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# Minimal changes in rhizobacterial population structure following root colonization by wild type and transgenic biocontrol strains

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**Abstract**

*Pseudomonas fluorescens* strains 2-79, Q8r1-96, and a recombinant strain, Z30-97, produce the antibiotics phenazine-1-carboxylic acid (PCA), 2,4-diacetylphloroglucinol (DAPG), or both antibiotics, respectively. Rhizosphere colonization by these strains and subsequent alterations of bacterial community structure were assayed over multiple growth cycles of wheat under controlled conditions. While added to soil at just log 4 cells per gram prior to planting, all four strains subsequently colonized germinating wheat roots to levels in excess of log 6.5 cells per g (f.w.). Strain-specific differences in rhizosphere competence were observed, but these were not generally related to the chromosomal insertion of the *phz* genes. Multiple differences in bacterial community structure were detected among treatments in each cycle; however, the large majority of changes were not consistently related to the abundance of inoculant strains in the rhizosphere nor the genetic make-up of the inoculant strains. Nonetheless, T-RFLP profiles of amplified 16S eubacterial sequences indicated that, when compared to the untreated samples, inoculation with Z30-97 resulted in several shifts in rhizosphere bacterial community structure previously associated with decreased levels of root disease.


## 1. Introduction

Microbial inoculants are playing an increasingly important role in integrated pest management systems aimed at controlling plant diseases [1]. Antibiotic production is a key mechanism by which some rhizosphere bacteria can inhibit plant pathogens and suppress disease [2]. Two of the best-studied antibiotics responsible for the activity of *Pseudomonas* biocontrol agents are the phenolic compound 2,4-diacetylphloroglucinol (DAPG) and phenazines. The DAPG biosynthetic locus, *phl*, includes six genes [3], and the phenazine-1-carboxylic acid (PCA) biosynthetic locus, *phz*, includes seven genes [4,5]. Both antibiotics can be isolated from the rhizosphere environment after introduction of DAPG- or phenazine-producing bacteria such as Q8r1-96 or 2-79, respectively [6]. The activity of these two antibiotics is broad spectrum, and thus their impact is potentially not limited to the target fungal pathogen [2]. For example, the DAPG producer *Pseudomonas fluorescens* F113 suppresses damping-off of sugar beet caused by *Pythium ultimum*, and cyst nematode and soft rot of potato caused respectively by *Globodera rostochiensis* and *Erwinia carotovora* subsp. *atroseptica* [7,8].

Molecular techniques have been used to construct rhizobacteria with increased or novel antibiotic production capacities to enhance biocontrol activity [9–13].

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Strains can now be constructed that produce an antibiotic or a combination of antibiotics that were previously never known to occur in a bacterial species or subspecies. If released in large densities into agricultural ecosystems, these recombinant strains are perceived to pose a threat to the indigenous soil microflora and their ecological activities. The impact of the introduced antibiotic biosynthetic locus on the ecological competence of the organism is also of concern. Production of antibiotics by biocontrol agents may affect the rhizosphere competence of the producer strain in different ways. For example, studies conducted with DAPG-producing *P. fluorescens* strains have shown that this antibiotic does not contribute to rhizosphere competence [14,15]. In contrast, PCA production is a rhizosphere competence factor in *P. fluorescens* 2-79 and *P. aureofaciens* 30-84 [16]. However, introduction of these loci individually into *P. putida* WCS358r did not change its rhizosphere competence [17,18]. Therefore, the impact of antibiotic synthesis on rhizosphere competence is thought to be strain specific.

A considerable number of studies have been conducted on the non-target effects on indigenous microflora following the introduction of biocontrol pseudomonads such as strains F113 [19], CHA0 [20], DR54 [21], and 06909 [22]. Of particular relevance to this work are three studies conducted with strain WCS358r, which was modified to produce either PCA or DAPG in the rhizosphere of wheat [17,18,23]. The purpose of our study was to determine the population dynamics and non-target effects in the wheat rhizosphere of the DAPG producer *P. fluorescens* Q8r1-96 and its recombinant derivative Z30-97, which contains the PCA biosynthetic locus introduced into the chromosome and has been shown to generate both PCA and DAPG [6,24]. Strain Q8r1-96 belongs to genotype D, one of 17 genotypes identified among DAPG-producing *P. fluorescens* strains by whole-cell repetitive sequence-based-polymerase chain reaction (rep-PCR) [25,26]. D-genotype strains such as Q8r1-96 are highly unusual among known rhizobacteria because, after being introduced onto seed or soil at low doses, they maintain large population sizes (at least log 5 CFU/g of root) in the rhizosphere as long as wheat or pea are grown ([26–28], B.B. McSpadden Gardener, S. Blouin Bankhead, A. Brown, K. Schroeder and D.M. Weller, unpublished data). This high level of rhizosphere competence on wheat is a major reason why they are primarily responsible for the natural biological control of take-all disease of wheat in the State of Washington known as take-all decline [29]. The recombinant strain Z30-97 also has enhanced biocontrol activity against Rhizoctonia root rot [24], a root disease that Q8r1-96 is ineffective at controlling.

As a first step toward assessing the impact of Z30-97 on the rhizosphere microflora and as a prelude to field testing, we conducted cycling experiments in growth chambers involving multiple growth cycles of wheat after introducing the test bacteria only once prior to the initial planting. We report that Q8r1-96 and Z30-97 are equally rhizosphere competent when introduced alone, but the wild type strain will tend to displace the recombinant when the two strains are co-inoculated. Furthermore, application of either the wild type or recombinant strains caused only small and transient disturbances in the structure of bacterial communities that colonize growing wheat roots.

## 2. Materials and methods

### 2.1. Soil and plants

Shano sandy loam was collected from an uncropped site covered by native vegetation near Quincy, Washington. The soil was collected in early December 2001 from the upper 30 cm of the soil profile, air dried at room temperature for one week, and sieved through a mesh screen (0.5 cm) before use. The spring wheat (*Triticum aestivum* L.) cultivar ''Penewawa'' was used throughout this study.

