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Genes Expressed in *Pseudomonas putida* during Colonization of a Plant-Pathogenic Fungus

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In vivo expression technology (IVET) was employed to study colonization of *Phytophthora parasitica* by a biological control bacterium, *Pseudomonas putida* 06909, based on a new selection marker. The *pyrB* gene, which encodes aspartate transcarbamoylase, an enzyme used for pyrimidine biosynthesis, was cloned from *P. putida* 06909. A *pyrB*-disrupted mutant did not grow in pyrimidine-deficient media unless it was complemented with *pyrBC′* behind an active promoter. Thirty clones obtained from *P. putida* 06909 that were expressed on fungal hyphae but not on culture media were isolated by IVET based on the promoterless transcriptional fusion between *pyrBC′* and *lacZ*. Nineteen of these clones were induced during late-stage bacterial growth in vitro, while 11 of the clones were expressed only when they were inoculated onto fungal hyphae. Restriction analysis of these 11 clones revealed that there were five unique clones. Sequence analyses of three of the five unique clones showed that the 3′ ends of the clones fused to *pyrB* were similar to genes encoding diacylglycerol kinase (DAGK), bacterial ABC transporters, and outer membrane porins. The sequences of the two other clones were not similar to the sequences of any of the genes in the database used. A LuxR family response regulator was found upstream of DAGK, and a LysR family response regulator was found upstream of the ABC transporter. The location of the inducible promoter of two clones suggested that DAGK and the ABC transporter are induced and may play a role in colonization of the fungus *P. parasitica* by *P. putida* 06909.

Pseudomonas putida 06909 suppresses populations of the root-rotting fungus *Phytophthora parasitica* in the citrus rhizosphere (50). This strain does not produce antibiotics in culture media. Mutagenesis has shown that both adherence to fungal hyphae and siderophore production by *P. putida* 06909 are important in the inhibition of *P. parasitica* (54). However, little else is known about the interaction between *P. putida* 06909 and *P. parasitica* during hyphal colonization. Continued interest in the use of this strain for biological control of citrus root rot (48) has prompted a more thorough analysis of this bacterium-fungus interaction.

We used in vivo expression technology (IVET) (25) to study the colonization of *P. parasitica* by *P. putida* 06909. IVET is a strategy for selecting cloned bacterial genes that are specifically induced in vivo. The in vivo-induced genes can be identified by their ability to express a promoterless selection marker gene that is essential for survival in vivo. Expression in vitro can then be monitored by studying the expression of another promoterless reporter gene cloned downstream from the selection marker as a transcriptional fusion. The IVET strategy has advantages over traditional mutagenesis techniques since there is positive selection for genes that are specifically induced by environmental cues in vivo and the procedure does not disrupt genes that may be essential in vivo.

In the original IVET system used for *Salmonella typhimurium*, a purine requirement for bacterial survival or antibiotic resistance was used as the selection criterion during bacterial infection of the host cells (25, 26). Other genes essential for bacterial survival were also used as selection markers to use the IVET system during various bacterial infections of animal cells (6, 13, 14, 24). In our study, since the target fungus, *P. parasitica*, is very sensitive to several antibiotics, antibiotic resistance genes were not useful as selection markers. In addition, we wanted to use the IVET strategy in rhizosphere and soil studies, in which antibiotic selection would not be feasible. A growth factor requirement, the pyrimidine requirement for bacterial growth, was used as a selection marker in our *Phytophthora-Pseudomonas* system.

The *pyrB* gene encodes aspartate transcarbamoylase (ATCase), an enzyme that is essential for biosynthesis of pyrimidines, and *pyrB* mutants cannot grow in pyrimidine-deficient culture media (42). Because the supply of pyrimidines is limited in many natural environments, the *pyrB* gene could provide selection in those environments. *lacZ* was used as a reporter gene for in vitro expression studies (9). To our knowledge, this is the first study in which the nondisruptive IVET strategy was used to identify bacterial genes induced during interaction with a plant-pathogenic fungus.

Our hypothesis is that bacterial genes that are specifically induced during fungal colonization are directly involved in the colonization of fungal hyphae and that some of these genes are important in the biocontrol activity of *P. putida*. These genes may ultimately be useful in novel biocontrol approaches. In this paper we describe application of the IVET strategy based on the pyrimidine requirement for bacterial growth of a mutant strain and identification of several genes specifically induced during colonization of *P. parasitica* by *P. putida* 06909. Five chromosomal loci of *P. putida* 06909 that are specifically expressed during colonization of *P. parasitica* were isolated. The sequences of two of the chromosomal loci of *P. putida* 06909 and potential promoter regions in these two loci induced during colonization of fungal hyphae were determined in this study.

