. It is unclear if this lack of consistency reflects a high degree of sequence diversity among the bacteria giving rise to the targeted TRF in our samples or results from amplification artifacts.

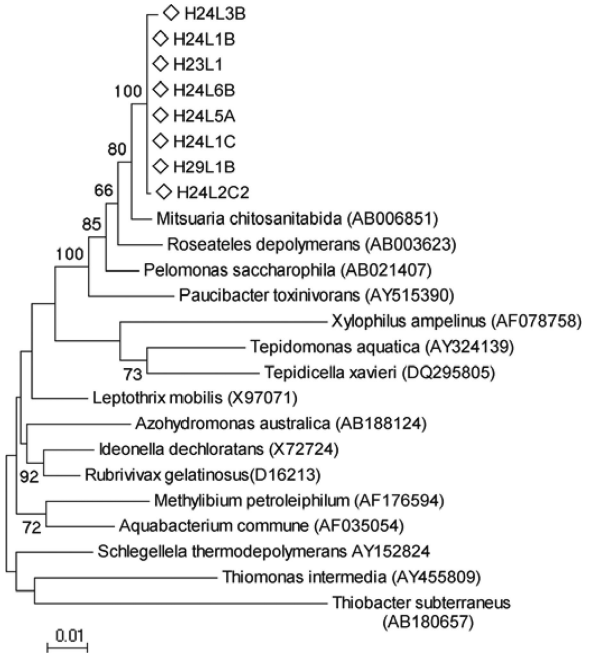

FIG. 1. Classification of M139-associated isolates (◇) as *Mitsuaria* sp. based on 16S rRNA gene sequence analyses. Included in the dendrogram are the sequences of the type strains representative of other species of genera incertae sedis of the order *Burkholderiales*. The phylogenetic relationships among taxa were inferred from ~1,200 bp of the 16S rRNA gene by using the neighbor-joining method from distances computed with the maximum composite likelihood algorithm. Bootstrap values of >60% (1,000 replicates) are shown next to the branches. GenBank accession numbers for each sequence are shown in parentheses. Scale bar shows number of base substitutions per site.

Culture collection screening for M139 and M141 isolates. Because no isolates with 100% sequence identity to the cloned TRFs and extended sequences had been previously identified, efforts were made to recover bacteria giving rise to the M139 and M141 markers. To do so, culture media favoring growth of *Burkholderiales* species related to the genera described above (i.e., sharing sequence identity with the various TRF clones) were selected, and the isolates were obtained from the mixture of hay species that had resulted in damping-off suppression. A two-step PCR-based approach was used to screen the isolate collection, first from pooled samples and then individually. Of the 704 isolates examined, 8, all isolated from LM (10^{-2}-dilution plates), had an exact sequence match to the M139 variable loop. The highest BLAST hit to a named species for all eight isolates was to *Mitsuaria chitosanitabida* (98 to 99% identity), followed by *Roseateles depolymerans* and *Pelomonas aquatica* or *Pelomonas saccharophila* (>97% identity), all belonging to the genera incertae sedis. Sequence identity within the group of isolates ranged from 98 to 100%, and their phylogenetic relationships to representative type strains of genera incertae sedis (*Burkholderiales*) are shown in Fig. 1. The type strain most closely related to the isolates retrieved from the mixed-species hay soils is *M. chitosanitabida* 3001 (2), but there is a clear distinction between known *Mitsuaria* species and the isolates from this study (see Fig. S6 in the supplemental material).

