The ISME Journal (2011) 5, 973–985
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www.nature.com/ismej

ORIGINAL ARTICLE

Transcriptional and antagonistic responses of *Pseudomonas fluorescens* Pf0-1 to phylogenetically different bacterial competitors

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The ability of soil bacteria to successfully compete with a range of other microbial species is crucial for their growth and survival in the nutrient-limited soil environment. In the present work, we studied the behavior and transcriptional responses of soil-inhabiting *Pseudomonas fluorescens* strain Pf0-1 on nutrient-poor agar to confrontation with strains of three phylogenetically different bacterial genera, that is, *Bacillus*, *Brevundimonas* and *Pedobacter*. Competition for nutrients was apparent as all three bacterial genera had a negative effect on the density of *P. fluorescens* Pf0-1; this effect was most strong during the interaction with *Bacillus*. Microarray-based analyses indicated strong differences in the transcriptional responses of Pf0-1 to the different competitors. There was higher similarity in the gene expression response of *P. fluorescens* Pf0-1 to the Gram-negative bacteria as compared with the Gram-positive strain. The Gram-negative strains did also trigger the production of an unknown broad-spectrum antibiotic in Pf0-1. More detailed analysis indicated that expression of specific Pf0-1 genes involved in signal transduction and secondary metabolite production was strongly affected by the competitors' identity, suggesting that Pf0-1 can distinguish among different competitors and fine-tune its competitive strategies. The results presented here demonstrate that *P. fluorescens* Pf0-1 shows a species-specific transcriptional and metabolic response to bacterial competitors and provide new leads in the identification of specific cues in bacteria–bacteria interactions and of novel competitive strategies, antimicrobial traits and genes.
The ISME Journal (2011) **5,** 973–985; doi:10.1038/ismej.2010.196; published online 13 January 2011
Subject Category: microbe–microbe and microbe–host interactions
Keywords: bacterial competition; inter-specific interactions; *Pseudomonas fluorescens*; transcriptional responses; antibiosis

Introduction

Soil microbial communities represent the world's greatest reservoir of biological diversity known so far (Curtis *et al*., 2002; Torsvik and Ovreas, 2002). Most known soil bacterial species are organotrophs that is, they obtain the energy for growth from the assimilation of organic compounds. However, the availability or accessibility of degradable organic compounds is limited in most soils (Alden *et al*., 2001; Demoling *et al*., 2007). In addition, there is a high degree of overlap in metabolic abilities of organotrophic bacteria (Strickland *et al*., 2009). This implies that a particular bacterial species is likely to encounter numerous taxonomically different competitors in different microsites in soil ecosystems.

Bacteria explore their biotic and abiotic environments by sensing and responding to a wide variety of chemical stimuli (Taga and Bassler, 2003; Ryan *et al*., 2008; Shank and Kolter, 2009; Straight and Kolter, 2009) possibly providing them with information on the identity of the nearest competitor(s). Indeed, recent studies revealed that bacteria alter their gene expression when confronted with another bacterial species (Garbeva and de Boer, 2009; Tai *et al*., 2009).

Most characterized bacterial species harbor multiple two-component signal transduction systems that enable the coupling of a diverse array of adaptive responses to neighboring macro- and microorganisms, and to abiotic environmental changes (Gao *et al*., 2007). *Pseudomonas* species, which are frequently isolated from complex environments, such as soil and rhizosphere, possess a range of two-component signal transduction

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Received 13 September 2010; revised 11 November 2010; accepted 12 November 2010; published online 13 January 2011

proteins (for example, 212 in *Pseudomonas fluorescens* Pf0-1, 223 in *P. fluorescens* Pf-5, 179 in *P. putida* KT2440 and 203 in *P. syringae* DC3000 (http://genomics.ornl.gov/mist)), which are thought to permit rapid responses to environmental changes (Heeb and Haas, 2001; Dubuis *et al*., 2007; Humair *et al*., 2010). In these complex natural environments, *Pseudomonas* species coexist with many other bacterial species. We hypothesize that signal transduction systems may, in part, have a role in fine-tuning their responses towards the nearest competing microbe and to optimize their competitive behavior.

Among the genes that are upregulated during interspecific bacterial interactions are often the genes involved in the production of antibiotics, suggesting that antibiosis may be a common defensive or offensive strategy in microbial interactions (Fong *et al*., 2001; Harrison *et al*., 2008; Garbeva and de Boer, 2009). In addition, genes that confer resistance against toxins were also found to be upregulated during competitive interactions, suggesting protection against self-intoxication or against toxins produced by other members of the microbial community (Dantas *et al*., 2008; Martinez 2008, 2009). Whether upregulation of genes involved in this 'chemical warfare' improves competitive success will depend on the sensitivity of the competitor to the antibiotic(s) and the resistance of the strain of interest to the competitor's toxins. Hence, it is likely that both toxin production and resistance may need to be adapted or tailored toward the identity of the competitor. Besides toxin production and resistance, modulating expression of other genes and cellular processes may also be required to sustain a population in a dynamic, competitive environment. To date, however, no reports have documented the variation in transcriptional responses of bacteria to different competitors.

In the present work, we investigated the ability of soil-inhabiting *P. fluorescens* strain Pf0-1 to cope with different competitors under nutrient-poor conditions. We examined the behavior and transcriptional responses of Pf0-1 during interaction with strains of three phylogenetically different bacterial genera, that is, *Bacillus*, *Brevundimonas* and *Pedobacter*. The results show that *P. fluorescens* Pf0-1 can distinguish between different competitors. Common transcriptional responses were also identified, including upregulation of cryptic genes in Pf0-1, which we demonstrate to be important for production of an antimicrobial compound.

Materials and methods

Bacterial cultures and growing conditions

The bacterial strains used in this study are summarized in Table 1.

These include *P. fluorescens* Pf0-1, *Pedobacter* sp. V48, *Brevundimonas* sp. V52 and *Bacillus* sp. V102. *P. fluorescens* Pf0-1 was isolated from an agricultural loam soil in Sherborn (MA, USA), whereas the other strains were isolated from a coastal dune site in The Netherlands (Compeau *et al*., 1988; de Boer *et al*., 2003, 2007). This coastal dune soil does also contain pseudomonads that are closely related to *P. fluorescens* Pf0-1 (de Boer *et al*., 2007). The strains were pre-cultured from frozen glycerol stocks on 1/10 strength Tryptic Soy Broth agar.

The interaction experiments were performed using the carbon-limited water-agar medium (WA-N), which has been used in a previous study (Garbeva and de Boer, 2009). This medium contained 20 $g l^{-1}$ of Agar, 5 $g l^{-1}$ of NaCl, 1 $g l^{-1}$ of KH_2PO_4 and 0.1 $g l^{-1}$ of $(NH_4)_2 SO_4$ The pH was adjusted to 6.5 before autoclaving. (Garbeva and de Boer, 2009). The WA-N agar plates (8.5-cm diameter) were divided into two zones (Supplementary Information Figure S1): zone B1, harbors *P. fluorescens* Pf0-1; and zone B2 contains one of the competing bacteria. Zones B1 and B2 were separated 1.5 cm from each other and all strains were inoculated with equal densities. Bacterial inocula, consisting of 20 μl washed cell suspensions in a 10 mM phosphate buffer (pH 6.5) and containing 10^7 cells per ml, were spread in the appropriate zone. All plates were incubated at 20 °C for 5 days. On the fifth day, bacterial cells were collected for enumeration (see below), whereas *P. fluorescens Pf0-1* was also collected for RNA extraction.

