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Application of PCR primer sets for detection of *Pseudomonas* sp. functional genes in the plant rhizosphere

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ABSTRACT

Plant growth promoting pseudomonads play an important role in disease suppression and there is considerable interest in development of biomarker genes that can be used to monitor these bacteria in agricultural soils. Here, we report the application of a PCR primer sets targeting genes encoding the main antibiotic groups. Distribution of the genes was variably distributed across type strains of 28 species with no phylogenetic grouping for the detected antibiotics genes, *phlD* for 2,4-diacetylphloroglucinol (2,4-DAPG) and *phzCD* for phenazine-1-carboxylic acid or *hcnBC* for hydrogen cyanide production. Analysis of field soils showed that primer sets for *phlD* and *phzCD* detected these genes in a fallowed neutral pH soil following wheat production, but that the copy numbers were below the detection limits in bulk soils having an acidic pH. In contrast, PCR products for the *phzCD*, *pltC,* and *hcnBC* genes were detectable in mature root zones following planting with wheat. The ability to rapidly characterize populations of antibiotics producers using specific primer sets will improve our ability to assess the impacts of management practices on the functional traits of *Pseudomonas* spp. populations in agricultural soils.

Keywords: PGPR (Plant Growth-Promoting Rhizosphere); *Pseudomonas*; PCR; 16S rDNA; Plant-Microbe Interactions

1. INTRODUCTION

Plant growth promoting rhizosphere bacteria improve plant growth through the production of hormones and by suppression of root disease through the production of antibiotics and siderophores. Using culture based methods to identify these bacteria, PGPR have been isolated from plants in soils from diverse geographical regions [1-5]. One of the most important groups of PGPR are strains of antibiotic-producing fluorescent *Pseudomonas* spp. that contribute to suppression of fungal diseases including take-all of wheat, black root rot of tobacco, or fusarium wilt of tomato [6-9]. The genes that are responsible for production of antibiotics are variably dispersed among different genotypes, which vary in their efficacy for disease suppression [5,8,10-12]. Thus there is interest in development of PCR primer sets that can be used to detect the presence of these genes in environmental samples and to monitor the populations of bacteria that carry these genes [6,7,13-15].

Genes encoding the antibiotics pyoluteorin (*pltC*) [1], pyrrolnitrin (*prnD*) [1], phenazine-1-carboxylic acid (*phzCD*) [3], and 2,4-diacetylphloroglucinol (2,4-DAPG, *phlD*) [3] are a major focus of research in biological control of disease by pseudomonads. Other genes that are relevant to biocontrol include the *gacA* and *hcnBC* genes encoding hydrogen cyanide production. The *gacA* gene (global activator of cyanide production) is a response regulator that interacts with quorum regulated genes to control expression of small RNAs and antibiotics in various strains of PGPR pseudomonads [16]. In the PGPR bacterium *P. chlororaphis*, *gacA* also functions as a negative regulator of indole-3-acetic acid hormone production. The genes encoding aminocyclopropane carboxylic acid deaminase (*acc*) are still other potential targets, functioning for the destruction of ethylene [17,18].

Molecular methods for the detection of these genes have been described, but are often utilized in combination with culture based methods to quantify the population sizes of *Pseudomonas* strains carrying specific genes [19]. The threshold population density at which 2,4-DAPG producing strains are effective for disease sup-

pression is 10^5 CFU g^{-1} root for protection against take-all disease caused by *Gaeumannomyces graminis*. 2,4-DAPG producing bacteria collected from various locations from around the world can be further grouped into at least 18 BOX-PCR genotypes that reflect the selection of local genotypes in different soils [20]. In general, the population densities of antibiotic producing *Pseudomonas* are highly dynamic, and generally increase by at least two orders of magnitude in the plant rhizosphere [21] and higher in the vicinity of plant roots than in unplanted soil [22]. The population size of different genotypes is further influenced by intrastrain competition as well as interactions with other rhizosphere bacterial species [23].