While the novel *Mitsuaria* isolates recovered from the disease-suppressive soil were found to have 16S sequences similar to those of the initial M139 clones, they were not identical. Also, the isolates shared 99% identity to a *Mitsuaria*-like extended-sequence clone. *Mitsuaria* species, however, do not produce an M139 TRF in vitro or in silico. In contrast, the MspI TRF for the isolates was 487 nt (488 nt expected from sequence). Interestingly, M488 and M489 TRFs were common in the TRF profiles of the soils studied, and positive associations between M488 and M489 and soilborne-disease suppression were observed in two of the contexts studied (8). Given the sequence similarity between *Mitsuaria* isolates and M139 clones and the identity at the first variable loop sequence of M139, it seems likely that these isolates represent bacteria very closely related to those giving rise to the M139 TRF associated with disease suppression in the system studied. Variation in TRF size could also relate to amplification artifacts resulting from sampling complex mixtures of closely related bacteria, as well as to the presence of pseudo TRFs in the samples (24).

A similar isolation strategy led to the recovery from R2A medium (10^{-4} dilution) of eight pure cultures with an M141-like amplification profile. The 16S sequences amplified from these isolates shared 24 out of the 26 nt of the M141-derived variable loop sequence. The highest BLAST hit for all eight was to unclassified *Burkholderia* spp. (i.e., 99% identity to sequences under GenBank accession numbers AY238505, AB025790, and AB298718). Sequence identity within the eight isolates was >99% but was only 96% identical to the type strain of the genus, *B. cepacia* (GenBank accession number U96927). The isolates from this study form a phylogenetically distinct cluster within the genus (Fig. 2), with their closest relatives being "*Candidatus* Burkholderia" spp., noncultured endosymbionts from leaf galls (97% identical). Sequence analysis revealed 97% identity between our *Burkholderia* isolates and the initial M141 clones but only 72 to 88% sequence identity with clones of the ~450-nt extended sequences. Still, the predicted 16S rRNA gene MspI TRF for these isolates was 141 nt. The observed 16S rRNA gene MspI TRF for the eight isolates, however, was a 139-/141-bp double peak, where both peaks added corresponded to >90% of the fluorescence in each sample. The presence of multiple peaks in T-RFLP profiles of individual isolates is not uncommon (e.g., see references 4 and 14) and could result from inefficient enzyme cleavage or variation within multiple copies of the 16S rRNA gene. In this case, however, template characteristics seem to also have an effect on individual profiles for all eight isolates. Individual profiles obtained from isolate DNA tend to have a greater 139-bp fluorescent peak (ranging from 45 to 55%), and from colony PCR runs, the 141-bp peaks tend to be higher (49 to 60% sample intensity).

Characterization of pathogen inhibition and lesion-suppressing activities. The statistical association of the M139 and M141 TRFs with in situ soilborne-disease suppression (8) led us to hypothesize that the novel *Mitsuaria* and *Burkholderia* isolates obtained would express antagonistic activities toward diverse soilborne pathogens. Initially, the capacity of the isolates to reduce pathogen growth in vitro against multiple fungal and oomycete plant pathogens was assayed. For the *Mitsuaria* isolates, inhibition was observed regardless of the pathogen tested (Fig. 3A), with the greatest frequency of in-