Enumeration of bacteria

Two agar discs (1-cm diameter) were cut from the bacterial zones on WA-N plates and shaken in 10 mM phosphate buffer (pH 6.5) for 90 min. Serial dilutions were plated on 1/10 Tryptic Soy Broth agar

Table 1 Bacterial strains used in this study

Bacterial strain	*Description*	*Reference*
P. fluorescens Pf0-1	Wild type, soil isolate, Gram-negative, Class: *γ-proteobacteria*	Compeau *et al*. (1988)
Pedobacter sp. V48	Wild-type Gram-negative, Phylum: *Bacteroidetes*, Class: *Sphingobacteria*	de Boer *et al*. (2007)
Brevundimonas sp. V52	Wild-type Gram-negative, Class: *α-proteobacteria*	de Boer *et al*. (2007)
Bacillus sp. V102	Wild-type Gram-positive, Class: *Bacilli*	de Boer *et al*. (2007)
P. fluorescens Pf0-1 mutants:		
Pf0-1 Δ3463-66	Pf0-1 with deletion of Pfl01_3463 to Pfl01_3466 (bases 3947327-3952129)	This study
Pf0-1 Δ3655-59	Pf0-1 with deletion of Pfl01_3655 to Pfl01_3659 (bases 4136447-4143889)	This study

plates. Plates were incubated at 20 °C and colonies were counted after 48 h.

Microarray experiment, RNA isolation and cDNA synthesis

Total RNA was extracted from *P. fluorescens* Pf0-1 growing on WA-N plates with or without (control) the presence of competing bacteria. For total RNA extraction, the WA-N agar zone carrying *P. fluorescens* Pf0-1 was cut from the plate (to prevent contamination by the competing species during recovery of cells) and the cells were suspended in 1.5 ml of sterile phosphate buffer. All suspensions were diluted in sterile phosphate buffer to the same optical density (OD; 600 nm) to obtain equal amounts of cells for RNA extraction. The cells were centrifuged at $16\,000 \times g$ for 3 min. RNA was extracted from the cell pellets with the Artrum Total RNA Mini Kit (Bio-Rad, Veenendaal, The Netherlands cat No. 732-6820) according to the manufacturer's recommendations. The extracted total RNA was treated with the TURBO DNA-free Kit to remove DNA (Ambion, Nieuwerkerk a/d lJssel, The Netherlands; cat No. 1907). Before creating complementary DNA (cDNA), the RNA concentrations in the samples were quantified with a NanoDrop Spectrophotometer (Isogen Life Science, IJssestein, The Netherlands). RNA quality was verified using the Experion System (Bio-Rad).

First strand cDNA was synthesized from 10 μg total RNA with random hexamer primers from Invitrogen (Breda, The Netherlands; cat No. 48190-011) using SuperScript Double-Stranded cDNA Synthesis kits (Invitrogen cat No. 11917-010). The cDNA synthesis was carried out according to the NimbleGen protocol for synthesis of double-stranded cDNA.

Microarray design and data analysis

On the basis of the sequence and annotation data for *P. fluorescens* Pf0-1, high-density, multiplex (4×72 K) microarrays were designed and produced by Roche NimbleGen (NimbleGen Systems of Iceland, Reykjavík, Iceland; cat No. A7241-00-01). The arrays consisted of 60-mer probes covering 5735 genes, 6 probes per gene and 2 replicates. Labeling of cDNA with Cy3 dye and hybridization was performed by Roche NimbleGen.

On each 4×72 K format microarrays two replicate interactions and two replicate controls were performed. The lists of differential expressed genes were extracted by comparison of each interaction to the control. The Robust Microarray Analysis-normalized gene expression data were analyzed with the Array Star 2 software for microarray analysis (DNASTAR, Madison, WI, USA). Analysis was performed, with application of false discovery rate (Benjamini–Hochberg method) and multiple testing corrections.

Quantitative Real-Time PCR

Quantitative Real-Time PCR (qRT-PCR) was performed on the original RNA extracts to verify the gene expression detected by microarray analysis. First-strand cDNA was obtained as described above. A volume of 2 μl of cDNA was subjected to RT-PCR using SYBR Green PCR master mix (Applied Biosystem, Warrington, UK). For each target gene (35 differentially expressed genes and 3 control genes- non differentially expressed), forward and reverse primers (Supplementary Information Table S1) were designed using Primer Express software (PE, Applied Biosystem). All primers used for real-time PCR were first tested using conventional PCR with DNA isolated from *P. fluorescens* Pf0-1. Real-time PCR was performed using a Corbett Research Rotor-Gene 3000 thermal cycler (Westburg, Leusden, The Netherlands) with the following conditions: initial cycle 95 °C for 15 min and 40 cycles of: 95 °C for 15 s; 56 °C for 50 s; and 72 °C for 50 s. The incorporation of SYBR Green in double-stranded DNA allowed the determination of the threshold cycle, which identifies the moment of the PCR cycle at which PCR amplicons exceed the detection limit. Standard curves were established for each cDNA sample.

Extraction of antimicrobial compounds

The extraction of antimicrobial compound(s) was carried out according to the methods described by Raaijmakers *et al.* (1999). Briefly, the WA-N agar zone carrying *P. fluorescens* Pf0-1 was removed carefully from the plate and cut in small (1-cm diameter) pieces. These pieces were vigorously shaken in 20 ml of 80% (v/v) acetone for 1 h at room temperature. The acetone solution was centrifuged for 10 min at $4000 \times g$ and the acetone was evaporated under air flow. The water fraction was acidified with trifluoroacetic acid (0.1% (v/v)), mixed with 2 volumes of ethylacetate and shaken vigorously for 5 min.

After incubation for overnight at −20 °C the unfrozen (ethylacetate) fraction that contains the active compounds was carefully transferred to a new flask and dried under air flow. The dried extract was dissolved in 150 μl of 50% (v/v) methanol and subjected to reverse phase high pressure liquid chromatography analysis.

Construction and use of Pf0-1 mutants

On the basis of the microarray and qRT-PCR analyses, several gene clusters were selected for site-directed mutagenesis. Genes were deleted from the *P. fluorescens* Pf0-1 genome by SOE-PCR (Horton *et al.*, 1989) and allele exchange using suicide plasmid pSR47s (Matthews and Roy, 2000) as described previously (Silby and Levy, 2004). Two of the deletion mutants obtained, Pfl01_3463-66 (bases 3947327-3952129) and Pfl01_3655-59 (bases 4136447-4143889), were used for comparison of antibiotic production with the wild-type strain. Each deletion was confirmed by PCR analysis using at least one primer that anneals outside of the regions used in the allelic exchange to create the deletions.