### 2.2. Bacterial strains and maintenance

All reagents were from Sigma Chemical Co. (St. Louis, MO) unless noted otherwise. Three biocontrol strains were used in this study: *Pseudomonas fluorescens* Q8r1-96, 2-79, and Z30-97 [24,30,31]. Strain 2-79 produces phenazine carboxylic acid (PCA) [32] and strain Q8r1-96 produces DAPG [30]. Both strains are biocontrol agents of take-all disease of wheat [30,31]. Spontaneous rifampicin-resistant derivatives of 2-79 and Q8r1-96 were used throughout this study. An additional variant of Q8r1-96, resistant to naladixic acid ($Q8r1\text{-}96_{nal}$) was used in competition assays with the recombinant strain Z30-97. Strain Z30-97 was obtained from L.S. Thomashow and constructed as described by Huang et al. [24] by inserting the seven-gene *phz* locus into the rifampicin-resistant strain of Q8r1-96. The strains were maintained in one-third strength King's medium B supplemented with ampicillin (40 μg/ml), chloramphenicol (13 μg/ml), and cycloheximide (100 μg/ml) ($KMB^{+++}$) as described by McSpadden Gardener et al. [33]. All strains were stored at −80 °C in one-third strength media with glycerol. To prepare inoculum for addition to the soil, cells were grown on 1/3 $KMB^{+++}$ agar with rifampicin (100 μg/ml) ($KMB^{+++r}$) or rifampicin and nalidixic acid (100 μg/ml) ($KMB^{+++rn}$). Bacteria were recovered from the rhizosphere using broth of the same media as described below.

### 2.3. Soil inoculum and plant cultivation

To prepare bacterial inoculum for introduction into the soil, cells were taken from −80 °C storage and streaked onto 1/3 $KMB^{+++r}$ agar. The plates were incubated at 25 °C for 48 h. Cells of a strain were thoroughly washed to minimize the carry over of PCA or 2,4-DAPG by adding a loopful of bacteria to a microcentrifuge tube prefilled with 1 ml molecular biology grade water. The suspension was then vortexed and spun at 8000*g* for 4 min. The supernatant was discarded, and the tubes were refilled with 1 ml water. The suspension was vortexed to resuspend the bacterial pellet, and 200 μl of the suspension was added to 800 μl of water. The new bacterial suspensions were vortexed and 100 μl in duplicate were added to a 96-well plate. Cell densities were determined by turbidity at $A_{600}$ with a microplate spectrophotometer (Dynatech MR5000; Dynatech Laboratories, Burlington, MA) to determine the amount of bacteria of each strain to add to the soil. Bacteria were added to the soil as described by Landa et al. [26]. Briefly, cells were resuspended in 1% methylcellulose and introduced into the soil to yield a final population density of log 4 CFU per g. The actual density in the soil was confirmed by assaying soil samples before planting. Square PVC pots (8 cm high, 7.5 cm wide) were filled with 200 g of soil inoculated with a single strain or 1% methylcellulose alone (control) immediately prior to planting. A total of eight pots were inoculated with each strain in each of two series of experiments, and each pot served as a replicate. In parallel, eight additional pots were inoculated with Q8r1-$96_{nal}$ and Z30-97 to a final density of log 4 of each strain per g, to study the competition between the strains.

Ten wheat seeds (cv. Penewawa) were sown into each pot and covered with a 1 cm layer of non-inoculated soil. Plants were grown in a growth chamber at 15 ± 1 °C and 40–60% relative humidity with a 12 h photoperiod. Pots were watered every other day and received fertilizer (Miracle Grow 1.0 g/l, 15-30-15, Scott's Miracle Grow Products, Port Washington, NY) two times per week. The plants were harvested after three weeks. At the end of the cycle, the contents of each pot was decanted into a plastic bag. In the bag, the plants were removed from the soil and gently shaken to remove loosely adhering soil from the roots. One wheat plant was selected randomly from each pot, and the roots with adhering rhizosphere soil were severed from the plant and processed as described below. Two series of experiments were performed in this study. In the first series, the soil was allowed to remain "fallow" for one week after harvest prior to planting the next cycle. The soil was placed in the growth chamber in open bags and allowed to air dry. In the second series, the soil was immediately sown after harvest. The process of growth, harvesting, and determination of population densities was repeated five times (cycles).

### 2.4. Enumeration of bacterial populations

Roots were processed as described by Landa et al. [26]. Roots plus tightly adhering rhizosphere soil from single plants were placed into 50 ml screw cap centrifuge tubes containing 15 ml of sterile, distilled water. Rhizosphere inhabiting bacteria were dislodged from the roots by vortexing and subsequent incubation in a sonication bath. Population densities were enumerated using the PCR-based dilution endpoint method described by McSpadden Gardener et al. [33].

Briefly, 100 μl per well of the rhizosphere root washes were serially diluted 1:3 in microtiter plates prefilled with 200 μl sterile, distilled water per well. Twenty-five μl of each dilution was then transferred to a well of a 96-well plate containing either one-tenth strength tryptic soy broth plus 100 μg/ml cycloheximide (1/10 $TSB^{+}$) (for estimating total culturable heterotrophs), 1/3 $KMB^{+++r}$ (for estimating root colonization by 2-79, Q8r1-96 and Z30-97) or 1/3 $KMB^{+++rn}$ (for Q8r1-$96_{nal}$ in competition assays). The microtiter plates were incubated at room temperature in the dark. Plates were assessed for bacterial growth after 48 ± 4 h (1/10 $TSB^{+}$) and 72 ± 4 h (1/3 $KMB^{+++r}$ and 1/3 $KMB^{+++rn}$). Bacterial growth was assayed spectrophotometrically ($A_{600}$) with an optical density of 0.05 scored as positive. These media plates were then stored at −80 °C for further analysis. The population densities of the introduced strains were determined from the highest dilution scored positive for growth, termed the terminal dilution culture (TDC). Aliquots of the TDCs were used to verify the identities of the inoculant strains by PCR analysis. A portion of the *phlD* gene present in Q8r1-96 and Z30-97 was amplified with the primers B2BF and BPR4 [33], and a portion of the *phz* locus present in 2-79 and Z30-97 was amplified using the primers PCA2a and PCA3b [6]. In the parallel experiments where the wild type and transgenic strains were coinoculated, Q8r1-$96_{nal}$ was enumerated as described above based on growth in $KMB^{+++rn}$ media, while the Z30-97 populations were determined using a PCR-based enumeration of $phlD^{+}$ $phzBC^{+}$ bacteria growing on 1/3 $KMB^{+++r}$. In all cases, the dilutions scored positive for growth of Z30-97 were found to be positive for the assayed portions of both the *phlD* gene and the *phz* gene cassette.

