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ORIGINAL ARTICLE

Pseudomonas putida 06909 genes expressed during colonization on mycelial surfaces and phenotypic characterization of mutants

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Abstract

Aims: The main focus of this study was to gain an overall view of *Pseudomonas putida* 06909 genes involved in the *Pseudomonas–Phytophthora* interaction as a biological control mechanism, and to understand the roles of these genes.
Methods and Results: Sixteen *Ps. putida* genes with increased expression on *Phytophthora* mycelial surfaces were identified using *in vivo* expression technology (IVET) screening. Sequence analysis of these *Phytophthora* mycelium-induced (*pmi*) genes revealed that many of them display similarity to genes known or predicted to be involved in carbohydrate catabolism, energy metabolism, amino acid/nucleotide metabolism, and membrane transport processes. Disruption of three *pmi* genes encoding succinate semialdehyde dehydrogenase, a dicarboxylic acid transporter, and glyceraldehyde-3-phosphate dehydrogenase showed significant phenotypic differences involved in the colonization processes, including motility, biofilm formation on abiotic surfaces, colony morphology, and competitive colonization of fungal mycelia. All three of these *pmi* genes were induced by glycogen and other substances, such as organic acids and amino acids utilized by *Ps. putida*.
Conclusions: The IVET screening and mutant characterization can be used to identify bacterial genes that are induced on the mycelial surface and provide insight into the possible mechanisms of mycelial colonization by this bacterium.
Significance and Impact of the Study: The IVET screening through a bacterial genome library might be a huge task. However, because the genes involved in direct interaction with *Phytophthora* and in bacterial adaptation can be identified, the IVET system will be a valuable tool in studying biocontrol bacteria at the molecular and ecological levels.

Introduction

Currently, there is a great interest in the study of colonization mechanisms in bacteria useful in biological control of plant diseases, to develop disease control to a greater extent (Simons *et al.* 1997; Bloemberg and Lugtenberg 2001). Soil bacteria belonging to the species *Pseudomonas fluorescens* and *Pseudomonas putida* have been well studied, because they have the potential for agricultural use (O'Sullivan and O'Gara 1992; Lugtenberg and Dekkers 1999; Haas *et al.* 2000; Walsh *et al.* 2001). One of the most attractive characteristics is that they can colonize root surfaces (rhizosphere), where diverse environmental factors and microbial interactions exist (Dekkers *et al.* 1998; Espinosa-Urgel *et al.* 2002). Through extensive studies by various genetic and physiological approaches, the molecular mechanisms involved in the attachment and colonization of plant roots by these fluorescent pseudomonads in the rhizosphere are being identified (Haas *et al.* 2000; Walsh *et al.* 2001). In the rhizosphere, these

biocontrol bacteria compete for nutrients and niches with endogenous micro-organisms, such as other bacteria and fungi. Therefore, to gain a better understanding of how biocontrol bacteria function in the rhizosphere, the interactions with other microbes, such as root pathogenic fungi, should also be taken into account.

In this study, we focused on a bacterial–*Phytophthora* interaction in an ongoing programme to identify the factors influencing biological control by *Ps. putida* 06909, a strain that has shown effectiveness in the control of citrus root rot caused by *Phytophthora* (Turney 1995; Steddom *et al.* 2002). In the earlier study using an *in vivo* expression technology (IVET) strategy based on *pyrB* as a selection marker, which is driven by fusions with active promoters from a random genomic library, five genes were identified in *Ps. putida* 06909, which were induced in the presence of *Phytophthora parasitica*. Sequence analysis indicated that three of these were related to genes with previously described functions, including a diacylglycerol kinase, an ABC transporter, and an outer membrane porin (Lee and Cooksey 2000). Recently, using IVET, the identification of the *Ps. fluorescens* genes, encoding traits such as two-component sensing systems, sugar transporters and metabolism, amino acid transporters, stress response, a type III secretion system, and a range of novel genes which are induced in the rhizosphere, provided a view of bacterial traits for rhizosphere adaptation (Rainey 1999). In this study, we identified 16 additional genes that were expressed at a higher level on *Phytophthora* mycelial surfaces using IVET screening, and many of them were predicted to be involved in carbon catabolism, amino acid/nucleotide metabolism and membrane transport processes. In a follow-up study, *Phytophthora*-induced genes encoding succinate semialdehyde dehydrogenase, a dicarboxylic acid transporter, and glyceraldehyde-3-phosphate dehydrogenase, were disrupted by marker exchange mutagenesis, and their mutants were phenotypically characterized. In addition, the nutritional factors stimulating the expression of these genes were studied.

Materials and methods

Bacterial strains, plasmids, media and culture conditions

Escherichia coli cultures were grown at 37°C on Luria-Bertani (LB) medium (Sambrook *et al.* 1989) supplemented with the appropriate antibiotics. The following antibiotic concentrations were used for the *E. coli* strains: tetracycline, 20 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; gentamycin, 15 μg ml^{-1} and ampicillin, 100 μg ml^{-1}. *Pseudomonas putida* strains were grown at 28°C on mannitol-glutamate (MG) medium (Kahane *et al.* 1978) supplemented with yeast extract (0·25 g l^{-1}) (MGY). The following antibiotic concentrations were used in the MGY medium: tetracycline, 20 μg ml^{-1}; kanamycin, 30 μg ml^{-1}; gentamycin, 15 μg ml^{-1} and ampicillin, 200 μg ml^{-1}. When it was necessary for bacterial growth in a rich medium, *Ps. putida* strains were cultured on *Pseudomonas* agar F (PAF) (Difco, Detroit, MI, USA) under the same conditions. The growth rates of the *Ps. putida* mutant strains were tested in M9 minimal medium (Sambrook *et al.* 1989) supplemented with $MgSO_4$ (1 mmol l^{-1}) and glucose (0·4%, w/v) as a carbon source. *Phytophthora parasitica* M191 was usually grown at 28°C on V8C agar (Miller 1955), and synthetic growth (SG) medium (Andrew 1980) was used for preparation of *P. parasitica*-spent medium. Agar discs (7 mm in diameter) were cut from the edge of the mycelium and used as inocula for all the assays on agar plates. In the plasmid pGEM-Km, a kanamycin gene flanked by *AscI* restriction sites was produced by polymerase chain reaction (PCR) with vector pGEM-T Easy (Promega, Madison, WI, USA) as a template.