Assay for testing antimicrobial activity of the extract
The extracts of antimicrobial compound dissolved in methanol were tested for activity against hyphal growth of the fungi *Rhizoctonia solani, Fusarium culmorum*, *Trichoderma harzianum* and *Mucor hiemalis* in 12-well open-chamber plates (Cat No. 665180 Greiner bio-one, Frickenhausen, Germany) with 250 µl WA-N agar in each well. Each well was inoculated on one side with 6-mm diameter agar discs containing fungal hyphae. A volume of 10 µl of the extracts were added to sterile filter paper discs (Whatman No.1, Whatman, Whatman Nederland BV, Hertogenbosch, The Netherlands; 6-mm diameter) and placed on the opposite side of the wells. The 12-well plates were inoculated for 48 h at 20 °C and checked for fungal inhibition by measuring hyphal extension. Filter paper discs containing 10 µl of 50% methanol were used as control.

The effect of the antimicrobial compound(s) on *Bacillus* sp. V102, *Pedobacter* sp. V48 and *Brevundimonas* sp. V52 growth was tested by mixing 5 µl of the extracts with 25 µl of bacterial cell suspensions containing 10^7 cells per ml and plating immediately the bacterial suspension on 1/10 Tryptic Soy Broth agar plates. The plates were incubated at 20 °C and enumeration was carried out after 72 h. As a control 5 µl of 50% methanol was added to the 25 µl bacterial cell suspensions.

Statistical analysis
For each experiment, replicates were used: two for the microarray experiments and three for all other experiments. The statistical analysis of microarray data were performed with the Array Star 2 software for microarray analysis (DNASTAR).

The statistical analyses of bacterial enumeration, antagonistic tests and qRT-PCR data were carried out with XLStat 2010 (Addinsoft, New York, NY, USA) using a two-tailed *t*-test, assuming unequal variance between data sets. Data were considered to be statistically different at $P \leqslant 0.05$.

Results

Effect of interspecific interactions on P. fluorescens *populations*
The numbers of bacteria that had developed in the different zones (B1 and B2 see Supplementary Information Figure S1), on WA-N agar were estimated to confirm that competitive interactions were occurring between *P. fluorescens* Pf0-1 and the other strains. The density, expressed as colony forming units (CFUs), of *P. fluorescens* Pf0-1 in the inoculation zone B1 was significantly reduced by the presence of another bacterial species (Figure 1a). Cell density of Pf0-1 was most negatively affected by the presence of the *Bacillus* strain. The latter was more abundant than *P. fluorescens* Pf0-1 during interaction whereas numbers of *P. fluorescens* Pf0-1 remained higher than those of the *Brevundimonas* and *Pedobacter* strains. The CFU of *Brevundimonas* and *Pedobacter* monocultures (Figure 1b) were significantly higher than the CFU of *Brevundimonas* and *Pedobacter* during interaction with *P. fluorescens* Pf0-1. There was no significant difference between CFU of *Bacillus* monoculture and CFU of *Bacillus* interacting with *P. fluorescens* Pf0-1.

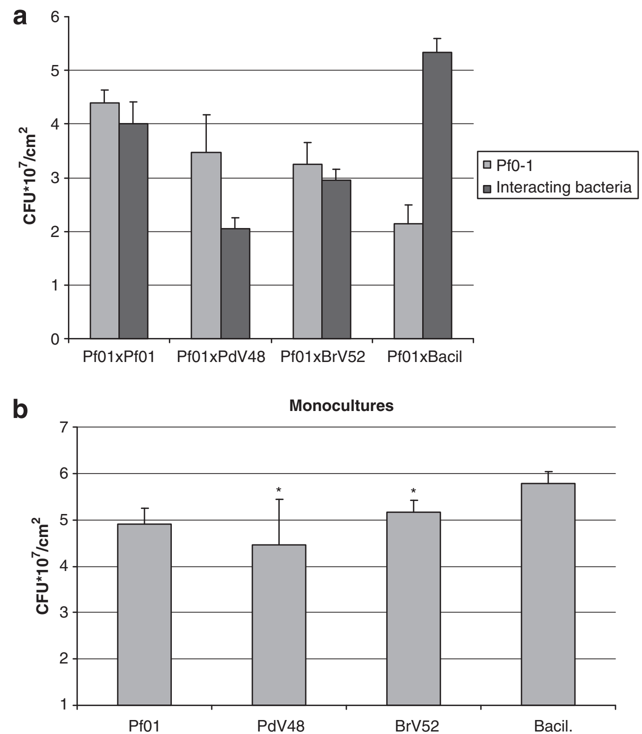

Figure 1 (**a**) Number of bacterial colony forming units (CFUs) produced during competitive interactions of *Pseudomonas fluorescens* Pf-01 with other bacteria on nutrient-poor agar: Pf0-1 × Pf0-1, interaction with *P. fluorescens* Pf0-1; Pf0-1 × PdV48, interaction with *Pedobacter* sp. V48; Pf0-1 × BrV52, interaction with *Brevundimonas* sp. V52; and Pf0-1 × Bacil, interaction with *Bacillus* sp. V102. Details of the experiment are given in Material and methods. Presented values are means of three replicates, error bars indicate s.d. and different letters indicate significant differences ($P < 0.05$) in Pf0-1 CFU. (**b**) Number of bacterial CFUs produced by monocultures of Pf0-1, *P. fluorescens* Pf0-1; PdV48, *Pedobacter* sp. V48; BrV52, *Brevundimonas* sp. V52; and Bacil, *Bacillus* sp. V102. *Indicates significant differences in CFU between monoculture and interaction with *P. fluorescens* Pf0-1 ($P < 0.05$).

Transcriptional responses of P. fluorescens *Pf0-1 to other bacterial species*
The response of *P. fluorescens* Pf0-1 to different bacterial competitors was studied by determining gene expression profiles. The presence of phylogenetically different soil bacteria in physically separated zones on nutrient-poor agar caused numerous changes in Pf0-1 gene expression (Table 2). Application of false discovery rate (Benjamini–Hochberg method) and multiple testing corrections revealed that each competing strain caused significant ($P < 0.05$) transcriptional changes of twofold or

Table 2 Differentially* expressed genes in *P. fluorescens* Pf0-1 during competitive interactions with other bacterial species

P. fluorescens Pf0-1 interacting with	*Upregulated genes*	*Downregulated genes*	*Total number of differentially expressed genes*
Pedobacter sp. V48	198	224	422
Brevundimonas sp. V52	159	166	325
Bacillus sp. V102	398	173	571

*Identified using the false discovery rate (Benjamini Hochberg method) correction method ($P<0.05$).

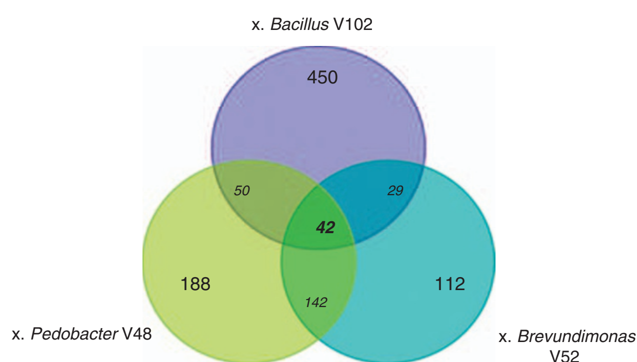

Figure 2 Venn diagram representing the number of differentially expressed genes in *P. fluorescens* Pf0-1 during inter-specific competitive interactions. Each circle represents the number of differentially expressed genes during interspecific competitive interaction with a single strain: green circle, interaction with *Pedobacter* sp. V48 (total 422 genes); blue circle, interaction with *Brevundimonas* sp. V52 (total 325 genes); and light indigo circle, interaction with *Bacillus* sp. V102 (total 571 genes). Italic and bold numbers represent the common differentially expressed genes in all two and three interactions, respectively.

higher; qRT-PCR analysis of 35 selected differentially expressed genes confirmed the microarray data (data not shown). The presence of *Bacillus* sp. caused the highest number (571) of gene expression changes. The presence of *Pedobacter* sp. and *Brevundimonas* sp. caused changes in the expression of 422 and 325 genes, respectively (see Supplementary Information Tables S2, S3 and S4 for complete lists).