### 2.5. Terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rDNA

Rhizosphere bacterial populations were assayed over five different cycles (i.e. 1–5) in two independent series. Rhizobacterial population structure was characterized following the method of McSpadden Gardener and Weller [34]. Briefly, rhizosphere washes were serially

diluted 1:3 in distilled water. Plates were frozen at −80 °C promptly after dilution and thawed at room temperature immediately prior to use as a template. A volume of 2.5 μl of the first dilution was used as template for PCR-based amplification of the 16S rDNA sequences using primers 8F-HEX and 1492R. The amplification products were digested with the restriction enzymes *Msp*I and *Rsa*I (New England Biolabs, Beverly, MA) and the HEX-labeled terminal restriction fragments (TRFs) were separated on an ABI 373 sequencer (Applied Biosystems, Forest City, CA). GeneScan software 2.1 (Applied Biosystems) was used to evaluate the results. The relative abundance of the defined TRFs were determined using the peak areas of signals presented by the GeneScan software. Two criteria were used to define TRFs in these assays. First, a threshold fluorescence of 2× the background noise (which ranged from 8 to 11 fluorescent units) was used to rapidly screen the profiles for the presence of uniquely sized TRFs. Second, the definition of a unique ribotype was limited to size classes that could be clearly distinguished from one another upon replicate gel runs. The limits used to define size classes were ±1 bp for TRFs < 300 bp, ±2 bp for TRFs between 300 and 600 bp, and ±4 bp for TRFs > 600 bp in size. Because of the natural variation in bacterial population structure between replicates of the same treatment, comparisons between treatments were performed only when TRFs appeared in a minimum of 4 out of 8 replicate samples of at least one treatment. This criteria assures that if differences in median peak areas are observed, the significance of those differences can be calculated using the Mann–Whitney test statistic ($P \leqslant 0.10$ for all cases). Online analyses of TRF identity were conducted using the TAP TRFLP software (http://rdp.cme.msu.edu/html/TAP-trflp.html#program).

### 2.6. Statistical analysis

The abundance of cultured populations was compared with STATISTIX (version 7, Analytical Software, St. Paul, MN). Standard one-way analysis of variance was used to determine differences in population densities, while mean comparisons among treatments were performed using Fisher's protected least significant difference test. Differences in the relative abundance of fluorescently-labeled TRFs were compared using Mann–Whitney test statistics for each set of peak areas.

## 3. Results

### 3.1. Rhizosphere colonization by wild type and transgenic inoculant strains

To determine the relative importance of antibiotic producing ability to rhizosphere colonization by the inoculant strains, the abundance of inoculant populations in the rhizosphere of 3-week old wheat seedlings was assayed at the end of each cycle. Though inoculated into the soil at only log 4.0 CFU per g, each bacterial strain colonized the rhizosphere of wheat to population densities between log 6.5 and log 8.6 by the end of cycle 1 (Fig. 1). These levels were two orders of magnitude higher than those attained by the otherwise uncharacterized, indigenous rifampicin-resistant bacteria that were detected in our assays. In both series of experiments, *Pseudomonas fluorescens* strain 2-79 colonized to a significantly lower population level than Q8r1-96 ($P < 0.05$). The populations of strain Q8r1-96 and Z30-97 generally were equal in each of the 5 cycles, and these near-isogenic strains tended to maintain their populations above log 5 CFU per g of root throughout the experiment. In contrast, populations of strain 2-79 tended to diminish more dramatically after the first cycle. The presence of the *phz* genes in Z30-97 did not appear to have a significant effect on its colonization ability. In 8 out of the 10 cycles examined, there was no significant

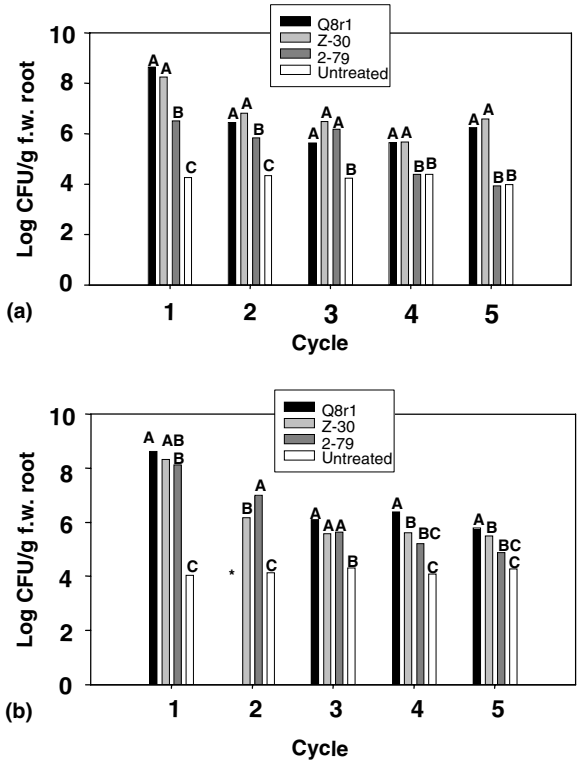


Fig. 1. Population dynamics of biocontrol inoculant strains in the rhizosphere of wheat. Each strain was introduced at approximately log 4 CFU per g of soil at the beginning of the first cycle, and the abundance of root-colonizing populations was determined after each three-week cycle. Mean abundance values ($n = 8$) of the log CFU per g of root are presented. For each cycle, means with the same letter are not significantly different ($P < 0.05$) by Fisher's LSD test. The data obtained from the first series of experiments (panel (a)) and the second series of experiments (panel (b)) are shown separately. The asterisk (*) in panel (b), cycle 2 indicates a processing error which prevented data collection.

difference in the abundance of wild type Q8r1-96 and transgenic Z30-97 populations in the rhizosphere.