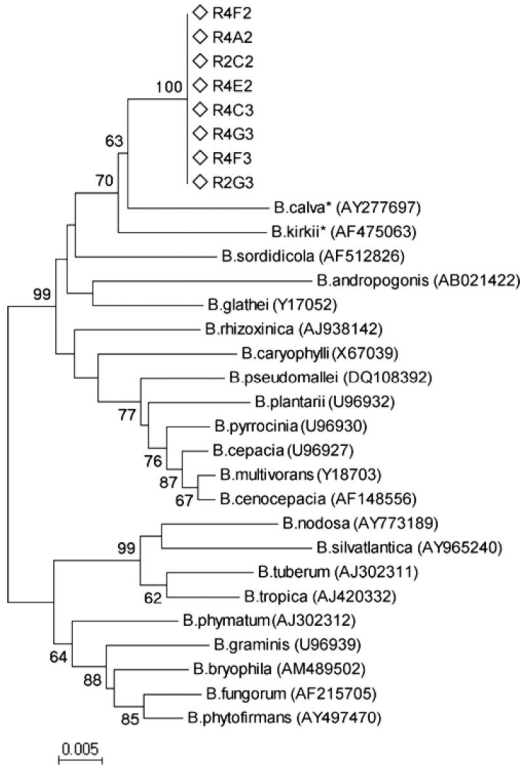

FIG. 2. Classification of M141-associated isolates (◇) as representing a novel *Burkholderia* sp. based on 16S rRNA gene sequence analyses. Included in the dendrogram are the sequences of the other 22 named *Burkholderia* species. The phylogenetic relationship among taxa was inferred from ~1,300 bp of the 16S rRNA gene by using the neighbor-joining method from distances computed with the maximum composite likelihood algorithm. Bootstrap values of >60% (1,000 replicates) are shown next to the branches. GenBank accession numbers for each sequence are shown in parentheses. Scale bar shows number of base substitutions per site. *, "*Candidatus* Burkholderia" species with no cultured isolate.

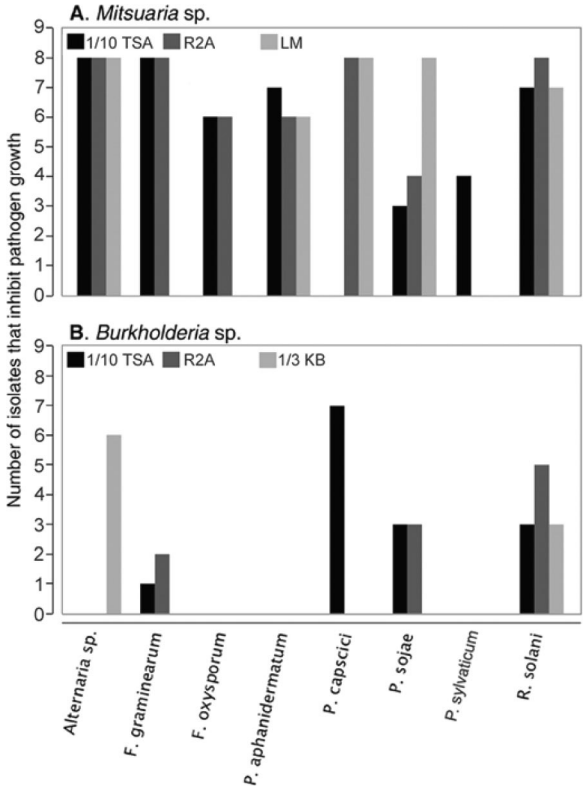

FIG. 3. Frequency of positive in vitro inhibition activity of *Mitsuaria* (A) and *Burkholderia* (B) isolates identified in this study against multiple fungal and oomycete tomato and soybean pathogens. In vitro inhibition activity was tested for eight isolates of each genus on three different media and was scored as positive or negative.

hibition expressed against *Pythium aphanidermatum*, *Phytophthora sojae*, *Rhizoctonia solani*, and *Alternaria solani* and the least against *Pythium sylvaticum*. All of the *Mitsuaria* isolates from this study have chitinolytic activity in vitro (see Fig. S7 in the supplemental material), which may explain the broad-spectrum inhibition observed against the various fungi, but other mechanisms must be involved in the inhibition of the oomycetes, which do not harbor chitin as a major component in their cell walls (6). Similar assays were performed with other *Mitsuaria* spp., including multiple chitosan-degrading strains isolated from soils in Japan (ATCC type strain *M. chitosanitabida* 3001, strain 12, and strain 13) (2) and gallic acid-degrading strains associated with freshwater plants (*Mitsuaria* spp. strains FBTS 25 and FBTS 19) (40). Of these, chitosan-degrading strains 12 and 13 showed a similar spectrum of inhibition, whereas the type strain 3001 gave a positive result for inhibition in only about half of the assays. While the sequence identity with the Japanese strains tested was ≥98%, the antagonistic phenotype of our isolates was less variable. The *Mitsuaria* strains recovered from freshwater plants expressed no pathogen inhibition in most cases. Among the *Burkholderia* isolates, in vitro pathogen inhibition was less frequent and more variable (Fig. 3B). Significant variation in the expressed inhibitory capacities was observed among isolates, with six isolates inhibiting at least three pathogens but none of these inhibiting the same three pathogens. In contrast to *Mitsuaria* isolates, all eight *Burkholderia* isolates tested negative for chitinolytic activity.