We subsequently searched for common transcriptional changes in Pf0-1 in response to the presence of the three different competing bacterial species. Despite the large number of genes that were differentially expressed in each interaction, only 42 were common to all three interactions, with putative functions in energy production and conversion (eight genes), amino acid transport and metabolism (six genes), signal transduction mechanisms (six genes), inorganic ion transport and metabolism (four genes), nucleotide transport and metabolism (four genes) and cell motility and secretion (three genes) (Figure 2, Table 3).

Comparison of the interactions of *P. fluorescens* Pf0-1 with *Pedobacter* sp. and *Bacillus* sp. revealed that the expression of 92 genes was affected by both bacteria (43 up- and 49 downregulated), whereas changes in 71 genes (43 up- and 28 downregulated) were common to the interaction with *Brevundimonas* sp. and *Bacillus* sp. However, the common response to the two Gram-negative bacteria was far greater (Figure 2), comprising 184 genes (87 upregulated and 97 downregulated genes; Supplementary Information Table S5).

Comparison of differentially expressed genes related to signal transduction

During interspecies interaction, expression of several genes related to two-component signal transduction systems was found to be altered compared with monoculture (Table 4), with six common to all three interactions.

The highest number of affected genes related to two-component signal transduction systems was detected during the interaction of Pf0-1 with *Bacillus* sp. (31 up- and 5 downregulated). The presence of *Pedobacter* sp. and *Brevundimonas* sp. altered expression of 16 and 15 genes related to two-component signal transduction systems, respectively. However, only seven of these were common for both interactions.

Differential expression of ribosomal proteins and phage-related genes

In all three interactions differential expression of genes encoding ribosomal proteins was observed. During the interactions with *Pedobacter* sp. and *Brevundimonas* sp., a nearly identical set of ribosomal protein genes showed altered expression, with similar directions (upregulation and downregulation). The interaction with *Bacillus* sp. led to downregulation of 13 ribosomal protein genes, of which only two were seen in other interactions (Supplementary Information Figure S2).

During the interaction with *Bacillus* sp., increased expression of phage-related genes was noted. In particular, expression of every gene (apart from Pfl01_1136 and 1163) in the putative prophage region Pfl01_1136 to Pfl01_1173 was elevated in the range of 2.1–8.5 with most of the expression increased by at least fourfold (all upregulated phage-related genes are indicated in bold in Supplementary Table S2 in Supplementary Information). None of these putative prophage genes was up- or downregulated during the interactions with *Brevundimonas* sp. and *Pedobacter* sp.

Interspecies interactions induce genes involved in antimicrobial compound production in P. fluorescens *Pf0-1*

During interaction with *Pedobacter* sp. and *Brevundimonas* sp. several genes were upregulated in

Table 3 Common genes differentially expressed in *P. fluorescens* Pf0-1 during interspecific competition with three bacterial strains

SEQ_ID	*Gene description*	*Possible function*	*Fold change with* Pedobacter *V48*	*Fold change with* Brevundimonas *V52*	*Fold change with* Bacillus *V102*
Pfl_0360	Urocanate hydratase	Amino acid transport and metabolism	6.3	3.9	19.3
Pfl_0651	Response regulator receiver domain protein (CheY)	Signal transduction mechanisms	3.3	2.3	2.6
Pfl_0657	Chemotaxis sensory transducer, cache sensor	Signal transduction mechanisms	3.4	2.4	3.2
Pfl_1499	Phenylalanine 4-monooxygenase	Amino acid transport and metabolism	30.5	4.9	4.4
Pfl_1529	Flagellar hook-associated 2-like	Cell motility and secretion	2.6	2.4	2.7
Pfl_1534	Two component, sigma-54 specific, transcriptional regulator	Signal transduction mechanisms	2.4	2.6	2.7
Pfl_1562	Cobyrinic acid a,c-diamide synthase	Cell motility and secretion	4.2	3.2	2.3
Pfl_1563	Flagellar biosynthesis sigma factor	Cell motility and secretion	3.3	2.9	2.3
Pfl_1702	Protein of unknown function DUF989	Unknown	3.4	2.7	2.5
Pfl_1703	Ureidoglycolate hydrolase	Nucleotide transport and metabolism	4.1	2.7	2.3
Pfl_2321	Ring hydroxylating dioxygenase, α-subunit	Energy production and conversion	3	6.6	2.1
Pfl_2322	2-chlorobenzoate 1,2-dioxygenase	Energy production and conversion	4.3	5.2	4.9
Pfl_2323	Benzoate 1,2-dioxygenase ferredoxin reductase subunit	Energy production and conversion	3.9	2.2	2.1
Pfl_2324	1,6-dihydroxycyclohexa-2,4-diene-1-carboxylate dehydrogenase	Energy production and conversion	5.8	2.2	2.7
Pfl_2601	Chemotaxis sensory transducer	Signal transduction mechanisms	3	2.8	5.4
Pfl_2964	Hypothetical protein	Unknown	7.6	2.3	2.3
Pfl_2968	Ring hydroxylating dioxygenase, α-subunit	Energy production and conversion	2.8	2.1	9.8
Pfl_3441	Dihydropyrimidinase	Nucleotide transport and metabolism	3.4	2.3	5.4
Pfl_3768	Chemotaxis sensory transducer	Signal transduction mechanisms	3.4	3.1	5.5
Pfl_4431	Chemotaxis sensory transducer, cache sensor	Signal transduction mechanisms	3.9	2.8	3.7
Pfl_5297	Phosphoribosyl-dephospho-CoA transferase	Lipid metabolism	6.1	2.2	10.5
Pfl_0193	Hypothetical protein	Unknown	−36.9	−6.5	−33.9
Pfl_0194	Thiosulphate-binding protein	Inorganic ion transport and metabolism	−45.6	−6.1	−49.5
Pfl_0221	Protein of unknown function DUF1234	Unknown	−6.3	−2.4	−9.6
Pfl_0223	NLPA lipoprotein	Inorganic ion transport and metabolism	−31.1	−2.1	−31.6
Pfl_0256	Taurine ABC transporter, periplasmic-binding protein	Inorganic ion transport and metabolism	−5.6	−3.2	−39.1
Pfl_0877	Sulfate adenylyltransferase subunit 2	Amino acid transport and metabolism	−15.6	−6.2	−5.7
Pfl_1610	Succinate dehydrogenase, cytochrome b subunit	Energy production and conversion	−3.4	−2.3	−2.6
Pfl_1611	Succinate dehydrogenase, hydrophobic membrane anchor protein	Energy production and conversion	−3.1	−2.6	−2.3
Pfl_2205	ABC transporter, periplasmic substrate-binding protein	Inorganic ion transport and metabolism	−3.5	−4.1	−3.3
Pfl_2346	Transcriptional regulator, XRE family with cupin sensor domain	Transcription	−4.8	−2.7	−2.7
Pfl_2801	Amino acid ABC transporter, permease protein, 3-TM region	Amino acid transport and metabolism	−5.2	−7.2	−9.4
Pfl_2802	Extracellular solute-binding protein, family 3	Cell envelope biogenesis, outer membrane	−9.4	−3.2	−23.1
Pfl_3594	Isocitrate dehydrogenase (NADP+)	Energy production and conversion	−6.5	−5.4	−2.9
Pfl_4370	Extracellular solute-binding protein, family 1	Carbohydrate transport and metabolism	−6.6	−2.7	−6.3
Pfl_4376	Glyceraldehyde-3-phosphate dehydrogenase	Carbohydrate transport and metabolism	−5.6	−4.7	−10.1
Pfl_4517	Argininosuccinate synthase	Amino acid transport and metabolism	−3.4	−2.3	−2.1
Pfl_5065	30s ribosomal protein S8	Translation, ribosomal structure and biogenesis	−2.7	−2.9	−2.5
Pfl_5621	Phosphoribosylaminoimidazole carboxylase ATPase subunit	Nucleotide transport and metabolism	−2.9	−2.2	−2.1
Pfl_5622	Phosphoribosylaminoimidazole carboxylase	Nucleotide transport and metabolism	−7.4	−3.1	−2.3
Pfl_5626	Sodium:alanine symporter	Amino acid transport and metabolism	−4.2	−2.5	−3.2
Pfl_5640	Acetyl-CoA carboxylase	Lipid metabolism	−12.3	−3.4	−5.4