The patterns of colonization also differed somewhat between the two series of experiments. In the first series, the population sizes of Q8r1-96 and Z30-97 were generally similar (Fig. 1(a)), but, in the second series, the populations of Z30-97 were significantly less abundant than those of Q8r1-96 after the fourth cycle (Fig. 1(b)). Such differences may have resulted from a change in the experimental protocol (i.e. where the soil remained fallow for a week before re-sowing in the first series but was re-sown immediately after harvest in the second series).

### 3.2. Relative colonization ability of co-inoculated wild type and transgenic strains

In order to more fully determine the impact of the *phz* transgenes on the rhizosphere competence of *P. fluorescens* strain Q8r1-96, a parallel series of cycling experiments (in which Q8r1-96$_{nal}$ and Z30-97 were co-inoculated into soil) were examined (Fig. 2). After the first cycle, both populations colonized the rhizosphere of wheat to approximately log 8 CFU per g. However, no significant differences ($P > 0.05$) were detected in the abundance of the wild type and transgenic populations until subsequent cycles when the wild type strain became more dominant. Populations of Q8r1-96$_{nal}$ were significantly higher ($P < 0.05$) than those of Z30-97 by the end of cycle 2 in series 1 and by the end of cycle 4 in series 2. In series 2 cycle 2, the mean population size of Z30-97 was greater than that of the Q8r1-96$_{nal}$ (Fig. 2(b)) however, it should be noted that a single sample with an inordinately large Z30-97 population (replicate number 6) in that cycle was responsible for that non-significant difference.

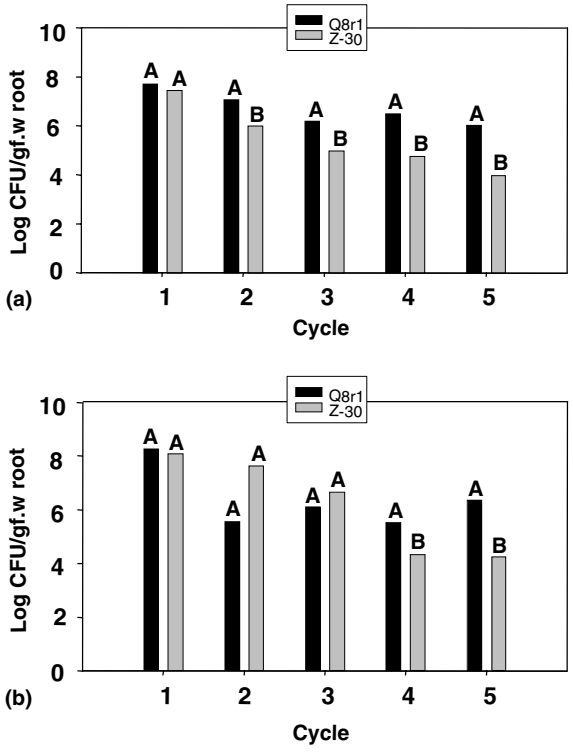


Fig. 2. Population dynamics of the wild type and transgenic *Pseudomonas fluorescens* strains, Q8r1-96$_{nal}$ and Z30-97, following co-inoculation. The two strains were introduced into the soil in a 1:1 ratio at a final population density of log 4 CFU per g of soil for each. Mean abundance values ($n = 8$) of the log CFU per g of root are presented. For each cycle, means with the same letter are not significantly different ($P < 0.05$) by Fisher's LSD test. The data obtained from the first series of experiments (panel (a)) and the second series of experiments (panel (b)) are shown separately.

### 3.3. Impact of inoculation on rhizobacterial population structure

Terminal restriction fragment length polymorphism (T-RFLP) analyses of amplified eubacterial 16S sequences was used to characterize the impact of the introduced strains on the structure of bacterial populations colonizing the roots of wheat seedlings. Visual inspection of the full-scale profiles showed that the profiles generated were remarkably similar across treatments, although one or more differences could be observed in the profiles coming from each individual cycle (Fig. 3). Fine-scale evaluations of the profiles provided the basis for making statistical comparisons of the abundance of defined size classes of TRFs among treatments (Fig. 4). Close inspection of the multiple profiles revealed that portions of the spectra were shifted occasionally due to sizing miscalls by the software. In those instances, the topology of the majority of replicates in the surrounding region was used as a guide in determining signal identity. In Fig. 4, one such instance is shown where signals located at 304 and 309 bp were interpreted to be R307 and R311 in the analysis. Over a dozen peaks, corresponding to distinct TRF size classes, were observed in each sample profile. For any given cycle, there were 13–18 TRFs in the *Msp*I-generated profiles and 10–15 TRFs in the *Rsa*I-generated profiles. Across all cycles, there were over two dozen distinct TRFs generated with each enzyme.