Lesion development was suppressed by inoculation with the novel *Mitsuaria* isolates (Table 2). All the isolates tested reduced lesion severity in soybeans challenged with *P. aphanidermatum* ($P = 0.03$ and 0.005) and in tomatoes challenged with *P. aphanidermatum* ($P = 0.0003$ and 0.002) and *R. solani* ($P = 0.27$, 0.02, and 0.0007). Although not significant for most experiments, the lesion severity caused by *R. solani* was also reduced by the *Mitsuaria* isolates in three separate assays. In addition, lesion severity was also reduced in soybean seedlings treated with *Mitsuaria* isolates when challenged with *P. sojae*, though only once was a disease differential observed between challenged and nonchallenged control plants. Overall, lesion severity reductions ranged from 5 to 20%. In 7 out of the 11 tests, treatment with *Mitsuaria* isolate H24L5A resulted in smaller lesions than in the water-treated control, whereas isolates H23L1 and H29L1B resulted in lesion severity reduction in 4 out of the 11 tests, with the greatest variation observed in the soybean bioassays (see Tables S5 to S8 in the supplemental material).

Similarly, lesions caused by *R. solani* were reduced on tomatoes and soybeans inoculated with *Burkholderia* isolates (Table

TABLE 2. Lesion severity in soybean and tomato seedlings treated with *Mitsuaria* isolates and challenged with damping-off pathogens

Crop	Treatment (strain and/or pathogen)	Lesion severity[a] with:	
		P. aphanidermatum	*R. solani*
Soybeans	H23L1	44.2**	22.9
	H24L5A	62.5*	26.7
	H29L1B	64.3	33.3
	Pathogen only	91.0	32.2
	No pathogen	27.0	11.7
Tomatoes	H23L1	57.8**	45.0**
	H24L5A	41.7***	33.3***
	H29L1B	60.4**	33.3***
	Pathogen only	91.7	55.6
	No pathogen	47.7	34.8

[a] Lesion severity is expressed as percentage of root length marked by a lesion. Median values are reported; $n = 16$ (soybean/*R. solani*) or $n = 12$ (others). Nonparametric Kruskall-Wallis test was used to assess differences among all five treatments; for soybean/*P. aphanidermatum*, $P < 0.0001$, and for soybean/*R. solani*, $P = 0.0002$. For tomato/*P. aphanidermatum*, $P = 0.019$, and for tomato/*R. solani*, $P = 0.006$. Significant pairwise comparisons between treatment and pathogen-only control: ***, $P < 0.01$; **, $P < 0.05$; *, $P < 0.1$ (Wilcoxon two-sample test).

TABLE 3. Lesion severity in soybean and tomato seedlings treated with *Burkholderia* strains and challenged with *Rhizoctonia solani*

Crop	Treatment (strain and/or pathogen)	Lesion severity[a]
Soybeans	R2C2	36.1***
	R2G3	34.5***
	R4F2	34.2**
	Pathogen only	56.1
	No pathogen	29.1
Tomatoes	R2C2	46.2***
	R2G3	42.3***
	R4F2	48.3***
	Pathogen only	63.6
	No pathogen	45.2

[a] Lesion severity is expressed as percentage of root length marked by a lesion. Median values are reported; $n = 12$. Nonparametric Kruskall-Wallis test was used to assess differences among all five treatments; for soybeans, $P = 0.003$, and for tomatoes, $P = 0.002$. Significant pairwise comparisons between treatment and pathogen-only control: ***, $P < 0.01$; **, $P < 0.05$ (Wilcoxon two-sample test).