Pf0-1, which may be involved in the valine, leucine and isoleucine degradation pathway. Two gene clusters within this pathway, Pfl01_3463-3466 and Pfl01_3655-59, are described in Figure 3. The gene cluster Pfl01_3463-3466 may encode for components of the branched-chain α-keto acid dehydrogenase complex. In *Streptomyces avermitilis,* a branched-chain α-keto acid dehydrogenase gene cluster is essential for polyketide backbone synthesis (Denoya, 1995; Yoon *et al.*, 2004). Moreover, it was demonstrated that branched-chain amino acids can serve as precursors for Type II polyketide synthesis through pathways that are nutrient dependent (Stirrett *et al.*, 2009). The upregulation of

Table 4 Differentially expressed genes in *P. fluorescens* Pf0-1 related to two-component signal transduction during competitive interaction with three bacterial strains

Interaction with Bacillus *sp. V102*		*Interaction with* Brevundimonas *sp. V52*		*Interaction with* Pedobacter *sp. V48*	
Gene description	*Fold changes*	*Gene description*	*Fold changes*	*Gene description*	*Fold changes*
Pfl0222 response regulator receiver domain protein (CheY)	−9.4	**Pfl0651 chemotaxis sensory transducer, cache sensor**	**2.5**	Pfl0222 response regulator receiver domain protein (CheY)	−3.4
Pfl0378 chemotaxis sensory transducer	3.2	**Pfl0657 chemotaxis sensory transducer, cache sensor**	**2.4**	Pfl0354 chemotaxis sensory transducer, cache sensor	−3.1
Pfl0529 chemotaxis sensory transducer	2.6				
Pfl0640 Two component transcriptional regulator, LuxR family	3.3	Pfl1533 PAS/PAC sensor signal transduction histidine kinase	3.9	**Pfl0651 chemotaxis sensory transducer, cache sensor**	**3.3**
Pfl0651 chemotaxis sensory transducer, cache sensor	**2.6**	**Pfl1534 two component, sigma-54 specific, transcriptional regulator, Fis family**	2.6	**Pfl0657 chemotaxis sensory transducer, cache sensor**	**3.4**
Pfl0657 chemotaxis sensory transducer, cache sensor	**3.2**	Pfl1564 response regulator receiver domain protein (CheY)	2.8	Pfl1363 anti sigma-E protein, RseA	−3.9
Pfl1533 PAS/PAC sensor signal transduction histidine kinase	2.1	Pfl1565 chemotaxis phosphatase, CheZ	2.0	Pfl1364 sigma-E regulatory protein, MucB/RseB	−3.3
Pfl1534 two component, sigma-54 specific, transcriptional regulator, Fis family	2.6	Pfl1566 CheA signal transduction histidine kinases (STHK)	2.2	**Pfl1534 two component, sigma-54 specific, transcriptional regulator, Fis family**	**2.4**
		Pfl1567 chemotaxis-specific methylesterase	2.8	*Pfl1567 chemotaxis-specific methylesterase*	2.7
Pfl1541 anti-sigma-factor antagonist (STAS)	4.2	**Pfl2601 chemotaxis sensory transducer**	2.8	Pfl1981 response regulator receiver domain protein (CheY)	−2.9
Pfl1833 chemotaxis sensory transducer, Pas/Pac sensor	2.1	Pfl2650 response regulator receiver domain protein (CheY)	−2.0	**Pfl2601 chemotaxis sensory transducer**	**3.0**
Pfl1912 carbon storage regulator, CsrA	2.9	*Pfl2907 chemotaxis sensory transducer*	2.0	*Pfl2907 chemotaxis sensory transducer*	4.0
Pfl2000 periplasmic sensor signal transduction histidine kinase	3.3	**Pfl3768 chemotaxis sensory transducer**	3.0	**Pfl3768 chemotaxis sensory transducer**	**3.5**
Pfl2004 two component, sigma-54 specific, transcriptional regulator, Fis family	2.9	Pfl3770 chemotaxis sensory transducer	2.1	Pfl3871 transcriptional regulator, TetR family	−6.0
Pfl2100 chemotaxis sensory transducer	4.3	*Pfl4804 transcriptional regulators, TraR/DksA family*	−2.2	Pfl4308 chemotaxis sensory transducer, Pas/Pac sensor	3.9
Pfl2176 putative diguanylate cyclase (GGDEF domain) with GAF sensor domain	3.9	**Pfl4431 chemotaxis sensory transducer, cache sensor**	2.8	**Pfl4431 chemotaxis sensory transducer, cache sensor**	**3.2**
Pfl2249 two component transcriptional regulator, LuxR family	2.9			Pfl4625 histidine kinase	3.3
				Pfl4804 transcriptional regulators, TraR/DksA family	−3.9
Pfl2554 chemotaxis sensory transducer	2.5				
Pfl2601 chemotaxis sensory transducer	**5.4**				
Pfl2652 histidine kinase	−2.7				
Pfl3133 anti-sigma-factor antagonist (STAS)	3.4				
Pfl3345 transcriptional regulator, Crp/Fnr family	2.9				
Pfl3636 sigma-54 specific transcriptional regulator containing GAF, and Fis DNA-binding domains	2.5				
Pfl3768 chemotaxis sensory transducer	5.5				
Pfl3770 chemotaxis sensory transducer	4.1				
Pfl3918 sigma-54 specific transcriptional regulator, Fis family	−3.7				
Pfl4240 two component transcriptional regulator, winged helix family	−2.1				
Pfl4261 response regulator receiver domain protein (CheY)	2.6				
Pfl4273 carbon storage regulator, CsrA	5.3				
Pfl4308 chemotaxis sensory transducer, Pas/Pac sensor	−2.8				
Pfl4431 chemotaxis sensory transducer, cache sensor	3.7				
Pfl4550 response regulator receiver domain protein (CheY)	3.1				
Pfl4766 chemotaxis sensory transducer, Pas/Pac sensor	4.3				
Pfl4883 carbon starvation protein CstA	5.4				
Pfl4889 transcriptional regulator, LuxR family	5.1				
Pfl4988 sigma-54 specific transcriptional regulator containing GAF, and Fis DNA-binding domains	3.4				

*Genes in bold are common for all three interactions. Genes that are in italic are common for the interactions with the Gram-negative strains *Pedobacter* sp. V48 and *Brevundimonas* sp. V52.