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MATERIALS AND METHODS

Microorganisms, plasmids, and culture conditions. All of the organisms and plasmids used in this study are listed in Table 1. *Escherichia coli* cultures were

TABLE 1. Microorganisms and plasmids used

Strain or plasmid	Relevant characteristics[a]	Source or reference
E. coli strains		
DH5α	F⁻ *recA1* Δ(*lacZYA-argF*)*U169 hsdR17 thi-1 gyrA66 supE44 endA1 relA1* ϕ80d*lac* Δ(*lacZ*)*M15*	40
HB101	F⁻ *hsdA20* (r⁻ m⁻) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ⁻	2
P. putida strains		
06909	Wild type; Amp^r	54
06909u1	*pyrB*; uracil auxotroph; Amp^r Kan^r	This study
06909u2	*pyrB*; uracil auxotroph; Amp^r Kan^r	This study
P. parasitica M191		54
Plasmids		
pUC119	Ap^r; cloning vector	55
pRK415	Tc^r; RK2-derived broad-host-range cloning vector	20
pHRP309	Gm^r; RSF1010-based broad-host-range transcriptional fusion vector	34
pHSK728	Ap^r Sm^r; Tn*7* delivery plasmid	44
pHSK828	Ap^r Sm^r; pHSK728 derivative; a *lux* operon in pHSK728 was replaced with an ice nucleation gene	Unpublished data
pDSK509	Km^r; RSF1010-derived broad-host-range cloning vector	20
pRK2013	Km^r; mobilization helper	10
pDAC41	Ap^r; *pyrB* cloned in pUC119 at *Hin*cII site	This study
pDAC42	Ap^r Km^r; 1.2-kb kanamycin cassette inserted in pDAC41	This study
pDAC43	Ap^r; *pyrBC′* cloned in pUC119 at *Hin*cII site	This study
pDAC54	Ap^r; Φ(*pyrBC′-lacZ*) cloned in pHSK828	This study
pRIV11	Tc^r; pRK415 carrying a 1.8-kb *Sph*I-*Eco*RI fragment (blunt ended) from pDAC42 containing the *pyrB*::*km* fragment	This study
pRIV13	Tc^r; pRK415 carrying a 2.4-kb *Hin*dIII-*Bam*HI fragment from pDAC43 containing *pyrBC′*	This study
pRIV16	Tc^r; pRK415 carrying a 6.1-kb *Hin*dIII-*Bam*HI fragment from pDAC54 containing a promoterless fusion; Φ(*pyrBC′-lacZ*) in opposite orientation compared to *lac* promoter	This study
pRIV16C	Tc^r; pRK415 carrying a promoterless fusion; Φ(*pyrBC′-lacZ*) in same orientation as *lac* promoter	This study
pRIVA1	Tc^r; pRIV16 carrying a constitutive promoter	This study
pRIVS1	Tc^r; pRIV16 carrying a clone expressed on fungal plates	This study
pRIVS2	Tc^r; pRIV16 carrying a clone expressed on fungal plates	This study
pRIVS3	Tc^r; pRIV16 carrying a clone expressed on fungal plates	This study
pRIVS4	Tc^r; pRIV16 carrying a clone expressed on fungal plates	This study
pRIVS5	Tc^r; pRIV16 carrying a clone expressed on fungal plates	This study
pRIVS13	Tc^r; 3.9-kb *Eco*RI fragment from pRIVS1 transferred into pRIV16	This study
pRIVS20	Tc^r; pRIV16 carrying a 1.4-kb *Pst*I fragment from pRIVS2; the fragment has the same orientation as Φ(*pyrBC′-lacZ*) of pRIV16	This study
pRIVS26	Tc^r; pRIV16 carrying a 1.4-kb *Pst*I fragment from pRIVS2; the fragment has the opposite orientation compared to Φ(*pyrBC′-lacZ*) of pRIV16	This study
pUIVS1	Ap^r; pUC119 carrying a 5.4-kb *Kpn*I fragment from pRIVS1	This study
pUIVS11	Ap^r; pUC119 carrying a 1.4-kb *Eco*RI-*Hin*dIII fragment from pUIVS1	This study
pUIVS2	Ap^r; pUC119 carrying a 5.5-kb *Eco*RI fragment from pRIVS2	This study
pUIVS21	Ap^r; pUC119 carrying a 2-kb *Sph*I fragment from pUIVS2	This study
pUIVS3	Ap^r; pUC119 carrying a 4.2-kb *Eco*RI fragment from pRIVS5	This study
pUIVS4	Ap^r; pUC119 carrying a 2-kb *Eco*RI-*Sph*I fragment from pRIVS4	This study
pUIVS5	Ap^r; pUC119 carrying a 4.5-kb *Eco*RI fragment from pRIVS5	This study