3). As a group, lesion severity was reduced by at least 15% on soybean ($P = 0.0001$ and 0.0005) and 20% on tomato seedlings ($P < 0.0001$ for both tests). For the *Burkholderia* isolates tested, no apparent variation in their ability to reduce lesion severity was observed. Overall, these data support the hypothesis that multiple isolates of novel *Mitsuaria* and *Burkholderia* species contribute to the general soilborne-disease suppression induced by the mixed-hay cropping system.

DISCUSSION

We successfully used molecular-profile data of bacterial community structure to rapidly identify and direct the isolation of novel and ecologically important microbial populations. Specifically, we recovered two novel groups of rhizosphere bacteria by using molecular markers (16S TRFs and first variable loop sequences) previously associated with the ecologically important function of soilborne plant disease suppression. The validity of this approach is evidenced by the pathogen-suppressing activities expressed by all of our isolates in vitro and in situ. The described community profiling approach led to a clear enrichment for pathogen-suppressing organisms involved in general disease suppression because all of the novel *Mitsuaria* and *Burkholderia* isolates obtained were active against multiple soilborne pathogens on different crops. This contrasts with traditional random screens, which typically show fewer than 10% of isolates to be active against any single pathogen and even fewer having activity against multiple pathogens. For example, our laboratory's screen of *Bacillus subtilis* and *Bacillus pumilus* isolates found fewer than 1% of isolates to be active against a similar spectrum of pathogens in vitro (data not shown). The efficient recovery of active strains is not surprising when one considers that the correlative evidence for the involvement of M139- and M141-marked bacteria in disease suppression was generated in multiple experimental contexts with various levels of disease pressure (7, 8). Indeed, reductions in damping-off of tomato and soybean seedlings have been observed with seeds treated with the recovered isolates (data not shown). The general utility of these new bacterial species as biocontrol inoculants, in different soils and on different crops, is currently under investigation.

The novel bacteria identified in this study probably represent just a small percentage of the total soil- and root-inhabiting bacteria present in the hay-amended soils. Based on our TRF data, the relative abundance rarely exceeds 10% of the total 16S signal (8), and here, based on our culturing methods, these bacteria represented <1% of the sampled population and were isolated based on sequence identity to TRF clones. While it may be surprising that such a small percentage of the total bacteria can have a significant functional impact on soilborne diseases, past research has correlated similarly small percentages of native and introduced populations of 2,4-diacetylphloroglucinol-producing pseudomonads with significantly higher stands and yields of crops (37, 47). Now, by definition, general soilborne-disease suppression involves the activities of multiple members of the community through multiple mechanisms of action (54). Therefore, it is likely that the two isolated populations are just a subset of the whole involved in the suppression differential described previously (7, 8). The identities and distribution and how the relative abundance of these other pathogen-suppressing populations might vary in the different environmental contexts remains unclear. However, it seems likely that multiple components are involved. Here we noted that another TRF associated with damping-off pathogen suppression (i.e., M148) (8) was represented by high sequence diversity spread along multiple taxonomic groups (Table 1). Thus, the marker-assisted recovery of functionally important microbes described is limited to identifying only a portion of the diverse pathogen-suppressing antagonists that are likely present in the tested soils. However, that portion represents generalist pathogen antagonists because the profiling was conducted under multiple environmental contexts (8). The value of this screening strategy is that the isolates recovered are more likely to be active pathogen suppressors under a broad range of environmental conditions, a result of much practical significance.