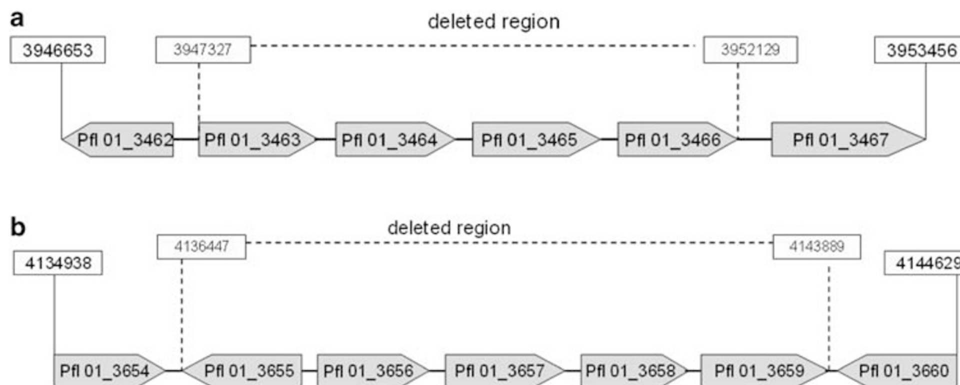

Figure 3 Graphic representation of gene clusters deleted in non-antibiotic producing mutants. (**a**) Pfl01_3463-3466 (Pfl01_3463 and Pfl01_3464, two branched-chain α-keto acid dehydrogenase E1 components; Pfl01_3465, branched-chain α-keto acid dehydrogenase subunit E2; and Pfl01_3466, dihydrolipoamide dehydrogenase). (**b**) Pfl01_3655-3659 (Pfl01_3655, AMP-binding domain protein; Pfl01_3656, isovaleryl-CoA dehydrogenase; Pfl01_3657, propionyl-CoA carboxylase; Pfl01_3658, γ-carboxygeranoyl-CoA hydratase; and Pfl01_3659, 3-methylcrotonoyl-CoA carboxylase, α subunit). In both panels, dashed lines indicate the deleted region and numbers in boxes refer to the nucleotide position in the Pf0-1 genome.

this gene cluster was observed only in the Pf0-1 interactions with *Brevundimonas* and *Pedobacter*, but not with *Bacillus*.

The role of upregulated branched-chain amino acid degradation genes in antimicrobial compound production

The first step to determine whether antimicrobial compounds are produced by Pf0-1 during interactions with the competing bacteria involved in organic extractions of the agar surrounding Pf0-1 when confronted with each of the three bacterial strains, followed by assays in which the antimicrobial effects of the extracts were tested. The densities of *Pedobacter*, *Brevundimonas* and *Bacillus* were indeed significantly reduced when exposed to the extract obtained from *P. fluorescens* Pf0-1 cocultured with *Pedobacter* relative to the extract from Pf0-1 monoculture (Figure 4a). We subsequently investigated whether the antimicrobial compound(s) produced during the interaction of Pf0-1 with *Pedobacter* also had activity against soil-borne saprophytic and pathogenic fungi. Activity bioassays showed that the extract inhibits mycelial growth of several fungi, including *Rhizoctonia solani, Mucor hiemalis* and *Trichoderma harzianum* (Figure 4b). No such inhibitory effect was observed for the extract produced by Pf0-1 during the interaction with *Bacillus* (Figure 4c).

To determine whether the upregulated genes in the Pfl01_3463-3466 and Pfl01_3655-3659 clusters are indeed involved in the production of the observed antimicrobial activity, each of the two gene clusters in Pf0-1 were deleted by site-directed mutagenesis (Table 1 and Figure 3). In contrast to the extracts obtained from wild-type strain Pf0-1 when interacting with *Pedobacter*, the extracts from the deletion mutants Δ3463-66 and Δ3655-59 under the same interaction conditions did not exhibit activity against *R. solani* (Figure 5). Subsequent analysis of these extracts by high pressure liquid chromatography showed the absence of at least three specific peaks in extracts of the mutants that were present in extracts of the wild-type strain (Supplementary Information Figure S3). High pressure liquid chromatography analysis followed by photodiode array analysis of the chromatograms (288 nm) further showed highly similar metabolite patterns for *P. fluorescens* Pf0-1 extracts when cocultured with *Pedobacter* or *Brevundimonas*. In contrast, the metabolite pattern of extracts obtained from *P. fluorescens* Pf0-1 cocultured with *Bacillus* was different from that of extracts obtained from Pf0-1 interacting with the other two strains (Supplementary Information Figure S3). The metabolite profiles of all extracts were also different from those obtained from the control extract, that is, extract obtained from Pf0-1 monoculture.

Discussion

In soil and rhizosphere environments, *Pseudomonas* species coexist with many other bacterial species. Several of these species compete for the same ecological niche and nutrient resources (Demoling *et al*., 2007). Therefore, the ability to cope with the presence of a range of competing microbial species is essential for growth and survival in soil ecosystems. The results of this study showed that the identity of the competing bacterium is an important determinant of the behavioral and genetic response of *P. fluorescens* Pf0-1. The genome-wide microarray analyses revealed a specific transcriptional response of Pf0-1 to each competitor as only a small percentage of the differentially expressed genes was common to all three

interactions. Both *Brevundimonas* and *Pedobacter*, but not *Bacillus*, induced the production of antimicrobial metabolites in *P. fluorescens* Pf0-1, further supporting the specific nature of the response of *P. fluorescens* Pf0-1 to different competitors.

Most bacteria, including Pseudomonads, possess two-component signal transduction systems that help them to adapt to fluctuating environmental conditions (Gao *et al.*, 2007). The expression of several *P. fluorescens* Pf0-1 genes involved in two-component signal transduction increased during interspecific interaction. This suggests an adaptive response of *P. fluorescens* Pf0-1 to the presence of unrelated microorganisms. Among the two-component regulatory genes that were significantly upregulated in all three interactions was the regulatory gene Pfl01_1534. This gene is an ortholog of *fleR* in *P. aeruginosa*, which is a regulator of genes for flagella biogenesis, chemotaxis, fimbrial biogenesis, and several genes annotated as 'hypothetical' (Dasgupta *et al.*, 2003). Elevated expression of this regulator may suggest that motility and/or chemotaxis are important and general components of the *P. fluorescens* response to other bacteria. Unlike Pfl01_1534, most of the differentially expressed 'signal transduction genes' were specific for a particular interaction (only six were common among the three interactions studied), suggesting that each competing bacterial species may produce specific cues that are perceived by *P. fluorescens* Pf0-1, triggering specific responses in addition to general ones. When comparing the transcriptional responses of *P. fluorescens* Pf0-1 to the presence of the different competing strains, the highest similarity was found for the two Gram-negative bacteria *Brevundimonas* and *Pedobacter.* This was the case for all genes including those related to two-component signal transduction systems. Whether the Gram-negative bacteria tested in this study exhibit a higher degree of overlap in specific cues as compared with those produced by the Gram-positive *Bacillus* strain remains to be tested.