With the advent of rapid screening methods for detection of marker genes that are associated with plant health and growth in agricultural soils, we were interested in determining the utility of PCR based methods to detect a suite of genes for plant growth promoting pseudomonads in wheat soils in S.E. Australia where there is still little information on the ecology and importance of these bacteria in soil health. The research reported here examined selected PCR primer sets from the literature for detection of specific marker genes, and the association of antibiotic genes among different type strains of *Pseudomonas* spp. The primer sets were evaluated for three different soil classes representing the major soils used for wheat cultivation in Victoria, Australia. We examined both the field soil and adjacent remnant areas that had not been cultivated and further compared soils that were collected during the fallow period and in the rhizosphere after cultivation with wheat. The results demonstrate the utility of molecular methods to compare soils, but also illustrate the highly dynamic changes that occur in managed soils.

2. MATERIALS AND METHODS

2.1. Soil Description

Soil samples were collected in autumn from three soil classes according to the Australian soil classification system (Isbell, R.F., 2002. The Australian Soil Classification. CSIRO Publishing, Melbourne), including a dermosol (NE Victoria, Rutherglen), a calcarosol (NW Victoria, Birchip) and a chromosol (SW Victoria, Hamilton) collected in southeastern Australia. Within each of these regions, soils were collected from paired sites representing two contrasting land-uses, a "remnant" uncultivated site where indigenous plant species were present and a 'managed' site where the soils were subjected to normal agricultural practices with fertilizer and herbicide input regimes, grazing and cropping activities. Soil chemical and physical characteristics for the six sites are summarized in **Table 1**. Soils were collected during the wheat fallow period and were air-dried and shipped to the University of California, Riverside, for the experimental study. Subsamples of the six soils were planted with wheat (*Triticum aestivum* L. cv. Yecora rojo) in 1 cm thick root boxes, with a detachable side plate, and were watered daily to maintain constant 30% moisture content by weight. The plants were cultivated in a glasshouse under ambient conditions. Soil was collected from the root boxes after 3 weeks and included the bulk soil fraction, and the base of older plant roots and the root tips. Soil samples were passed through a 2-mm sieve to homogenize

Table 1. Land-use and soil chemical and physical characteristics.

Soil class	Location/ landuse (sample code)	pH		EC	C	N	S	C/N	Particle size(%)			Soil	Exchangeable Cations (cmol(+)/kg)				NO_3-N	NO_2-N	NH_4-N	PO_4-P	Fe	Zn	Cu	Mn
		1:1 ($CaCl_2$)	1:5 (H_2O)	(µs/cm)	(%)	(%)	(%)	ratio	Clay	Sand	Silt	texture	Ca	Mg	K	Na	(ppm)				(mg/kg)			
Calcarosol	Birchip/ managed (BM)	6.6	8.5	88	1.62	0.1	0.05	11.9	18.9	53.9	27.3	Sandy loam	3.81	0.63	1.08	8.31	7.25	2.45	0.33	0.58	25,980	31.2	10.6	243.3
Calcarosol	Birchip/ remnant (BR)	6.8	8.4	121	2.72	0.2	0.04	11.9	16.1	57.8	26.1	Sandy loam	3.71	0.54	2.11	5.73	8.08	1.95	0.10	0.33	23,310	26.2	10.0	284.4
Chromosol	Hamilton/ managed (HM)	4.6	4.9	388	2.39	0.2	0.04	11.7	8.3	34.7	56.9	Silt loam	13.60	3.86	2.05	8.21	133.83	UD	6.30	UD	14,370	10.8	5.4	115.2
Chromosol	Hamilton/ remnant (HR)	4.4	5.7	113	4.73	0.4	0.05	12.9	11.0	42.4	46.6	Loam	0.99	0.47	0.56	5.88	16.83	UD	0.18	0.25	42,070	21.5	11.3	291.3
Dermosol	Rutherglen/ managed (RM)	5.9	6.0	204	2.48	0.2	0.03	11.3	8.2	32.2	59.6	Silt loam	9.58	1.64	1.82	4.51	81.03	1.18	1.60	0.63	14,400	16.6	10.6	705.0
Dermosol	Rutherglen/ remnant (RR)	4.3	5.2	75	2.07	0.3	0.02	12.0	13.0	32.2	54.8	Silt loam	0.67	0.16	0.85	1.82	12.23	0.30	1.08	0.05	19,260	25.4	13.0	522.0