In most cycles, the large majority of defined TRFs did not differ significantly in abundance among the treatments. Typically, only one to three TRFs differed significantly in abundance (i.e. median fluorescent peak areas). One notable exception to this lack of disturbance was observed. In cycle 3 of series 1 over one-third of the identified TRFs differed among treatments (Figs. 3 and 4). In that cycle, differences were found in eight *Msp*I-generated signals and four *Rsa*I-generated signals ($P < 0.10$ for each). In nine of those cases, the median signal size differed between the two wild type treatments (i.e. 2-79 and Q8r1-96). M72, M88, and M498 were

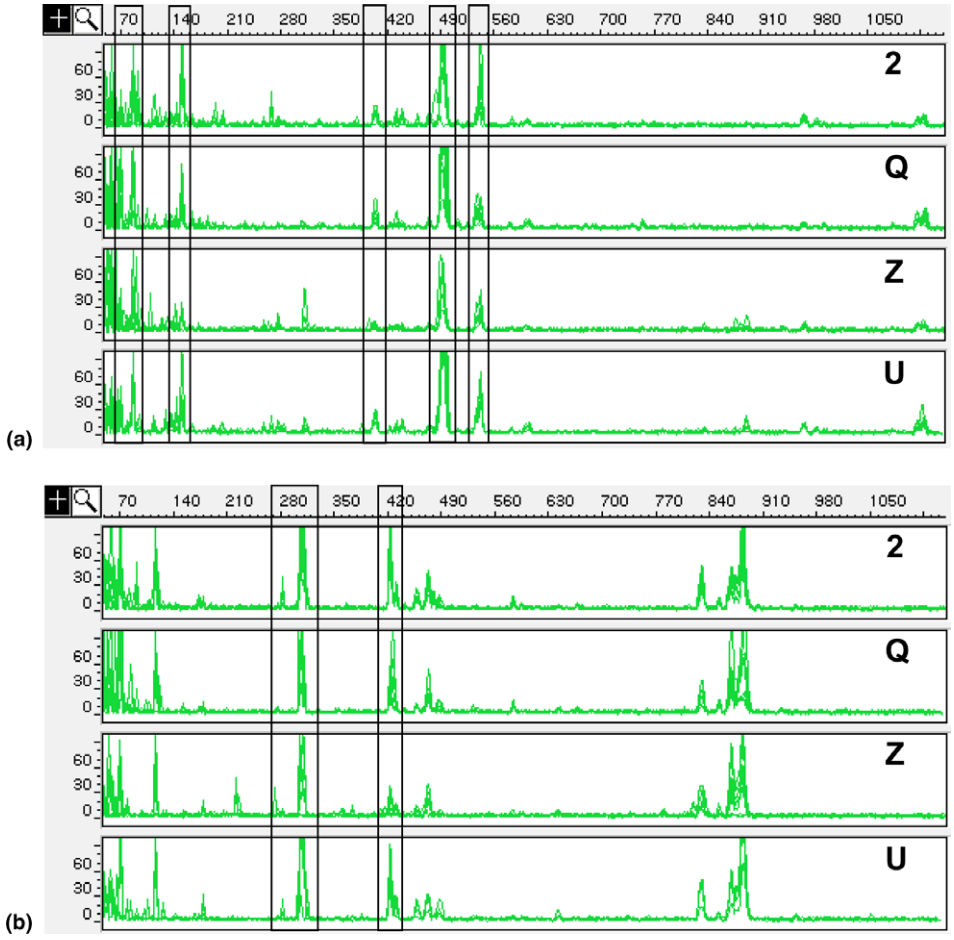


Fig. 3. Full spectrum T-RFLP profile of 16S sequences amplified from bacterial populations inhabiting the rhizosphere of wheat grown in soils inoculated with different biocontrol strains. Soil was inoculated with $10^4$ CFU per g of soil with *Pseudomonas fluorescens* strain 2-79 (2), Q8r1-96 (Q), Z30-97 (Z) or left untreated (U). Data from cycle 3 of series 1 are shown. The T-RFLP profiles were generated from amplified 16S sequences digested with either *Msp*I (panel (a)) or *Rsa*I (panel (b)). In each panel, overlaid chromatographic traces from multiple independent samples ($n \geqslant 6$) are displayed. The size of each TRF in base pairs is indicated by the horizontal scale at the top of the GeneScan results display, and the relative abundance of each was determined using the peak area given in arbitrary fluorescence units on the vertical scale. Regions of the profile containing TRFs whose abundance (i.e. median peak areas) differed significantly ($P < 0.10$) among one or more treatments are outlined by boxes.

significantly more abundant in the profiles of the Q8r1-96-treated samples while M151, M153, M544, R284, R311, R316, and R424 were more abundant in the 2-79-treated samples.

The abundance of relatively few of the TRFs detected changed over the course of these cycling experiments. Across the entire study, only 12 of the *Msp*I- and 9 of the *Rsa*I-generated TRFs were found to differ in abundance among treatments in at least one cycle. The identity of those TRFs, the frequency of their occurrence, and the patterns of their relative abundance in the four different treatments are shown in Table 1. Most of these TRFs were detected in multiple cycles of each cycling series. However, no significant differences in their abundance were noted in the majority of those cycles where they were detected. One interesting exception was R307, a signal that varied among treatments in 5 of the 7 cycles in which it was detected (Table 1). In total, 41 instances were noted where the abundance of a TRF differed significantly ($P < 0.10$) between two or more treatments. These instances were almost evenly split between the two sets of profiles; 21 in *Msp*I- and 20 in *Rsa*I-generated profiles. In 29 of these 41 instances, significant differences in population structure were observed between the uninoculated control and one or more of the inoculated treatments. For 12 of the 21 TRFs noted, significant differences were observed in multiple cycles, but the patterns of variation were consistent for only four of those twelve TRFs (M492, M544, R284, and R316). In those four cases, the abundance of the TRFs was greater in the profiles of the untreated samples as compared to those treated with Z30-97 (Table 1). This pattern was also observed in three of the nine instances where differences in the abundance of a