The competing strains had also specific effects on Pf0-1 genes encoding ribosomal proteins. During the interaction with *Brevundimonas* and *Pedobacter,* a nearly identical set of ribosomal proteins were up- or downregulated, whereas only downregulated ribosomal proteins were found during the interaction with *Bacillus* (Supplementary Information Figure S2). Ribosomal proteins may have various functions apart from ribosome and protein synthesis (Wool, 1996). Downregulation of ribosomal proteins has been linked to various stress responses as well to a decrease of cellular growth (Ishige *et al.*, 2003; Stintzi, 2003; Silberbach and Burkovski, 2006). Moreover, de Carvalho *et al.* (2010) recently reported antimicrobial activities of ribosomal proteins.

Growth of *P. fluorescens* Pf0-1 was most strongly reduced when confronted with *Bacillus.* Apart from the downregulation of ribosomal proteins also other stress-related genes were differentially regulated, such as the prophage genes in the cluster Pfl01_1136 to Pfl01_1173. This prophage region carries two genes annotated as Pyocin R2_PP, holin (Pfl01_1137) and Pyocin R2_PP, lytic enzyme (Pfl01_1172), which may be related to pyocin biosynthesis. Pyocins are proteinaceous, narrow-spectrum antibacterial bacteriocins that are generally effective against closely related species. Whether this prophage region is functional or degenerated in Pf0-1 is not known, but the induction of nearly all genes in this region during the interaction with *Bacillus* may be an indication of a response to a particular stress factor not encountered by Pf0-1 in the other two interactions. Prophage induction after exposure to DNA-damage-causing agents, oxidative stress, or to other stress factors like antibiotic treatment, heat and starvation has been well documented (Frye *et al.*, 2005; Garcia-Russell *et al.*, 2009), leading to our suggestion that *Bacillus* induces a specific stress response in Pf0-1.

Organic extracts contained antimicrobial activity when isolated from cultures where *P. fluorescens* Pf0-1 was interacting with *Brevundimonas* or *Pedobacter*, but not *Bacillus*. The observation that antibiotic production is only triggered during interspecific interactions suggests a cost-effective strategy whereby the antibiotic is produced only when needed, or a strategy to avoid adaptation of competitors to the antibiotic (Garbeva and de Boer, 2009). Evidence that gene clusters *Pfl01_3463-66* and *Pfl01_3655-59* are indeed involved in the production of an antimicrobial compound was obtained by site-directed mutagenesis followed by activity bioassays. A strain carrying a deletion of these gene clusters did not show antimicrobial activity when confronted with *Pedobacter* sp. or *Brevundimonas* sp. In addition, high pressure liquid chromatography analysis confirmed that the mutants *Pfl01_Δ3463-66* and *Pfl01_3655-59* are defective in the production of compound(s) specifically induced in the wild-type strain when cocultured with *Pedobacter* or *Brevundimonas.* The nature and identity of these antimicrobial compound(s) produced by *P. fluorescens* Pf0-1 are not yet known and will require large-scale extractions, purifications and extensive analytical-chemical analyses (for example, liquid chromatography-mass spectrometry and nuclear magnetic resonance). The observed inhibition of both bacterial and fungal growth suggests that these antimicrobial compound(s) produced by *P. fluorescens* Pf0-1 can be effective in competitive interactions with many different bacterial and fungal species. It is interesting to note that the antibiotic compound(s) were not produced by *P. fluorescens* Pf0-1 during confrontation with the *Bacillus* strain even though growth of *Bacillus* is affected when exposed to the extracted ccompound(s). This could point to the absence of appropriate cues produced by *Bacillus* to induce production of the antimicrobial compound(s) by Pf0-1 or to deregulation of its production.