the soil fractions, after which the soil and root were processed for DNA extraction.

2.2. Chemical and Physical Analysis

Soil pH and salinity (electrical conductivity, EC) were measured in soil extracts using 1:5 soil water and 1:1 soil water: 1 mM $CaCl_2$ extraction solutions. Exchangeable cations, and heavy metals (iron, zinc, copper, and manganese) were quantified by atomic absorption spectrophotometer (Perkin-Elmer AAnalyst 800). Carbon and nitrogen was analyzed using a C-N-S analyzer (NA 1500 series 2, Carlo Erba). The soil particle size was analyzed using the hydrometer method. Nitrate, nitrite, ammonia and soluble reactive phosphorus were quantified with a Technicon TRAACS 800 Autoanalyzer (Tarrytown, NY).

2.3. DNA Extraction

DNA was extracted from 0.5 g samples of each soil and root using the FastPrep™ Soil DNA Extraction kit (Qbiogene, Carlsbad, CA) according to the manufacturers' recommendations. Five replicates for each soil and root sample were prepared from each soil and were stored at −80˚C until use for subsequent analyses.

2.4. Primers and PCR Reaction Conditions

Table 2 lists the gene targets and PCR primer sets that were used to detect genes encoding the selected antibiotics, regulatory genes, and markers for fluorescent pseudomonads. The 50 µL PCR reaction mixtures consisted of 1 µl of DNA template, 50 mM Tris (pH 8.3), 250 µg of bovine serum albumin (BSA) per ml, 2.5 mM $MgCl_2$, 200 nM deoxynucleoside triphosphates, 200 nM each SSU rDNA primer and 3 U of *Taq* DNA polymerase. All primers were obtained from Invitrogen (Carlsbad, CA.). PCR was performed on a PTC 200 (Bio-Rad Laboratories, USA). The PCR program was 5 min at 95˚C; 30 cycles of 30 s at 94˚C, 30 s at 50 or 55˚C, and 1 min at 72˚C; and a final extension for 10 min at 72˚C. Optimal annealing temperatures were decided after gradient PCR or testing two different temperatures, 48˚C and 55˚C. Detection of PCR products was determined for ethidium bromide (EtBr) stained agarose gels using Gel-doc (Bio-Rad) and image analysis using Quantity One (Bio-Rad).

2.5. Phylogenetic Analysis

PCR was conducted with 10 µl reaction volumes containing: 1 µl of DNA, 2 µl of Terminator Ready Reaction Mix, and 200 nM each of T7 and SP6 primers. The reagents were combined and heated to 96˚C for 1 min. Twenty-five cycles of PCR were run at 96˚C for 10 s, 50˚C for 10 s, and 60˚C for 4 min. Approximately 1 kb (position 289-1258 [24] were sequenced using the ABI 377 sequencer (Applied Biosystems, Foster, CA, USA)).

16S rDNA sequences were aligned with greengenes [25,26]. Evolutionary distances were calculated by the method of Kimura 2-parameter and a phylogenetic tree was constructed by the neighbor-joining method [27] with MEGA4 for Windows, including bootstrap analysis [28].

The nucleotide sequence data reported in this paper will appear in the GenBank/EMBL/DDBJ nucleotide sequence databases with the accession number(s) AY860450-AY860454.