DNA techniques

Plasmid preparation, restriction endonuclease cleavage, ligation, electrophoresis, and other recombinant DNA techniques were carried out using standard methods (Ausubel *et al.* 1987). Chromosomal DNA of *Ps. putida* wild-type strain 06909 and its mutants was prepared with a Genomic DNA Preparation Kit (Promega). Southern blot analysis was performed using the DIG DNA labelling and detection kit (Boehringer Mannheim, Mannheim, Germany), as described in the manufacturer's instructions. DNA sequencing was carried out at the Genomic Institute of the University of California, Riverside, CA, USA. The primers used in this study were synthesized commercially (Genosys Biotechnologies, Inc, The Woodlands, TX, USA). Sequences were analysed and compared with the Genbank databases by using BLAST (Basic Local Alignment Search Tool) programs. Sequence data for the *Ps. putida* KT2440 and *Pseudomonas aeruginosa* PA01 genomes were obtained from The Institute for Genomic Research (http://www.tigr.org) and the *Pseudomonas* Genome Project (http://www.pseudomonas.com), respectively.

IVET screening

A new genomic library of *Ps. putida* 06909 for *Phytophthora*-inducible IVET clones was constructed in pRIV16 as described in an earlier study (Lee and Cooksey 2000). Briefly, 2–8 kb *Sau*3A size-fractionated DNA fragments were ligated into the *Bam*HI site of pRIV16 to generate the library. About 20 000 clones with inserts were

constructed, which was designed to represent all the DNA present in *Ps. putida* in both orientations with respect to the marker gene (*pyrBC*′). Recombinant plasmids were maintained in *E. coli* DH5α, introduced into the *pyrB* mutant strain *Ps. putida* 06909u2 by tri-parental mating with pRK2013 as a helper plasmid, and grouped in pools of ≈1000 transconjugants. Each pool of the transconjugants carrying recombinant clones was applied to V8C agar plates completely colonized by *P. parasitica*, at levels of 60 000 CFU of bacteria per plate. After incubating for 24 h at 28°C, the bacterial cells were harvested with 2 ml of sterile water and reapplied to V8C agar fungal plates to further enrich for *Phytophthora*-induced clones. However, enrichment more than twice resulted in the selection of clones containing constitutive promoters, and not *Phytophthora*-induced clones. Finally, the cells were recovered from *Phytophthora* plates and were selected on V8C agar supplemented with uracil (50 μg ml^{-1}), tetracycline, kanamycin and 80 μg of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per millilitre. As a promoterless *lacZ* gene in pRIV16 was cloned downstream from *pyrBC*′ as a transcriptional fusion, blue colonies contained genes expressed *in vitro* and were eliminated. Only white colonies that contained cloned promoters induced specifically during colonization of *P. parasitica* were retained. To confirm *Phytophthora* induction and to eliminate sibling clones, the plasmids were recovered from the selected clones and analysed by determining the restriction enzyme digestion patterns obtained with *Eco*RI, *Sph*I, *Kpn*I, *Xba*I, *Xho*I, *Cla*I and *Sma*I. The plasmids of 16 unique clones were reintroduced into *Ps. putida* 06909u2 to confirm their induction by *Phytophthora*.

Gene disruption in *Pseudomonas putida* 06909 by marker exchange mutagenesis

Marker exchange plasmids for disruption of Phytophthora mycelium-induced (pmi) genes in the Ps. putida chromosome were constructed by crossover PCR for pmi-73 and pmi-81 (Yang *et al.* 2002), or with a commercial EZ::TN Insertion Kit (Promega) for pmi-41 (Fig. 1). Briefly, the crossover PCR strategy consisted of two rounds of PCR amplification by using two sets of primers: primers A and B for amplification of the left region flanking the sequences targeted for disruption, and primers C and D for amplification of the right region. Each primer set was designed to allow the amplification of more than 500 base pairs (bp) for efficient homologous recombination. Primer B contained, in the 5′ to 3′ direction, a 20-base sequence complementary to the 5′-end of primer C, an AscI endonuclease cleavage site (eight bases) for insertion of a kanamycin gene, and 21 bases homologous to the region close to the ATG translation initiator. Its complementary partner, primer C, also contained the 20-base

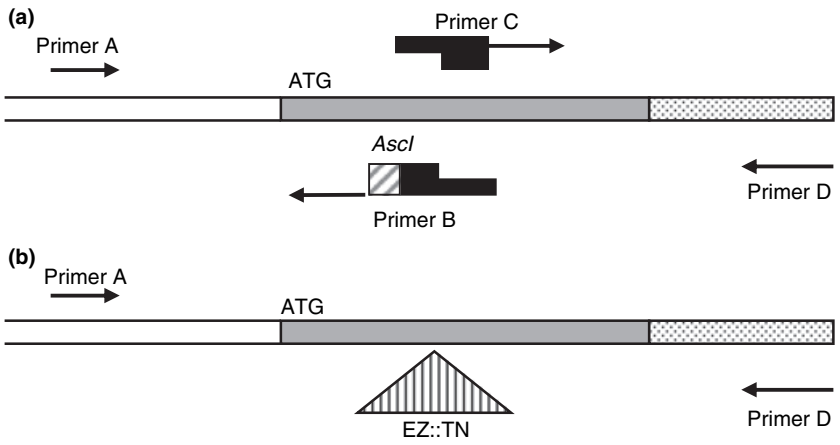

Figure 1 Construction of marker exchange plasmids for marker exchange mutagenesis in *Pseudomonas putida* 06909 by using crossover polymerase chain reaction (PCR) (a) and a commercial EZ::TN insertion kit (b). Four specific primers were designed (primers A, B, C and D), where each primer had a region (arrow) homologous to the genomic sequence, and primers B and C had a sequence (dark block) complementary to each other. Each primer set (A/B and C/D) was used for the first round of PCR amplification, respectively, and primers A/D were used for the second round of PCR amplification. The gray region indicates the truncated *pmi* gene region and the dotted region indicates the downstream gene which is not included in the *pmi* fusion. The sequences of primers used were: 73-A: 5′-GGCTGGCGGTGGTCAAGGATA-3′, 73-B: 5′-GTAAACCGGGAAAGCCGCAAGGCGCGCCTAGTAGTGGCCGAGCAGGATA-3′; 73-C: 5′-TTGCGGCTTTCCCGGTTTACGCATGAAGTCGGTCGGTAAAA-3′; 73-D: 5′-GAGTAGCCAGTCGGGATAACC-3′, 81-A: 5′-ATGTCCTGGTGCGGCGAGAAC-3′; 81-B: 5′-TAATAATGAACGAGCACACCGGCGCGCCGCCTTGAGGATCGAGATAACC-3′; 81-C: 5′-GGTGTGCTCGTTCATTATTAGGCCAACAAGTTCAAGCAGGA-3′; 81-D: 5′-GATGTCGCCGTGGTTGATGCC-3′; 41-A: 5′-ACAGCATCATCGTTTCAGCA-3′; and 41-D: 5′-AGCTCCAGGGAAACCTTCTT-3′. Bold sequences in primers B and C are complementary to each other, and the sequence underlined indicates the *Asc*I restriction site.