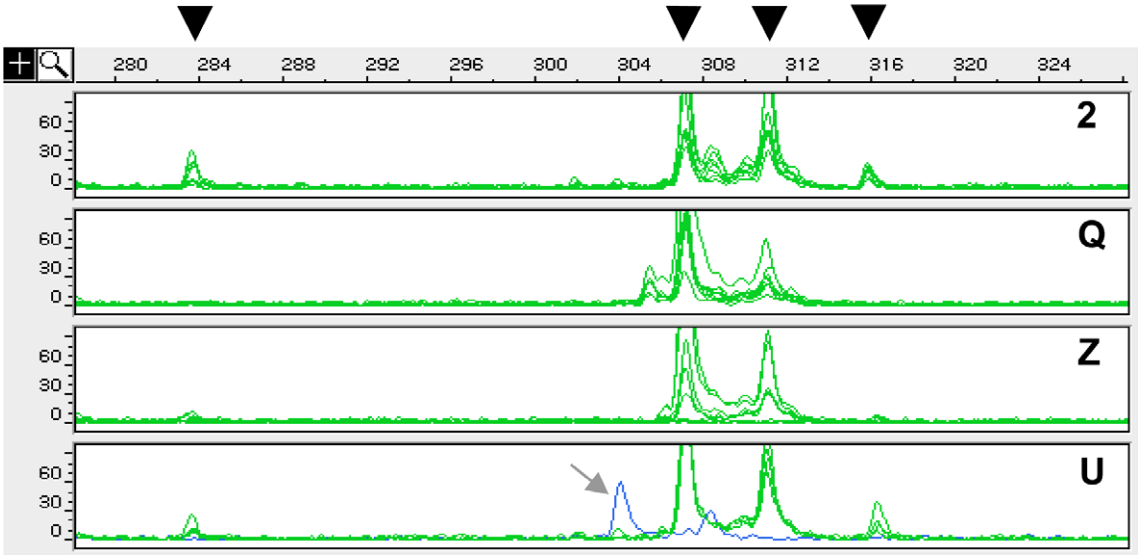


Fig. 4. Enlarged view of T-RFLP profile data presented in Fig. 3(b). Samples, conditions, and labeling are as described for Fig. 3, except that only profiles of *Rsa*I-generated TRFs ranging in size from 278 to 328 bp are shown. The large arrows at the top of the figure indicate TRFs that differ in abundance ($P < 0.10$) among one or more treatments. The small arrow in the bottom panel indicates the one sample profile that was interpreted to be improperly sized in this region.

single TRF in a single cycle occurred. Overall, changes in the abundance of individual TRFs among treatments were not consistently associated with inoculation, genomic background, or antibiotic biosynthesis genes (i.e. for DAPG and/or PCA) carried by the inoculant strain.

### *3.4. Likely sources of the TRFs responding to inoculant treatments*

The complex nature of the bacterial communities profiled precluded the identification of species based on

Table 1
Terminal restriction fragments differing in abundance among treatments

| Digest[a] | TRF | *N*[b] | Cycles[c] where significant differences were observed | Cycles[c] where no significant differences were observed | Significant differences between treatments when observed[d] | Overall impact |
|---|---|---|---|---|---|---|
| *Msp*I | M72 | 6 | 1.3, 2.1 | 1.1, 1.2, 1.5, 2.2 | Q > 2, Z, Z > U,2 | No pattern |
| | M88 | 7 | 1.1, 1.3, 2.4 | 1.2, 1.5, 2.2, 2.3 | 2,Q > U, Q > Z, U > Z,Q | No pattern |
| | M144 | 6 | 2.3 | 1.1, 1.2, 1.3, 1.5, 2.2, 2.4 | 2,Q,Z > U | |
| | M151 | 7 | 1.3, 1.5, 2.3 | 1.1, 1.2, 2.2, 2.4 | 2 > Q,Z, U > Q,2, Z > U,2 | No pattern |
| | M153 | 7 | 1.3, 1.5 | 1.1, 1.2, 2.1, 2.2, 2.3 | 2 > Q,Z, U > Q,2 | No pattern |
| | M205 | 2 | 1.2 | 1.5 | 2 > Q,Z | |
| | M404 | 8 | 1.3 | 1.5, 2.1, 2.2, 2.3, 2.4 | 2,Q,U > Z | |
| | M407 | 8 | 1.3 | 1.5, 2.1, 2.2, 2.3, 2.4 | 2,Q,U > Z | |
| | M492 | 8 | 2.2, 2.4 | 1.1, 1.2, 1.3, 1.5, 2.1, 2.3 | U > Z, U > Z,Q | U > Z |
| | M494 | 7 | 2.2 | 1.1, 1.2, 1.3, 2.1, 2.3, 2.4 | U > Z | |
| | M498 | 5 | 1.3 | 1.5, 2.1, 2.3, 2.4 | Q > Z | |
| | M544 | 8 | 1.3, 2.2, 2.4 | 1.1, 1.2, 1.5, 2.1, 2.3 | 2 > Q,Z, U > Z,2, U > Q,Z | U ⩾ Z |
| *Rsa*I | R118 | 7 | 1.2, 2.2, 2.4 | 1. 1, 1.3, 1.5, 2.1 | Q,Z > U, U > 2,Q,Z, U > Z,Q | No pattern |
| | R174 | 1 | 1.2 | | 2 > Q,Z | |
| | R284 | 2 | 1.2, 1.3 | | U > Z, 2 > Q,Z | 2,U ⩾ Z |
| | R307 | 7 | 1.1, 1.2, 1.5, 2.2, 2.4 | 1.3, 2.1 | Q > U, Q > 2,Z,U, U > Z,Q, 2,Q > Z, U,2 > Z | No pattern |
| | R311 | 7 | 1.3, 2.1, 2.2 | 1.1, 1.2, 1.5, 2.4 | 2,U > Q,Z, Q > Z,U, U > Z,Q,2 | No pattern |
| | R316 | 2 | 1.3, 1.5 | | 2 > Z,Q, U > Q,Z | 2,U ⩾ Q,Z |
| | R424 | 7 | 1.3 | 1.1, 1.2, 1.5, 2.1, 2.2, 2.4 | 2 > Q,Z | |
| | R431 | 5 | 1.2 | 1.1, 1.3, 1.5, 2.4 | 2 > Q,Z,U | |
| | R870 | 6 | 1.2, 2.4 | 1.1, 1.3, 1.5, 2.1 | Q > Z,U, U > Z,Q | No pattern |