The *Mitsuaria* isolates we describe in this work represent the first association of this genus and closely related species of the genera incertae sedis (Fig. 1) of the *Burkholderiales* (such as *Roseateles* and *Pelomonas*), with plant disease suppression. *Mitsuaria* strains from soils in Japan (2, 58) and the rhizosphere of a freshwater plant (40) have been described. These earlier studies, however, focused on exploring specific substrate degradation (i.e., of chitosan and gallic acid). And, while bacteria with sequences similar to *Mitsuaria* sequences were isolated from the rhizoplane of oil seed rape (28) and from hemodialysis fluid (20), no function was ascribed to them. Bacteria of the genera incertae sedis are known to exhibit a range of metabolic activities, including nitrogen fixation, photosynthesis, and metal oxidation (21, 33, 48, 56). Some sequences associated with these genera have been found in the rhizosphere and phyllosphere of crops (15, 27, 46) but were not previously associated with disease suppression. Other metabolic activities besides chitin degradation might be involved in the observed pathogen-suppressing phenotype. Further characterization of *Mitsuaria* isolates from this study will be required to better understand their metabolism, their contributions to plant disease suppression, and their potential utility as biological control agents. Work in progress has shown the ability of these isolates and culture filtrates to inhibit the growth in vitro of other plant pathogens in addition to those presented in this study, including plant-pathogenic bacteria (R. Raudales and B. McSpadden Gardener, unpublished data).

The other isolates described in this study likely represent a new species of the genus *Burkholderia.* These isolates share 99% sequence identity with several strains isolated from soils (23, 32) and insect guts (30). However, within validly named species, the closest relatives are plant-symbiotic "*Candidatus* Burkholderia" species (Fig. 2). Because the prefix *Candidatus* has been adopted for the description of noncultured bacteria with defined phylogeny and some phenotypic description (41), our isolates make further systematic work on these organisms possible for the first time. The *Burkholderia cepacia* complex is the major phylogenetic cluster associated with human diseases, with *B. multivorans* and *B. cenocepacia* (*B. cepacia* genomovars II and III, respectively) being most frequently isolated from cystic fibrosis patients (16). But our *Burkholderia* isolates share only 96% sequence identity with *B. cepacia* and are part of a distinct clade within the genus that is separate from the *B. cepacia* complex (Fig. 2). This lack of similarity may assuage some of the concerns about using these strains as microbial biopesticides, a problem that has limited the commercial interest of other species of the genus (44). Antibiotic production, nitrogen fixation, and production of indol acetic acid are expressed by some *Burkholderia* strains (11, 18, 45), and this study adds to the list of species with the potential for plant health promotion. Further analysis of the *Burkholderia* isolates described in this study will be aimed at understanding the mechanisms involved in plant pathogen suppression.

To our knowledge, this work represents the first direct connection of TRF-derived molecular markers to isolates capable of expressing an ecologically important microbial function, specifically, biocontrol. T-RFLP has been used extensively for comparing microbial community structure (24), including in plant disease suppression contexts (38). Yet, to date, no group has reported the use of such a technique to direct the recovery of novel disease-suppressive microbes. Methods used to classify microbes that generate specific TRFs include the analysis of isolates or clone collections for matching TRF sizes, as well as sequencing of selected TRF clones (e.g., see references 38 and 55). T-RFLP has been used to guide the isolation of methanogenic archaea (12) and biodegraders (25), but those investigations monitored solely for TRF size and did not consider sequence. Ribosomal-sequence-directed isolation of microorganisms involved in plant disease suppression was performed previously for fungal species, using data generated from oligonucleotide fingerprinting of rRNA (43, 57). In that system, however, disease suppression was specific, being caused by a parasite of nematode cysts. For that same system, bacterial populations were also analyzed and correlations with suppressiveness were observed (52), but suppressiveness was demonstrated only for the fungi *Dactylella oviparasitica* and *Fusarium oxysporum* (43). Such studies can lead to the discovery of novel microorganisms expressing a specific function by first coupling them to sequence data associated with that function. Because of this initial in situ association, it seems likely that the microbes recovered using this approach will have a much higher probability of being able to perform effectively when reintroduced through inoculation. Studies of novel microorganisms associated with plant disease suppression will provide insight into the diversity of metabolisms involved in this type of interaction (31). Our approach not only led to the discovery of two novel sets of bacteria that contribute to general soilborne-disease suppression, it further establishes the validity of using such an approach to better link structure and function of diverse microbial communities via sequence-directed isolations.

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