sequence complementary to the 5′-end of primer B and 21 bases homologous to an interior site of the target gene which determined the size of the deletion. By the complementary sequences contained in primers B and C, two fragments produced by first round of PCR were annealed to each other by heating at 94°C for 4 min and then slowly cooling to room temperature. After a short extension step at 72°C for 10 min, the second round of PCR amplification was performed by using primers A and D designed to anneal upstream and downstream of the target gene, respectively. Primer D in the pmi-73 clone was designed as a degenerate primer based on the dctA sequence from Ps. putida KT2440 and Ps. aeruginosa PA01. The final mutagenic PCR fragments obtained from the second PCR was cloned into pGEM-T Easy (Promega), and then a kamamycin gene from pGEM-Km was inserted into the AscI site. For the pmi-41 clone, after insertion of a commercial EZ::TN, including a kamamycin gene instead of using the crossover PCR strategy, PCR amplification using primers A and D was performed, and its product was cloned into pGEM-T Easy. The resulting construct was recloned into the NotI site of pJQ200SK (Quandt and Hynes 1993). Following tri-parental mating with pRK2013 as a helper plasmid on YDC (yeast-dextrose-calcium carbonate agar) plates for 20 h as described earlier (Yang *et al.* 1994), the cells were suspended in sterile distilled water, serially diluted and plated onto PAF agar medium containing ampicillin, kanamycin and 5% sucrose (Ried and Collmer 1987). Sucrose-resistant colonies were tested for gentamycin sensitivity to select for double-crossover recombinants (gentamycin-sensitive). Chromosomal DNA was isolated from putative mutant strains, and PCR was used to confirm the mutations using primers A and D. Southern hybridization using kanamycin and gentamycin genes as probes was also performed for the final confirmation. These double crossover mutants were named 41TN, 73 km and 81 km, respectively.

Promoter induction assays

A quantitative β-galactosidase assay of *pmi* genes was performed as described earlier (Lee and Cooksey 2000). Briefly, V8C agar discs covered by actively growing *P. parasitica* and supplemented with uracil were inoculated with *Ps. putida* 06909u2 transconjugants carrying selected plasmids. After incubation for 20–24 h, the agar discs were dropped into 1 ml of sterile water, resuspended to release the bacterial cells grown on the discs, and assayed for β-galactosidase activity, as described by Miller (1972). The controls included an agar disc that was not inoculated with bacteria and an agar disc that was inoculated with *Ps. putida* 06909u2 (pRIV16).

A β-galactosidase assay was qualitatively performed with *P. parasitica*-spent medium and the Biolog GN2 MicroPlates (Biolog, Hayward, CA, USA) to study the nutritional factors stimulating the *pmi* gene expression. Preparation of the *P. parasitica*-spent medium was performed as described earlier (Fedi *et al.* 1997). *Phytophthora parasitica* was grown on SG agar (Andrew 1980) to cover the whole plate for 7 days, and then was overlaid with 10 ml of SG soft agar (0·2% agar). After incubation for 3 days, the overlay was collected and centrifuged at 12 000 ***g*** for 20 min. The supernatant was adjusted to pH 7·0 and filter sterilized. The assay was performed in a 96-well polystyrene microtitre plate (Costar 3596; Corning Inc., Corning, NY, USA), to which 100 μl of *P. parasitica*-spent medium, 50 μl of M9 minimal medium supplemented with glucose, 1 μl of of X-gal solution (20 mg ml^{-1}) and 10 μl of bacterial suspension of a *pmi* fusion strain ($OD_{600} \approx 0{\cdot}02$) were added per well. When assayed in Biolog GN2 MicroPlates? (Biolog), each well included 125 μl of a suspension of a *pmi* clone suspended in M9 minimal medium supplemented with glucose, which was adjusted to OD $\approx$ 0·002, and 1 μl of X-gal solution (20 mg ml^{-1}). After 24 h of incubation, the plates were observed for expression of the *lacZ* reporter gene by the appearance of a blue colour.

Utilization of various carbon sources

Biolog GN2 MicroPlates? (Biolog) were used according to the directions of the manufacturer to determine the differences in the ability of mutants and wild type to oxidize 95 different carbon sources. The colour reaction was read by a Microplate Spectrophotometer (SpectraMax 340PC; Molecular Devices, Sunnyvale, CA, USA).

Colonization and growth inhibition on *Phytophthora* mycelia

Mycelial colonization of three mutants was observed along the edge of the mycelial colony. Two agar patches (3 mm × 3 mm) of actively growing *P. parasitica* on V8C were transferred onto each V8C plate. After the *Phytophthora* were allowed to grow for 2 days at 28°C, each mutant and wild type was spot-inoculated on the edge of one of the fungal colonies with sterile toothpicks and incubated for 24 h.

Phytophthora growth inhibition was assessed by directly inoculating these strains onto mycelial agar discs as described earlier (Yang *et al.* 1994). An agar disc containing mycelium of *P. parasitica* was transferred to the centre of two kinds of agar plates, PAF and V8C. Wild-type *Ps. putida* 06909 or each mutant was inoculated to the centre of the mycelial surface with toothpicks. All the

plates were incubated for 7–8 days at 28°C until the control plate containing only a mycelial disc without bacteria almost covered the whole plate. The *Phytophthora* growth on each plate was measured as a diameter of the *Phytophthora* colony. The diameter of each *Phytophthora* colony was the mean between the longest and the shortest diameters of the colony. Each treatment included three replications.