[a] Profiles of amplified terminal restriction fragments (TRF) were generated using either *Msp*I or *Rsa*I for each sample.
[b] The number (*N*) of cycles where the listed TRF was observed to occur in a minimum of five replicates of at least one treatment.
[c] The series (*x*) and cycle (*y*) are noted as $x \cdot y$ for each listing.
[d] Multiple comparisons of the abundance of the TRF between two or more treatments as determined by the Mann–Whitney procedure ($P < 0.05$). Only statistically significant differences are noted. Treatment abbreviations: inoculation with 2-79 (2), Q8r1-96 (Q), Z-30-97 (Z), or uninoculated (U).

the occurrence of equally abundant TRF signals in both *Msp*I- and *Rsa*I-generated profiles. However, an attempt was made to deduce the likely phylogenetic identities of bacteria giving rise to the TRFs noted above based on the level of precision attained in our assays (see Section 2.5). Of the 2374 sequences examined by the TAP TRFLP software, several dozen could be associated with the TRFs listed in Table 1. Most of these were predicted to have come from various genera of Flavobacteria and Sphingobacteria. These included *Flavobacterium* (M88, R307, R311, R316), *Capnocytophaga* (M88, R307), and *Cytophaga* (M88, M544, R310). Closely related genera, including *Gelidibacter*, *Pedobacter*, *Polaribacter*, and *Sphingobacterium* could also give rise to one or more of the identified TRFs. M492 and M494 most likely arose from *Pseudomonas* spp. (R72, R644, and R878), though *Klebsiella* (R882) and *Synechocystis* (R425) cannot be ruled out. M151 could have arisen from one or more bacterial genera, including *Bacillus* (R454), *Caulobacter* (R422), *Paenibacillus* (R487), *Rhodopseudomonas* (R424), or *Sphingomonas* (R422) species, all of which might reasonably be present in the rhizosphere of crop plants. M118 likely represents α or β proteobacteria belonging to the genera *Zoogloea* (M488), *Zymomonas* (R404), or *Sphingomonas* (R604). Also, the abundance of *Mycoplasma*- (M544, R473) and *Ureaplasma*- (R284, M546) like species may have fluctuated in response to the applied inoculants.

### *3.5. Impact of inoculation on the abundance of cultured rhizobacteria*

The abundance of bacteria cultured from wheat roots on 1/10 $TSB^+$ after two days of incubation ranged from log 8.0 to log 9.5 CFU per g (f.w.). In 8 of the10 cycles examined, the abundance of these bacteria did not differ significantly among treatments. Nonetheless, the mean culture counts differed significantly in two instances, series1 cycle 2 and series 2 cycle 3. In both of these cases, the populations of cultured bacteria were significantly lower in those treatments where biocontrol inoculants had been applied ($P < 0.05$). In the latter case, cultured populations were the lowest in the 2-79 treated samples. These differences did not correspond to the number nor the types of differences between treatments noted in the T-RFLP profiles of amplified 16S sequences.

## 4. Discussion

The engineering and deployment of microbial inoculants has led to much interest in the ecological impacts of their release [35,36]. Wild type and genetically modified bacterial inoculants can influence microbial community structure in the rhizosphere, as determined by culture dependent methods [37–39] or culture independent methods that rely on amplification of ribosomal RNA sequences from soil and rhizosphere DNA samples [40–42]. Most studies report measurable changes in microbial community structure, but the magnitude of such changes and their significance to ecological functions remains to be fully characterized. However, other studies found no significant alterations in soil microbial community structure following inoculation with bacterial antagonists of plant pathogens [43]. By definition, functionally significant shifts in microbial community structure have taken place wherever inoculation with a biological control agent effectively reduces plant disease development. However, the magnitude of such shifts in terms of taxonomic diversity and the functional activities of non-target populations is not well understood.

In this study, we characterized the impact of biocontrol strains which produce two very potent antibiotic compounds on the structure of rhizosphere bacterial communities. While the complete suite of antagonistic factors and activities of these strains remains unknown, the production of antibiotic compounds by the inoculant strains can be correlated with the biological control of plant pathogens [2]. Strains producing these compounds have been reported to be effective biocontrol agents of fungal root pathogens, indicating their capacity to disrupt the activities of at least one rhizosphere colonizing population. Recently, the effects of *Pseudomonas putida* strain WCS358r and genetically-modified derivatives capable of synthesizing PCA or DAPG were reported [18,23,44]. Transient changes in fungal and bacterial populations inhabiting the roots of field-grown wheat were observed during the growing season [23,44] and repeated introductions did not significantly increase the level of disturbance [18]. Other groups have shown that wild type PCA and DAPG producers can alter the structure and activities of rhizosphere inhabiting fungi [44–46], nematodes [10,13], and bacteria [7,19,44,47,48], but those same studies indicated that the measured changes were of small magnitude or limited duration. Our results reinforce the interpretation that while statistically significant treatment-specific disturbances in microbial community structure attributable to. antibiotic-producing biocontrol agents can be detected, the nature of such disturbances are not likely to be ecologically significant. In this study, the changes observed were transient, of small magnitude, and not generally reproduced across multiple cycles.