3. RESULTS

3.1. Distribution of Antibiotic Genes among *Pseudomonas* Type Strains

PCR primer sets for conserved sequences of genes involved in expression and regulation of six antibiotics were targeted against 28 type strains of *Pseudomonas* spp. Of the six sequences evaluated, antibiotic gene sequences for 2,4-DAPG (*phlD*) and phenazine (*phzCD*) were detected in 3 and 5 of the type strains, respectively (**Figure 1**). In all cases where a positive signal was obtained, the PCR products were of the correct predicted size. Where no signals were obtained, the PCR reactions were rerun at less stringent conditions, but did not produce signals, indicating that the failure to obtain PCR products for those targets were most likely due to low copy numbers rather than base substitutions in the target sequence. Of the antibiotics detected, none occurred simultaneously in the same types strains, nor were there apparent relationships between distributions of the genes with taxonomic groupings by 16S rRNA gene.

PCR products were obtained in all of the type strains with primers for 16S rRNA sequences, and in 25 and 23 of the strains using primers for the proposed phylogenetic classification markers *gyrB* and *rpoD*. The *hcnBC* genes for cyanide production and *gacA* for global activator cyanide, which are involved in a regulatory circuit affecting antibiotic production, were widely dispersed in 8 and 14 of the 28 type strains. In this case, the *gacA* gene was detected in 5 of the 8 type strains having positive signals for *hcnBC*. Marker gene sequences for aminocyclopropane deaminase (*acc*) and indole acetic acid production (*ipdc*) were not detected in any of the type strains tested here.

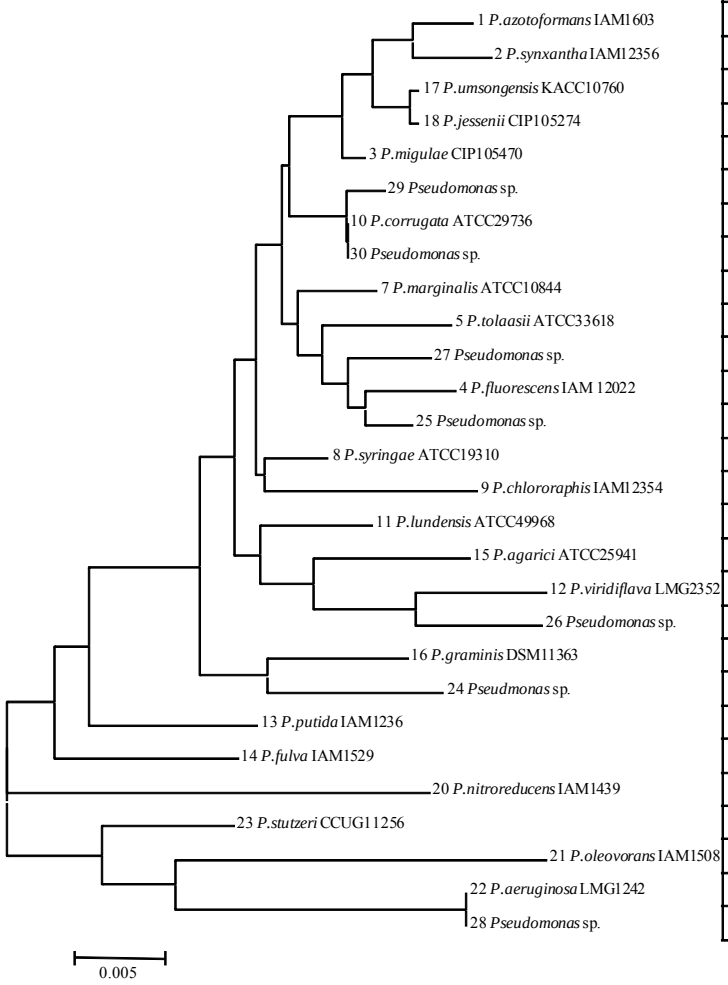
3.2. Detection of PCR Products for Antibiotic Genes of *Pseudomonas* in Soil and Root

The fully set of gene targets for *Pseudomonas* sp. and functional genes were tested in bulk soils and in root samples from wheat for managed and remnant soils

Table 2. Target genes and primers used in PCR analysis of soil DNA.