Competitive assay on mycelial mats

The growth of the mutants alone or mixed with the wild type was assessed on mycelial surfaces. Overnight cultures of the wild type and each mutant were diluted to $OD_{600} \approx 1{\cdot}0$ in water, and also mixed at a proportion of 1 : 1. *Phytophthora* mycelial colonies grown for 5 days on V8C agar were inoculated at the edge of the colony with 20 μl of each individual inoculum, or mixed inoculum of the wild type and each mutant strain. After incubation for 24 h at 28°C, bacteria were recovered from the mycelial colonies in 3 ml of sterile water, and viable cells from each population were determined by dilution plating on two kinds of selective media. Both the wild type and each mutant could grow on LB agar medium supplemented with ampicillin (200 μg ml^{-1}). However, only the mutants could grow on LB agar medium supplemented with kanamycin (50 μg ml^{-1}). Population data (CFU ml^{-1}) from three replications were averaged, and the standard error (SE) of each mean was calculated.

Motility and chemotaxis assays

Each strain was stab-inoculated to the centre of a LB soft agar (0·3% agar) plate with a sterile toothpick and incubated for 17 h at 28°C. Motility was then assessed through quantification, by examining the circular turbid zone formed by the bacterial cells migrating away from the point of inoculation. A chemotaxis assay was performed towards tryptone as described earlier (Espinosa-Urgel *et al.* 2000). A water-agar solution (0·2%, w/v) mixed with bacterial suspensions of the wild type or each mutant was poured onto the plates containing a drop of tryptone solution (1%, w/v) in the centre of the bacterial plate and incubated at 28°C (Taguchi *et al.* 1997). After 5 h, a concentric halo around the mycelium or the tryptone was observed as a positive.

Nucleotide sequence accession numbers

The DNA sequences determined in this study have been deposited in the GenBank database under accession no. AY214160 (clone in *pmi-41*), AY214161 (clone in *pmi-73*), AY214162 (clone in *pmi-81*) and AY214163 (clone in *pmi-4*).

Results

Isolation of *Pseudomonas putida* genes induced during colonization of *Phytophthora* mycelial surfaces and induction of their promoters

The library of *Ps. putida* DNA constructed in pRIV16, including a promoterless *pyrB′C-lacZ* fusion and introduced into *pyrB* mutant strain (*Ps. putida* 06909u2), was applied onto *Phytophthora* mycelial mats grown on pyrimidine-deficient V8C agar medium. The clones surviving in this interaction were considered as having actively expressed promoters, which drove the *pyrB* gene, from the random library. Plating of these clones onto the V8C agar medium including a pyrimidine source (uracil) and X-gal as a substrate for *lacZ* expression allowed the separation of clones with a promoter, which is specifically induced on *Phytophthora* mycelia (white colonies), from clones with a constitutive promoter (blue colonies). These promoters are referred to as *pmi* promoters in this study. From this initial screening, several hundred clones were selected as potential *pmi* clone candidates. For confirmation and quantification of *Phytophthora* induction, the expression of the *lacZ* gene of the *pmi* fusions was examined on a mycelia-covered agar plug compared with those grown on an agar plug without mycelia. Finally, 16 unique *pmi* fusions were selected by the quantitative β-galactosidase assay (Fig. 2) and the restriction enzyme digestion pattern analysis (data not shown). These *pmi* fusions exhibited at least 2–15-fold higher induction levels on *Phytophthora* mycelial surfaces compared with the growth on agar surfaces without mycelia (Fig. 2).

Sequence analysis of *pmi* fusions

To identify the upstream insert promoters driving expression of *pyrBC′* in the *pmi* clones by *Phytophthora* signals, at least 500 bp upstream of *pyrBC′* of each *pmi* clone was sequenced and amongst them, three clones (*pmi-41*, *pmi-73* and *pmi-81*) were completely sequenced for further study. The resulting sequence data were used to search for similarities with published sequences in the GenBank database and for functions in the Clusters of Orthologous Groups of proteins (COG) database. As summarized in Table 1, 13 of the *pmi* loci are predicted to be involved in metabolic pathways, such as energy production and conversion (*pmi-4* and *pmi-7*), carbohydrate metabolism and transport (*pmi-10*, *pmi-73*, *pmi-81*, *pmi-90*, *pmi-97* and *pmi-102*), amino acid metabolism and transport (*pmi-41*, *pmi-93* and *pmi-99*) and nucleotide metabolism (*pmi-85*, *pmi-86* and *pmi-91*). Most of these deduced amino acid sequences showed high similarities to the corresponding proteins in *Ps. aeruginosa* PAO1. Two other loci (*pmi-84*

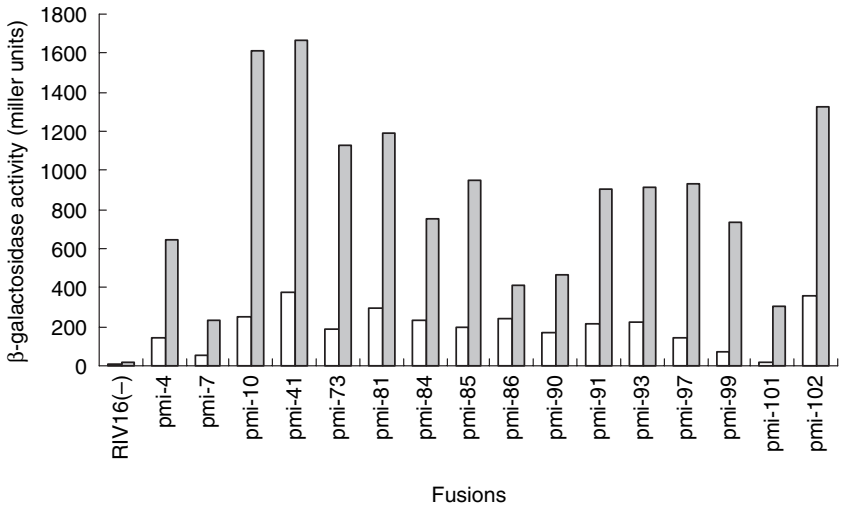

Figure 2 Induction of *pmi* genes on *Phytophthora* mycelia. Each clone was inoculated on both V8C agar discs supplemented with uracil (without fungal mycelia) and discs covered by *Phytophthora parasitica* (with fungal mycelia). After 24 h of incubation, each disc was resuspended in 1 ml of water to release the bacterial cells grown on discs. As a negative control, RIV16 (no promoter) was used. The *β*-galactosidase activity of the cells obtained from the discs was determined as described by Miller (1972), and the data are the average of two replications. □, w/o *Phytophthora* mycelia; ■, w/ *Phytophthora* mycelia.

and *pmi-101*) could not be assigned any probable function.