Like all PCR-based community profiling methods, our approach cannot be expected to detect all of the bacterial populations present in an environmental sample [49]. Indeed, relatively few ribotypes were observed in our bacterial community profiles because we used a moderately high-throughput approach to amplify 16S sequences from root washes. Rhizosphere samples were washed in roughly 10 ml of distilled water, but only 2.5 μl of a 1:3 dilution of such washes were used as template

for amplification (see Section 2). While this diluted out substances that can inhibit PCR, only bacterial populations occurring at densities of greater than log 5 cells per g of root could possibly be detected. Genomic DNA was released from bacteria by the combined stresses of an initial osmotic shock, a freeze-thaw treatment, and the high heat of the thermocycling conditions (see Section 2). This approach clearly biases our observations towards the detection of cells whose walls are most easily ruptured. More metabolically active bacterial cells would be preferentially lysed as compared to more dormant subpopulations of the same species, because starved cells and spores can better maintain cell integrity in the face of abiotic stresses such as those applied [50]. Because of differences in cell wall structure, Gram-negative bacteria are expected to lyse more easily than Gram-positive bacteria. In fact, most of the TRFs identified in this study were predicted to come from Gram-negative genera, but a few were consistent with some Gram-positive genera (e.g. *Bacillus*, *Paenibacillus*; see Section 3.4). Template DNA from more resistant bacterial populations may be recovered using harsher extraction methods, but doing so can result in excessive shearing of DNA released from easily-lysed cells [51]. Thus, our approach represents and empirical trade-off. On the one hand, numerically dominant, metabolically active, and Gram-negative bacteria were more likely to be detected in our assays. On the other, less abundant, more dormant, Gram-positive, and endophytic bacteria were less likely to be detected. The full extent and significance of this experimental bias remains unclear and is worthy of further investigation.

Despite such technical limitations, we did note a number of statistically significant differences among treatments in the relative abundance of several bacterial populations (Table 1, Fig. 3). In a number of instances, the abundance of multiple TRFs was reduced following treatment with the transgenic PCA-producing strain, Z30-97 (Table 1). Because this strain has the effective colonization phenotype of Q8r1-96 ([39]; Fig. 1), the production of PCA may have been of sufficient quantity to inhibit the growth of these other bacterial populations to some degree. Interestingly, several of the differences in TRF abundance noted in this study can be related to previous work examining the shifts in bacterial population structure in the rhizosphere of wheat produced following infection by a root pathogen [34]. Several TRFs noted in this study (i.e. M492, M544, R118, R311 and R870) were generally more abundant in uninoculated as compared to one or more of the inoculated treatments (Table 1). All of the significant differences noted were small quantitative variations in the relative abundance of amplified TRFs. While these data may indicate that the antibiotic-producing biocontrol strains had a small degree of antibacterial activity, the inconsistent variation indicates dynamic fluctuations were more significant than the impact of the inoculants themselves. Most of the TRFs whose abundance was reduced in the inoculated treatments were predicted to have arisen from Flavobacteria and Sphingobacteria (see Section 3.4). Populations of these bacteria may be particularly sensitive to perturbations in the soil and rhizosphere environments. Interestingly, similar populations of bacteria were previously shown to be more abundant on diseased as compared to healthy wheat roots [34]. Therefore, these data may also indicate that, in several cycles, the net effect of bacterial inoculants used in this study (especially Q8r1-96 and Z30-97) was to suppress the development of unobserved root infections. The soil used in this study had not been previously cropped to wheat and no root pathogens had been deliberately introduced. Therefore, we think that weakly virulent fungi were causing unobserved infections under our assay conditions. Because we examined an environment (i.e. the wheat rhizosphere) that was well colonized by the inoculant strains, we expect even less of an impact in the bulk soil where residual inoculant populations are expected to be less. Residual impacts of DAPG-producing inoculants on soil rhizobial populations have been noted, but they were found to be of lesser magnitude than alternative chemical treatments [48]. Therefore, we do not foresee any significant environmental risk arising from the use of Q8r1-96 or Z30-97 as biocontrol seed treatments.

The impact of introducing the *phz* genes from strain 2-79 into the genomic background of Q8r1-96 was also small but definable in terms of rhizosphere competence of the inoculant strains. Strain Q8r1-96 was chosen for this study because it is one representative of the D genotype of DAPG producers [25]. This genotype has been noted for its high degree of rhizosphere competence in the rhizosphere of wheat and other field crops ([26,28], B. McSpadden Gardener, D.M. Weller, unpublished data). We observed only minimal impact on the colonization ability of Z30-97. Any deleterious load imparted by the *phz* insertion may have been offset by the benefit of producing the antibiotic. Indeed, a positive correlation between rhizosphere colonization and PCA production has been reported for *P. fluorescens* strain 2-79 [16]. It is especially notable, however, that when Q8r1-96 and Z30-97 were co-inoculated, the wild-type strain had a tendency to out compete the recombinant strain. No doubt both strains share the same niche in the rhizosphere of wheat and research is ongoing as to the nature of this competition. The ability of the strains with the D genotype background to persist and colonize wheat roots makes it an excellent vehicle for the introduction of added traits related to the biological control of plant pathogens. The minimal impact of the wild type and transgenic strains on rhizosphere bacterial populations suggests that introductions of such

strains into the environment may result in improved biological control because the beneficial effects of indigenous bacteria will not be compromised.

The ecological significance of altering the structure of soil and rhizosphere microbial populations remains unclear. On the one hand, changes that reduce the abundance and activities of soil pathogens are desirable, but changes that alter other mutualistic associations or biogeochemical cycling may be undesirable. While such issues may appear to be particularly relevant to the application of new biotechnologies, they are equally relevant to the application of more conventional agricultural practices. Indeed, research has shown that a number of managed and unmanaged variables can have significant effects on microbial community structure and activities, including soil structure [52], fertilizer use [53], crop species [54], host genotype [46], host development [55], and climate [56]. However, the evidence for significant, reproducible, and lasting disturbances in rhizosphere microbial community structure is generally lacking, and that seems to indicate the tremendous resilience of the microbiological communities in agricultural systems to human disturbance [57]. The data presented in this study support the view that the introduction of transgenic, biocontrol *Pseudomonas* strains presents no significant a priori risk to non-target bacterial populations in the rhizosphere of treated wheat. Further investigation of the potential of these strains to influence microbial community structure and functioning under field conditions is currently underway (S. Blouin Bankhead, A. Brown, and D.M. Weller, unpublished data).

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