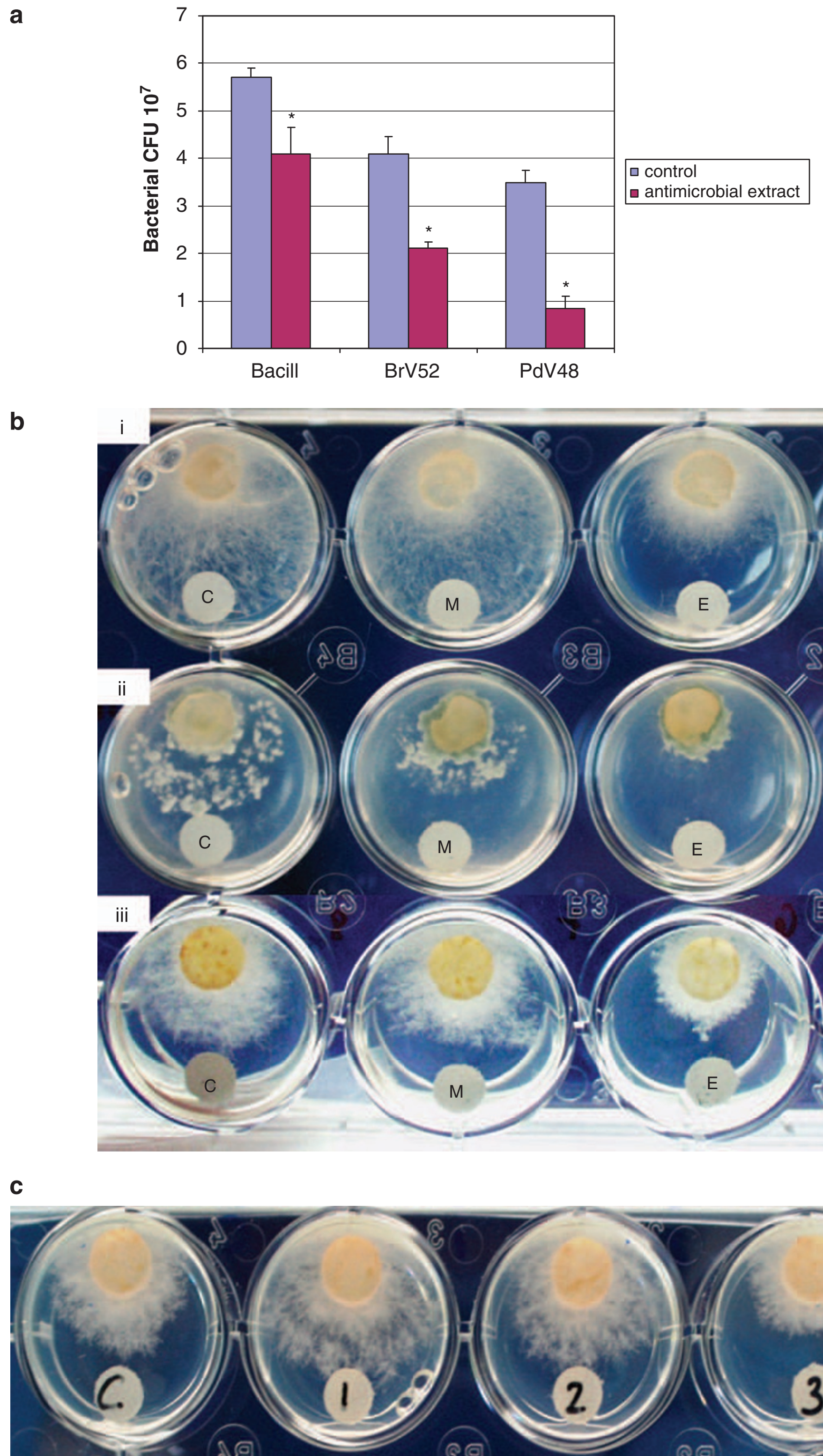

Figure 4 (**a**) Effect of extracts from *P. fluorescens* Pf0-1 zones on growth of bacteria: Bacill, *Bacillus* sp. V102; BrV52, *Brevundimonas* sp. V52; and PdV48, *Pedobacter* sp. V48. Control, extract from *P. fluorescens* Pf0-1 monoculture; Antimicrobial extract, extracts from the *P. fluorescens* Pf0-1 zone during interaction with *Pedobacter* sp. V48. *Indicates significant differences within column pairs ($P<0.05$). (Supplementary Figure 4) (**b**) Effect of extracts from *P. fluorescens* Pf0-1 zones on growth of fungi i, *Rhizoctonia solani*; ii, *Mucor hiemalis*; iii, *Trichoderma harzianum*. C, control extract from *P. fluorescens* Pf0-1 monoculture; M, 50% methanol; and E, extracts from the *P. fluorescens* Pf0-1 zone during interaction with *Pedobacter* sp. V48. (**c**) Effect of extracts from *P. fluorescens* Pf0-1 zones on *R. solani*. C, control 50% Methanol; 1, extract from Pf0-1 monoculture; 2, extract from Pf0-1 interacting with *Bacillus* sp. V102; and 3, extract from Pf0-1 interacting with *Pedobacter* sp. V48.

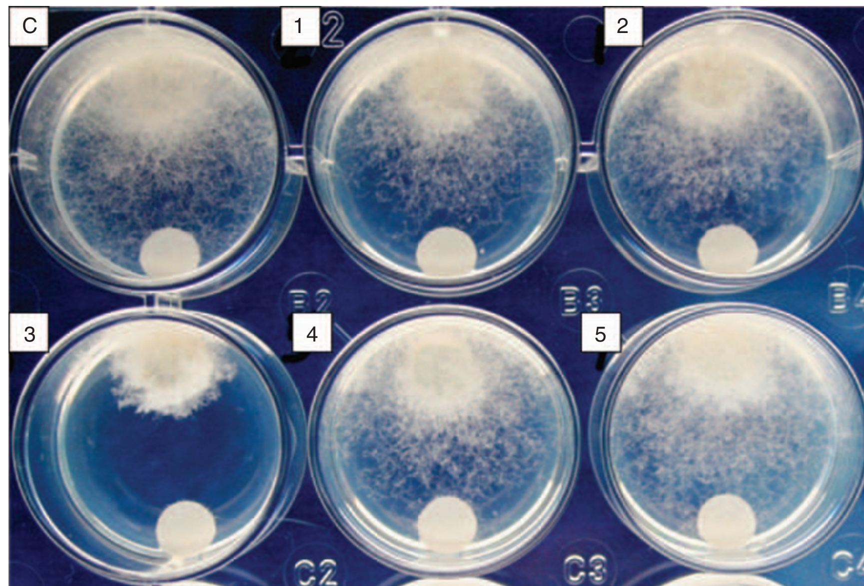

Figure 5 Antagonist assay against *Rhizoctonia solani* with extracts from: 1, *P. fluorescens* Pf0-1 monoculture; 2, *P. fluorescens* Pf0-1 interacting with *R. solani*; 3, *P. fluorescens* Pf0-1 interacting with *Pedobacter* sp.V48; 4, *P. fluorescens* Pf0-1 Δ3655 interacting with *Pedobacter* sp.V48; 5, *P. fluorescens* Pf0-1 Δ3463 interacting with *Pedobacter* sp.V48; and C, control 50% methanol.

This study did not reveal information on the identity of the cues that trigger the specific differential gene expression observed for *P. fluorescens* Pf0-1. However, these cues should either be diffusible or volatile metabolites as the differential expression occurred without direct cell–cell contact of *P. fluorescens* Pf0-1 with the competing strains. In an additional experiment (data not shown), a range of reporter constructs (responding to different *N*-Acyl Homoserine Lactones, Autoinducer-2 family compounds, or Diffusible Signal Factor compounds) were tested to resolve the putative nature of the signals produced by the interacting bacteria. *Brevundimonas* sp. was capable of triggering a positive response in a bioreporter strain that detects production of the Diffusible Signal Factor signal 2-dodecenoic acid. *P. fluorescens* Pf0-1 itself cannot produce Diffusible Signal Factor, but has the gene for perception (Pfl01_3253). Neither *Pedobacter* sp. nor *Bacillus* sp. stimulated a response from any of the tested bioreporters (unpublished data). This is again pointing at specificity in the response of *P. fluorescens* Pf0-1 to different competitors.

In the present work, we studied the response of *P. fluorescens* Pf0-1 to different bacterial competitors in a one-to-one confrontation. However, in natural environmental settings bacteria are likely to encounter several different competitors at the same time or in sequential events. In such situations, the production of a broad-spectrum antibiotic may be a beneficial strategy for Pf0-1 as it will target diverse competitors. It is even plausible that superior competitors, like *Bacillus* in the current study, can be inhibited by the antibiotic when it is triggered by another species in a microbial consortium. In this study, we investigated bacteria–bacteria interactions using an agar plate model system. Although these assays are somewhat artificial, they do allow an in-depth analysis of fundamental mechanisms and genes underlying microbial interactions. In addition, the experiments were performed under carbon-limiting conditions, which to some extent mimics the situation in most soils (Demoling *et al*, 2007; Rousk and Baath, 2007).

It is interesting to note that several of the differentially expressed genes during the inter-specific interactions have an unknown function. Some of these so-called orphan or cryptic genes are commonly found in all three interactions. For example, gene Pfl_1702 encodes a protein of unknown function (DUF989) that is upregulated in all three interactions. A related gene was also previously reported to be upregulated during inter-specific interactions in *Pseudomonas* sp. A21 (Garbeva and de Boer, 2009). Although our experiments did not reveal the functions nor the products of these genes, they do suggest that these genes have a role in the response to other microbes. It will be interesting in a further study to determine the precise function of such genes in bacterial interactions.

In summary, our results demonstrate that *P. fluorescens* Pf0-1 shows a species-specific transcriptional and metabolic response to bacterial competitors, which may provide new leads in the identification of specific cues in bacteria–bacteria interactions and of novel competitive strategies, antimicrobial traits and genes.

Acknowledgements

This work is supported by The Netherlands Organization for Scientific Research (NWO). The authors thank Saskia Gerards for her help in extraction of antimicrobial compounds. This is publication No.4568 of the Netherlands Institute of Ecology (NIOO-KNAW).

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