Growth properties of three *pmi* mutants *in vitro*

To characterize the three *pmi* mutants (41TN, 73 km and 81 km), we measured their growth rates in M9 minimal medium supplemented with glucose and LB medium. In no case was the growth rate in either medium significantly different from the wild type (data not shown), suggesting that the disrupted genes were not indispensable for bacterial growth *in vitro*. However, the mutant 81 km showed a slightly longer lag phase in LB medium, probably because the disrupted gene (putative glyceraldehyde-3-phosphate dehydrogenase) might be interconnected with other metabolic pathways supporting fast growth in a rich medium. As judged by the growth on minimal medium supplemented only by glucose, these three mutants are not auxotrophs. The ability of these mutants to oxidize various carbon substances provided by the Biolog microplates was also measured. The mutant 41TN showed a significantly weaker reaction for α-aminobutylic acid, while 73 km showed a weak reaction for *ρ*-hydroxyphenyl acetic acid and succinic acid and a stronger reaction for α-D-glucose. The reactions by the mutant 81 km were slightly different from that of the wild type for many carbon sources, such as L-arabonose, pyruvic acid methyl ester, succinic acid mono-methyl ester, acetic acid, malonic acid, propionic acid, L-alaninamide, L-leucine, phenylethly amide and 2-aminoethanol.

Colonization and inhibition of *Phytophthora* mycelia

As reported earlier (Lee and Cooksey 2000), *Ps. putida* 06909 colonizes along the edge of the mycelial colony from the inoculation spot and gradually spreads toward the centre of the mat. During incubation for 12 h, 81 km did not colonize further from the inoculation spot, while two other mutants, 41TN and 73 km, as well as the wild type, colonized the entire edge of the mycelial colony (data not shown). After 24 h of incubation, 81 km also colonized the entire edge of a mycelial colony. When each of these mutants mixed with the wild type was inoculated onto the mycelial colonies, the population of the mutant was reduced compared with that of the wild type (Table 2), suggesting that the mutants were not as competitive for *Phytophthora* colonization as the wild type. The reduced populations of the mutants was not likely owing to an impaired ability of the mutants to utilize nutrients in the V8C medium used in the assay, because when each of these mutants was inoculated individually onto the discs containing *P. parasitica* mycelia, all the bacteria grew similar to the wild type, fully colonizing *Phytophthora* mycelium and inhibiting *Phytophthora* growth (data not shown).

Other phenotypic characterization of *pmi* mutants

Motility has been shown to be important in establishing cell-surface contacts during colonization and further biofilm formation. However, it costs more energy to synthe-

Table 1 *Pseudomonas putida pmi* fusions expressed during colonization of *Phytophthora parasitica*

Locus	Similar protein, organism, per cent identity (GenBank ID)	Predicted function
Energy metabolism/carbohydrate catabolism/amino acid metabolism		
pmi-4	*glnK*, PA5288, *Pseudomonas aeruginosa*, 83% (AAG08673)	Nitrogen regulatory protein PII-2
pmi-7	*yhbW*, *Escherichia coli*, 34% (COG2141)	Flavin-dependent oxidoreductase
pmi-10	PA5046, *Ps. aeruginosa*, 94% (AAG08431)	Malic enyme
pmi-41	*gabD*, PA0265, *Ps. aeruginosa*, 89% (AAG03654)	Succinate-semialdehyde dehydrogenase
Pmi-81	PA3001, *Ps. aeruginosa*, 84% (AAG06389)	Probable glyceraldehyde-3-phosphate dehydrogenase
Pmi-90	*rpe*, PA0607, *Ps. aeruginosa*, 94% (AAG03996)	Ribulose-phosphate 3-epimerase
pmi-97	*tal*, PA2796, *Ps. aeruginosa*, 77% (AAG06184)	Transaldolase
pmi-102	*eno*, PA3635, *Ps. Aeruginosa*, 88% (AAG07023)	Enolase
Nucleotide biosynthesis		
pmi-85	*carA*, PA4758, *Ps. aeruginosa*, 85% (AAG08144)	Carbamoyl-phosphate synthase
pmi-86	*purM*, *E. coli*, 76%(AAA83898)	Phosphoribosylaminoimidazol (AIR) synthetase
pmi-91	*carB*, PA4756, *Ps. aeruginosa* 94% (AAG08142)	Carbamoylphosphate synthetase large subunit
Membrane proteins/transporter		
pmi-73	*dctA*, PA1183, *Ps. aeruginosa*, 82% (AAG04572)	C_4-dicarboxylate transport protein
pmi-93	*aotJ*, PA0888, *Ps. aeruginosa*, 59% (AAG04277)	Arginine-/ornithine-binding protein
pmi-99	*braC*,PA1074, *Ps. aeruginosa*, 61% (AAG04463)	Leucine-, isoleucine- and valine-binding protein
Other noncategorized		
pmi-84	*Pseudomonas fluorescens*, 71% (No COG related)	Hypothetical protein
pmi-101	*Ps. Fluorescens*, 71% (No COG related)	Hypothetical protein

Table 2 Competition between *Pseudomonas putida* strains*

	CFU ($\times 10^8$)	
Inoculants	+ Ap	+ Km
WT	137 ± 7·5	–
41TN	–	105 ± 1·4
73 km	–	111 ± 1·4
81 km	–	37 ± 0·5
WT/41TN	115 ± 6·1	17 ± 1·6
WT/73 km	103 ± 9·0	34 ± 0·5
WT/81 km	107 ± 4·2	4 ± 0·2

*Each *Pseudomonas putida* strain was spot-inoculated onto the edge of *Phytophthora* mycelial colony grown on V8C agar medium for 5 days with 20 μl (OD = 1·0) of bacterial inoculum. After 24 h of incubation, the bacterial cells were harvested in 2 ml of D/W (distilled water) and plated out on two selective media containing ampicillin [Ap, for wild type (WT) and mutant] or kanamycin (Km, for mutants only). Data are the averages of three replications. Standard deviations were below 10% of the given values.