Target gene	Target group	Primer	Sequence	Length	Reference
phlD (2,4-DAPG, diacetylphloroglucinol)	f. *Pseudomonas*[1]	Phl2a	GAGGACGTCGAAGACCACCA	745 bp	3
		Phl2b	ACCGCAGCATCGTGTATGAG		
phzCD (phenazine-1-carboxylic acid)	f. *Pseudomonas*	PCA2a	TTGCCAAGCCTCGCTCCAAC	1.15 kb	3
		PCA3b	CCGCGTTGTTCCTCGTTCAT		
prnD (pyrrolnitrin)	*Pseudomonas*	PRND1	GGGGCGGGCCGTGGTGATGGA	786 bp	1
	Burkholderia	PRND2	YCCCGCSGCCTGYCTGGTCTG		
pltC (pyoluteorin)	*Pseudomonas*	PLT C1	AACAGATCGCCCCGGTACAGAACG	438 bp	1
	Burkholderia	PLTC2	AGGCCCGGACACTCAAGAAACTCG		
ipdc(IAA, indolepyruvate decarboxylase)	*P. putide*	ipdc-F	GAAGGATCCCTGTTATGCGAACC	1.7 kb	30
		ipdc-R	CTGGGGATCCGACAAGTAATCAGGC		
pupA (pseudobactins)	*P. putide*	PupAf	TAYGARYTBGGBIIIAARG	500 bp	32
		PupAr	RTTVCGNGGIIIVCCRTA		
16S rDNA	f. *Pseudomonas*[1]	Psmn289	GGTCTGAGAGGATGATCAGT	957 bp	24
		Psmn1258	TTAGCTCCACCTCGCGGC		
gacA[2]	f. *Pseudomonas*[1]	gacA1	GBATCGGMGGYCTBGARGC	425 bp	16
		gacA2	MGYCARYTCVACRTCRCTGSTGAT		
acc (ACC[3] deaminase)	*P. fluorescens*	ACC4a	CAGCAGGAAAAGGATTTGGG	850 bp	18
	E. cloacae	ACC4b	ACTCCACTGAATTGAACCCG		
gyrB (DNA gyrase B subunit)	Bacteria	UP-1E	CAGGAAACAGCTATGACCAYGSNGGNGGNAARTTYRA	891 bp	31
		APrU	TGTAAAACGACGGCCAGTGCNGGRTCYTTYTCYTGRCA		
rpoD (Sigma factor70)	Bacteria	70F	ACGACTGACCCGGTACGCATGTAYATGMGNGARATG GGNACNGT	816 bp	31
		70R	ATAGAAATAACCAGACGTAAGTTNGCYTCNACCATYTCYT-TYTT		
hcnBC (hydrogen cyanide)	*Pseudomonas*	ACa	ACTGCCAGGGGCGGATGTGC	587 bp	33
		ACb	ACGATGTGCTCGGCGTAC		

[1]fluoresecent *Pseudomonas*; [2]global activator of cyanide and antibiotic production; [3]1-aminocyclopropane-1-carboxylic acid.



	phlD	*phzCD*	16S	*gacA*	*gyrB*	*rpoD*	*hcnBC*
1			O		O	O	
2		O	O		O	O	
17			O	O	O	O	O
18	O		O	O	O	O	
3			O	O	O	O	O
29			O		O		O
10		O	O	O	O	O	O
30			O		O		
7			O	O	O	O	
5		O	O	O	O	O	
27			O		O	O	
4			O	O	O	O	
25			O		O	O	
8			O		O	O	
9			O	O	O	O	
11			O		O	O	
15			O		O		
12		O	O		O	O	
26			O	O	O	O	
16			O		O	O	O
24			O		O	O	O
13			O		O	O	
14		O	O	O	O	O	O
20			O				
23	O		O	O	O	O	O
21	O		O	O	O	O	
22			O	O		O	
28			O	O			