Table 3 Phenotypes of three *pmi* mutants

	Motility (mm)		
Strain	in M9	in LB	Colony morphology†
WT	15·5 ± 0·7*	52·0 ± 2·0	Normal
41TN	16·0 ± 1·0	64·3 ± 6·4	Normal
73 km	17·6 ± 0·5	62·3 ± 0·1	Normal
81 km	13·6 ± 0·5	14·3 ± 1·1	Very small

*The diameters of motility halos (average ± standard deviation) calculated by three replications.
†Colony morphology was observed on Luria-Bertani (LB) agar medium after incubation at 28°C for 3 days.

size and assemble various components of flagella and pili components required for motility (Rashid and Kornberg 2000).Therefore, we tested the motility of the mutants, because their disrupted genes are predicted to be involved in metabolic pathways and transport processes for energy production (Table 1). All the strains were motile and indistinguishable in M9 soft agar medium supplemented with glucose. However, in the assay performed in the LB soft agar medium (Table 3), mutant 81 km formed a significantly smaller motility halo (14·3 ± 1·1 mm in diameter) in LB compared with the wild type (52·0 ± 2·0 mm in diameter) after 23 h of incubation, while the other two mutants, 41TN and 73 km, formed larger halos (64·3 ± 6·4 and 62·3 ± 6·1 mm, respectively). In a qualitative chemotaxis assay, all the strains formed clear chemotatic rings towards a droplet of tryptone solution deposited in the centre of a plate mixed with the bacterial solution (data not shown), indicating that the chemotaxis ability of these mutants was not impaired. Mutant 81 km also had an altered colony morphology, with small-sized colonies on both M9 minimal agar medium supplemented with glucose and on LB agar medium, and had a rough surface on LB agar medium (Table 3), while the colony morphology of the other two mutants was indistinguishable from that of the wild type. The slightly longer lag phase in LB medium observed for mutant 81 km

could account for a mild decrease in both motility and colony size, but probably would not account for the large differences observed between this mutant and the wild type.

Nutritional factors as *Phytophthora* signals

In order to test whether nutritional factors produced by *P. parasitica* can induce the *pmi* gene promoters, we first tested the induction of three *pmi* gene promoters using a *Phytophthora*-spent medium. As shown in Fig. 3, when the *pmi* clones were incubated in a microtitre plate containing M9 minimal medium including *P. parasitica*-spent medium and X-gal solution for 24 h, the promoter induction was observed by the appearance of blue colour. Two out of the three *pmi* gene promoters (*pmi-41* and *pmi-81*) showed a strong reaction (blue colour), and *pmi-73* presented a slight reaction, indicating that these *pmi* genes can be induced by the substances diffused from *P. parasitica* in culture. In the promoter induction assay using Biolog microplates containing 95 different carbon sources (Table 4), all three of these *pmi* genes were induced by glycogen, which suggested that they may be induced by *Phytophthora* cell wall components. These *pmi* genes were also induced by other substances, such as organic acids and amino acids, including some that were utilized by *Ps. putida*, suggesting that fungal constituents or exudates with similar composition might induce these *pmi* genes and provide substrates for the bacterium on mycelial surfaces.

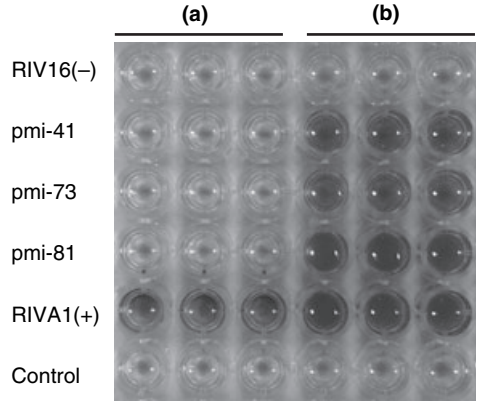

Figure 3 Promoter induction assay using *Phytophthora parasitica*-spent medium. Each well of a microtiter plate included 50 μl of M9 minimal medium supplemented with glucose, 100 μl of water (a) or *P. parasitica*-spent medium (b), 10 μl of bacterial suspension (OD_{600} = 0·02) and 1 ml of X-gal solution (20 mg ml^{-1}). RIV16 (no promoter, a negative control); *pmi-41*, *pmi-73*, and *pmi-81*: pmi fusions; RIVA1 (constitutive promoter, positive control); Control: no bacteria inoculated. The promoter induction was observed by the appearance of blue colour after 24 h of incubation at 28 °C. Three replications were performed per treatment.

Discussion

As an initial step for the better understanding of the molecular mechanisms involved in bacterial colonization of *Phytophthora* mycelia, we have used the IVET strategy (Handfield and Levesque 1999; Rainey and Preston 2000). This IVET-based strategy has been applied to the study of other plant-associated bacteria. For example, genes that are preferentially expressed in the rhizosphere have been identified in *Ps. fluorescens* and *Ps. putida* (Bayliss *et al.* 1997; Rainey 1999), and genes that are induced during infection of *Arabidopsis thaliana* have been identified in *Ps. syringae* pv. *tomato* (Boch *et al.* 2002). In our IVET screening using *pyrB′C-lacZ* fusion as a selection marker, we originally identified five genes in *Ps. putida* with increased expression on *Phytophthora* mycelial surfaces. Sequence analysis showed that three of these were related to genes with earlier described functions, including a diacylglycerol kinase, an ABC transporter, and an outer membrane porin (Lee and Cooksey 2000). In the present study, sequence analysis of an additional 16 strongly induced *pmi* genes revealed that many of them displayed similarity to genes known or predicted to be involved in diverse carbon catabolism, amino acid/nucleotide metabolism and transport processes. This study has therefore expanded our understanding of the differential gene expression patterns of *Ps. putida* under these conditions. We demonstrated earlier that the adherence to hyphae and siderophore production by *Ps. putida* 06909 are important in the inhibition of *Phytophthora* (Yang *et al.* 1994), but in the current study, no additional functions that provide obvious explanations for the observed biological control effect of *Ps. putida* 06060 on *Phytophthora* were identified (Turney 1995; Steddom *et al.* 2002). Instead, it appears that successful colonization and inhibition of *Phytophthora* by *Ps. putida* involves the induction of multiple metabolic pathways, in addition to adherence and competition for iron.

The discovery of a *glnK* homolog (*pmi-4*) suggests that *Ps. putida* on the mycelial surface is under metabolic stress, such as starvation for an essential nutrient, because the PII-like signal transduction protein encoded by *glnK* is known to be involved in nitrogen regulation under nitrogen starvation (van Heeswijk *et al.* 1996; Ninfa and Atkinson 2000). The same gene was also identified in another fusion (*pmi-33*, not shown in Table 1) which spans more DNA downstream of *glnK* to *amtB* that encodes a membrane-bound ammonium transport protein, and usually consists of an operon with *glnK* in several bacteria (Meletzus *et al.* 1998; Ninfa and Atkinson 2000; Thomas *et al.* 2000; Coutts *et al.* 2002).