Figure 1. Phylogenetic analysis of type strains and PCR signals detected by amplification with primers for a range of target genes.

represented 3 soil geomorphic classes in Victoria, Australia. These soils were of similar texture, but varied in pH and organic carbon (**Table 1**). Among the antibiotic genes, PCR products were obtained for *phlD* and *phzCD* in bulk soils from Birchip (neutral pH soil) but were not detected in the two acid soils. In contrast, positive signals were obtained for these gene products in all of the soils following growth of wheat plants for 3 weeks, indicating that the copy number increased to detectable levels in the plant root. The *phlD* gene was detected in both mature root zones and in the root tip region. In contrast, the *phzCD* gene was detected only in mature root zones, and not at the root tip. This same pattern was observed for *pltC*. In this case, the *pltC* gene was not detected in any of the fallow bulk soil samples, but was detected in mature roots for all three soil classes. The *prnD* gene was an anomaly, producing, with the PCR primers for this gene producing multiple products of variable size in all of the soils tested, even under high stringency melting conditions (67˚C) during the PCR reaction.

The *hcnBC* gene was detected in two of the six bulk soil samples, and in mature root zones in all three soil classes. The *gacA* gene was not detected in any of the bulk or root samples, nor were the two growth regulator genes, the *ipdc* and *acc* genes were not detected in any of the root samples. The phylogenetic group marker 16S rRNA gene target for *Pseudomonas* was detected in all soil samples, but the *gyrB* and *rpoD* genes were not detected in any of the soil samples.

4. DISCUSSION

The objective of this study was to evaluate a selected set of gene targets for detection of *Pseudomonas* sp. and functional genes for antibiotic production, regulation of cyanide and antibiotic production, and plant growth hormone production across different soil types representing a range of important soils used for wheat production in Australia. There is considerable interest in developing molecular methods to monitor these and other species of plant growth promoting bacteria as part of a strategy to

monitor soil health and the impacts of various management practices on the population densities of PGPR bacteria. Previous research has identified a number of candidate genes that are potential markers for antibiotic-producing *Pseudomonas* spp. It has also been shown that there is considerable variation in genotypes of specific antibiotic-producing *Pseudomonas* that vary in their ability to colonize the rhizosphere (Bergsma-Vlami *et al*., 2005). In general, population densities of pseudomonads are highly dynamic in soils and are highest in the plant rhizosphere, but are also influence by the host plant species [6]. To characterize the abundance and distribution of these marker genes in the literature, we used PCR-based methods to screening for the antibiotics, 2,4-DAPG (*phlD*) [3], phenazine-1-carboxylic acid (*phzCD*) [3], pyoluteorin (*pltC*) [1], pyrrolnitrin (*prnD*) [1], and hydrogen cyanide (*hcnBC*) [29]. Other gene targets included indole-3-acetic acid (*ipdc*) [30] and ACC deaminase (*acc*) which degrades a precursor for ethylene [18]; *gacA*, the global activator of cyanide and antibiotic production [16], and phylogenetic group markers (*gyrB*) [31], RNA polymerase beta-subunit encoding gene (*rpoB*) [31], and 16S rRNA genes with *Pseudomonas* specific primers [24]. Our results showed that among the antibiotic genes targets that were evaluated, the two that were detected with pure cultures, *gyrB* and *rpoD*, were broadly distributed among types strains of the 25 and 23 different *Pseudomonas* species that were tested in pure culture. The broad distribution of these genes in different species illustrates the difficulty of monitoring PGPR based solely on tracking species using 16S rRNA genes or other phylogenetic markers. In this manner, monitoring of PGPR may be better achieved by tracking the presence and copy number of specific functional genes. The *phlD* and *phzCD* genes were readily detected all three of the field soils, but detection was enhanced by use of root samples and was further influenced by sample location in the mature roots or the root tips. The *phlD* gene was found in all locations in the root in all of the soils, whereas the *phzCD*, *pltC* and *hcnBC* genes were detected only in mature root zones (**Table 3**). Altogether, these data suggests that sampling procedures must be highly standardized for using the presence, absence, or copy number of specific marker genes as parameters for measuring soil health and the impact of management practices on PGPR bacteria. The method used here in which wheat plants were cultivated in the soil for a defined period of time provides a standardized condi-