In *pmi-41*, the gene *gabD* encoding a succinate semialdehyde dehydrogenase homolog (P25526) is known in

Table 4 Carbon substances that induce three *pmi* genes*

Fusion	Not utilized as a carbon source	Utilized as a carbon source
pmi-41	Glycogen (A4) Sebacic acid (E11)	ACETIC ACID (D1), b-hydroxybutyric acid (D11), a-KETOGLUTARIC ACID (E4), D,L-LACTIC ACID (E6), quinic acid (E9), d-saccharic acid (E10), succinic acid (E12), d-ALANINE (F5), l-alanine (F6), l-alanylglycine(F7), l-ASPARAGINE (F8), l-aspartic acid (F9), l-GLUTAMIC ACID (F10), glycyl-l-aspartic acid (F11), glycyl-l-glutamic acid (F12), hydroxy-l-proline (G2), l-PROLINE (G6), l-PYROGLUTAMIC ACID (G7), γ-aminobutylic acid (G12), uridine(H3), PUTRESCINE (H6)
pmi-73	Glycogen (A4)	Succinic acid (E12), URIDINE (H3)
pmi-81	Glycogen (A4)	Tween 40 (A5), Tween 80 (A6), ACETIC ACID (D1), b-hydroxybutyric acid (D11), a-ketogluitaric acid (E4), d,l-lactic acid (E6), quinic acid (E9), succinic acid (E12), d-alanine (F5), l-alanine (F6), l-glutamic acid (F10), l-pyroglutamic acid (G7), γ-AMINOBUTYRIC ACID (G12), URIDINE (H3), putrescine (H6)

*The assay was performed by using a Biolog GN2 MicroPlate™ containing 95 different carbon sources. Each well had 125 ml (≈10^6 cells) of bacterial suspension in M9 medium supplemented with glucose and 1 μl of X-gal (20 mg ml^{-1}). After 24 h of incubation, blue colour wells had a higher intensity than that of the control well (water, A1) and were considered as positive inducers. The capitalized substances showed higher induction, more than three times when measured by microplate spectrophotometer at 340 nm.

some bacteria to be a gene of the *gab* cluster, *gabCDPT*, which specifies the synthesis of the enzymes of the γ-aminobutyrate (GABA) degradation pathway (Dover and Halpern 1972; Metzer and Halpern 1990) and is responsible for the conversion of succinic semialdehyde into succinate. The activation of the *gab* gene expression by nitrogen deprivation has been demonstrated earlier to be mediated by the *gln* regulatory system (Ntr) (Kahane *et al.* 1978; Zaboura and Halpern 1978). However, in *Ps. putida* KT2440 and *Ps. aeruginosa* PA01, only two genes, *gabD* and *gabT* (GABA aminotransferase, P22256), are known to appear in a cluster. The *gabT* protein catalyses the conversion of GABA into succinic semialdehyde (Bartsch *et al.* 1990), and was recently reported to be involved in lysine metabolism and to be rhizosphere-induced in *Ps. putida* KT2440 (Espinosa-Urgel and Ramos 2001). Therefore, it is possible that the fungal induction of *gabD* in this study is because of the exudates produced by *Phytophthora* mycelia and the nutrient (nitrogen) limitation on the mycelial surface.

Two loci (*pmi-73* and *pmi-10*) induced by *Phytophthora* mycelia are predicted to be involved in C_4-dicarboxylic acid transport and metabolism, respectively. The gene *dctA* (*pmi-73*), encoding a C_4-dicarboxylic acid transport protein, is known to be a gene of the *dct* system of rhizobia, where the organization of the *dct* locus is conserved and consists of a three-gene cluster together with two regulatory genes (*dctB* and *dctD*). However, in *Ps. putida* and *Ps. aeruginosa*, no similar genes were found upstream or downstream from *dctA* in the genome sequence. In rhizobia, the *dctB* and *dctD* gene products function as a two-component system, together with sigma factor σ^{54}, for the expression of *dctA* under free living conditions in response to the presence of dicarboxylic acids (Yarosh *et al.* 1989). As the C_4-dicarboxylic acids are known to be transported and metabolized in symbiotic associations between rhizobia and leguminous plants, the compounds might be acquired by the bacterium from *Phytophthora* in this interaction. In *E. coli*, C_4-dicarboxylic acids are known to be utilized as carbon and energy sources (Davies *et al.* 1999). A malic enzyme homolog encoded by *pmi-10* catalyses the reductive carboxylation of pyruvate to give malate as an anaplerotic pathway, where the citric acid cycle intermediates would be replenished to maintain the flux through the cycle. The induction by these two loci was shown to be about sixfold greater than those of the same clones in the absence of the fungus (Fig. 2). Thus, C_4-dicarboxylic acid transport and metabolism might be involved in this bacterial–*Phytophthora* interaction.

Carbon catabolism and the related energy metabolism seem to play important roles in the bacterial colonization on mycelial surfaces. Recently, the global carbon metabolism regulator catabolite repression control (CRC) was reported to be a component of the signal transduction pathway required for biofilm development by *Ps. aeruginosa* (O'Toole *et al.* 2000), which suggests that carbon regulation could be a part of the pathway for the bacterial colonization on mycelial surfaces. Four *pmi* genes (*pmi-81*, *pmi-90*, *pmi-97* and *pmi-102*), which encode glyceraldehyde-3-phosphate dehydrogenase, ribulose-phosphate 3-epimerase, transaldolase and enolase homologs, respectively, were shown to be highly expressed on the *Phytophthora* mycelia, and might be involved in energy production for colonization on them.

Three of the fusions (*pmi-85*, *pmi-86* and *pmi-91*) were predicted to be involved in nucleotide and amino acid biosynthesis (Table 1). The genes *carA* (*pmi-85*) and *carB* (*pmi-91*), encoding subunits of carbamoylphosphate synthase small chain, are involved in pyrimidine biosynthesis and glutamate metabolism, and *purM* (*pmi-86*) is involved in *de novo* purine biosynthesis. The *car* operon is known to be regulated by *ArgR*, the terminal gene product of the *aot* operon, which functions in control of

arginine metabolism in *Ps. aeruginosa* (Park *et al.* 1997a,b). As arginine metabolism is of considerable significance in *Ps. aeruginosa*, which can use utilize this amino acid as a good source of carbon, nitrogen and energy (Haas *et al.* 1990), the induction of these genes might be involved in energy metabolism or other gene expressions required for colonization of mycelia by *Ps. putida*.