Table 3. PCR signals detected using plant growth promoting and population specific-primers.

Soil sample	***phlD***	***phzCD***	***prnD***	***pltC***	***hcnBC***	**16S rDNA**
Birchip/ managed(BM)	+	+	+			+
Birchip/ remnant (BR)	+	+	+		+	+
Hamilton/ managed (HM)			+			+
Hamilton/ remnant (HR)			+			+
Rutherglen/ managed (RM)			+		+	+
Rutherglen/ remnant (RR)			+			+
Root sample	***phlD***	***phzCD***	***prnD***	***pltC***	***hcnBC***	**16S rDNA**
BM-tip	+		+			+
BM-base	+	+	+	+	+	+
BR-tip	+		+			+
BR-base	+	+	+	+	+	+
HM-tip	+		+			+
HM-base	+	+	+	+	+	+
HR-tip	+		+			+
HR-base	+	+	+	+	+	+
RM-tip	+		+			+
RM-base	+	+	+	+		+
RR-tip	+		+	+		+
RR-base	+	+	+		+	+

tion that may be preferable, allowing uniform conditions for establishment of a stable population size for comparative analysis of soils over time and across different soil types and management regimes. This is analogous to monitoring of chemical and physical variables used to describe soil quality, where specific reference locations, or "benchmarks", are used for comparison of changes in chemical and physical variables such as bulk density and hydraulic conductivity. In the case of soil biological variables, the benchmark conditions employ a standardized test plant species, plant age, and rhizosphere location.

The *gacA* sequence was of particular interest here for its role in regulation of both cyanide and antibiotic production. However, our study of the type strains for 28 species shows that the *hcnBC* and *gacA* genes were co-associated only in 30% of the strains. This could be due to variations in the sequence that was targeted. The *gacA* gene, which was detected in 3 of the 14 strains was consistently co-associated with the *phlD* gene for 2,4-DAPG production, but was inconsistently associated with phenazine production for 3 of the 5 *phzCD* containing type strains.

Several species of soil bacteria contain the *acc* gene [17,18]. The gene appears to undergo horizontal transfer and has been found in various *Pseudomonas* sp. The sequence used here was not detected in any of the type strains, or in the soils that were screened. This was unexpected as the target sequence we used is conserved. It is possible of course that our inability to detect this gene and others in our primer set such as the *pupA* gene for siderophores production is due to low copy numbers or interferences from PCR inhibitors in the soil extracts that lowered the detection efficiency.

Molecular methods for detection of specific gene sequences for monitoring bacterial species population densities and copy numbers of functional genes are advancing rapidly. Results of this research demonstrate that it will be necessary to empirically test many of the sequences that have been identified in the laboratory for their efficacy in actual field samples, and standardize the conditions that are to be used for screening soils. This may be best achieved by bringing soils to a standard reference state that reflects the conditions under which the PGPR perform their ecosystem function; in this case the presence of a plant rhizosphere of a particular plant species. The ability to rapidly characterize populations of antibiotics producers will greatly enhance our understanding of their role in enhancing plant health and yields, and the impacts of management practices on PGPR bacteria.

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