Two *pmi* fusions involved in amino acid transport processes were identified in this screen. The polypeptide of *pmi-93* gene has high similarity with a component (AotJ) of a periplasmic binding protein-dependent transporter for arginine and ornithine, which is usually organized in an operon (*aotJQMOP-argR*) with five other genes in *Ps. aeruginosa* (Nishijyo *et al.* 1998). The terminal gene, *argR*, in this operon is known to function in the control of expression of certain genes of arginine biosynthesis and catabolism, as well as in the control of expression of the catabolic glutamate dehydrogenase (Park *et al.* 1997a,b). As a sequence structure similar to that of the rho-independent terminator is known to be located in the *aotJ-aotQ* intergenic region (Nishijyo *et al.* 1998), other downstream components of the transport system and *argR* could be expressed differently from *aotJ*. The other fusion involved in amino acid uptake is *pmi-99*, for which the deduced amino acid sequence is highly similar to the sequence of the branched-chain amino acid transport protein BrcC in *Ps. aeruginosa*, and LIV-I and LS transport proteins in *E. coli* belonging to the hydrophobic amino acid transporter (HAAT) families of ABC transporters of amino acids (Saier 2000; Hosie and Poole 2001). These proteins are expected to be involved in the transport of neutral and aliphatic amino acids, such as leucine, isoleucine, valine, threonine and alanine. Recently, the Bra transporter of *Rhizobium leguminosarum* was reported to transport a broad range of solutes including acidic and basic polar amino acids, and GABA, as well as hydrophobic and neutral amino acids (Hosie and Poole 2001). Therefore, the induction of genes involved in the amino acid uptake of *Phytophthora* in this study suggests that the uptake of amino acids could be a bacterial trait involved in bacterial colonization, in addition to the ability of *Pseudomonas* to synthesize amino acids (Simons *et al.* 1997).

Some of the *pmi* genes, such as *gabD* (*pmi-41*), *dctA* (*pmi-73*) and *braC* (*pmi-99*) are correlated with rhizosphere-induced genes (Simons *et al.* 1997; Rainey 1999; Espinosa-Urgel and Ramos 2001), suggesting that similar or common mechanisms might be involved in bacterial colonization to mycelial surfaces as well as to root surfaces (rhizosphere). This is supported by the fact that some of the composition of root exudates, consisting of sugars, organic acids and amino acids, is similar to that of fungal constituents or exudates used for bacterial survival and adaptation (Bartnicki-Garcia 1966; Lugtenberg *et al.* 1999; Singh and Arora 2001). As some of the rhizosphere-induced genes of *Pseudomonas* biocontrol strains have been known to be highly expressed in response to root exudates (Lugtenberg *et al.* 1999; Vilchez *et al.* 2000; Espinosa-Urgel and Ramos 2001), *Phytophthora* constituents or exudates with a similar composition might be able to induce the *pmi* genes required for complete colonization, and provide substrates for the bacterium on mycelial surfaces. All the three *pmi* genes tested were highly expressed by *P. parasitica*-spent medium as well as various substances, such as organic acids and amino acids, suggesting that many of the *Phytophthora* signals stimulating the *pmi* gene expression might be involved in bacterial nutrition. It is notable that all the three genes are induced by glycogen that was not utilized by the *Ps. putida* strains. Glycogen is a type of branched homopolysaccharide produced as a storage product in *Phytophthora*. Related glucan polymers make up the cell wall of *Phytophthora*. Thus, the *pmi* gene expression might be stimulated by cell wall or storage components of *Phytophthora*. The diffusible signals produced by the phytopathgenic oomycete *Pythium ultimum* have been reported to repress the expression of *Ps. fluorescens* F113 genes (Fedi *et al.* 1997; Smith *et al.* 1999). However, the method by which the repression is recognized and processed by the bacterium is unknown. Thus, understanding the *in vivo* signals produced by *Phytophthora* and their target genes would provide deeper insight into the mechanisms on bacterial signal transduction and developmental colonization processes.

Although all the three *pmi* mutants appeared to be indistinguishable from the parental strain with respect to the growth rate, the Biolog assay showed differential oxidation of the different carbon substances. Particularly, the mutant 81 km differed in oxidation of several carbon sources, suggesting that the disrupted glyceraldehyde-3-phosphate dehydrogenase gene may affect different metabolic pathways. The mutant 81 km presented slower adaptation to mycelial surfaces as shown in Table 2, suggesting that the disrupted gene might be involved in the bacterial ability to utilize the substances on mycelial surfaces. When each of these mutants was mixed with the wild type and inoculated onto mycelial surfaces, their populations were reduced.Thus, these disrupted genes might be required for full competitive colonization of the mycelial surfaces.

Phytophthora exudates act as attractants and growth substrates for bacteria (Singh and Arora 2001), and bacterial growth and colonization on the mycelia might cause nutrient stress or reduced vigour to *Phytophthora*, affecting their growth (Lim and Lockwood 1988). None of these *pmi* mutants seem to be significantly affected in its

ability to inhibit *Phytophthora* growth on PAF agar medium (data not shown), suggesting that these disrupted genes might not be directly involved in antifungal activity. However, when assayed on the V8C agar medium under the same conditions, these mutants seemed to inhibit mycelia growth more than the wild type (data not shown), suggesting that these gene disruptions might cause differential colonization of *Phytophthora* mycelia depending on the nutritional environment.

A significant alteration of the colony morphology was observed in the mutant 81 km, but not in the other two mutants. Its smaller and rougher colony morphology might be caused by an effect on bacterial surface structure or surface association (Wai *et al.* 1998; Henderson *et al.* 1999; Deziel *et al.* 2001). A similar morphological alteration was observed in the phenotypic variants of *Pseudomonas* ssp., which were involved in motility, biofilm formation and various traits important for the rhizosphere colonization (Sanchez-Contreras *et al.* 2002).

Our results described earlier demonstrate that IVET screening and mutant characterization can be used to identify bacterial genes which are induced on the mycelial surface. The study of nutritional signals that originated from *P. parasitica* provided insight into the possible mechanisms of mycelial colonization by this bacterium. Disruption of the genes highly expressed during colonization of the fungal mycelia can cause various phenotypic changes affecting bacterial behaviour on mycelial surfaces.

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