[a] Amp^r, chromosomal ampicillin resistance; Kan^r, chromosomal kanamycin resistance; Ap^r, ampicillin resistance; Tc^r, tetracycline resistance; Sm^r, streptomycin resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance.

grown at 37°C on Luria-Bertani agar or in Luria-Bertani broth supplemented with the appropriate antibiotics (27). The following antibiotic concentrations were used for *E. coli* strains: tetracycline, 15 μg/ml; kanamycin, 50 μg/ml; and ampicillin, 100 μg/ml. *P. putida* strains were grown at 28°C on mannitol-glutamate medium (MG medium) (19) supplemented with yeast extract (0.25 g/liter) (MGY medium) or in MGY broth. The following antibiotic concentrations were used in MGY medium: tetracycline, 20 μg/ml; kanamycin, 30 μg/ml; and ampicillin, 200 μg/ml. MG medium or MGY medium was supplemented with uracil (50 μg/ml) for growth of pyrimidine auxotrophs of *P. putida* 06909. When it was necessary for bacterial growth in rich medium, *P. putida* strains were cultured on *Pseudomonas* agar F under the same conditions. *P. parasitica* M191 was grown at 28°C on V8C agar or in V8C broth (30). Plasmids pHRP309 and pHSK828 were kindly provided by Caroline Harwood, University of Iowa, Iowa City, and by Noel Keen, University of California, Riverside, respectively.

Recombinant DNA techniques and DNA sequencing. Plasmid preparation, restriction endonuclease cleavage, ligation, agarose gel electrophoresis, and other standard recombinant DNA techniques were carried out as described previously (40). Total DNA of *P. putida* wild-type strain 06909 and its mutants was prepared by the standard cetyltrimethylammonium bromide method described elsewhere (1). Southern blot analysis of digested genomic DNA from *P. putida* strains was performed with nylon membranes (MSI, Westboro, Mass.). A 1.2-kb *Ava*II fragment carrying a kanamycin resistance cassette and a 700-bp *Pst*I fragment of *pyrB* were used as probes to confirm the correct replacement of the *pyrB* locus of *P. putida* 06909 with *pyrB*::*km*. Probes were labeled with a digoxigenin-dUTP DNA labeling kit (Boehringer GmbH, Mannheim, Germany) and were detected with a chemiluminescent substrate, disodium 3-(4-methoxyspiro-{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate (Boehringer), as described in the manufacturer's instructions.

DNA sequencing was carried out at the DNA Sequencing Facility of the University of California, Berkeley. When automated sequencing was not successful for a segment containing a palindromic sequence, DNA sequencing was carried out manually by using the chain termination method (41) and a Sequenase version 2.0 DNA sequencing kit (U.S. Biochemicals, Braunschweig, Germany). The DNA sequences were analyzed by using a software package obtained from the Genetics Computer Group of the University of Wisconsin and the Blast programs provided by the National Center for Biotechnology Information. The primers used in this study were synthesized commercially (Genosys Biotechnologies, Inc., The Woodlands, Tex.).

Cloning of *pyrB* and marker exchange mutagenesis. Degenerate primers D1 (5′-dATGACGCCNATHGAYGCNAAR-3′) and D2 (5′-TCAYTGNGCRTT YTCYTGRTC-3′), based on the PyrB sequence from *P. putida* PPN and *Pseudomonas aeruginosa* PAO (42), were designed to amplify *pyrB* from *P. putida* 06909 (Y = T or C; N = A, T, G, or C; R = A or G; H = A, T, or C). DNA was amplified in a 100-μl (total volume) reaction mixture which contained 10 μl of Vent DNA polymerase 10× buffer (New England BioLabs, Inc., Beverly, Mass.), each deoxynucleoside triphosphate (New England BioLabs) at a concentration of 200 μM, 5 mM $MgSO_4$, 5% dimethyl sulfoxide, each primer at a concentration of 1 μM, and 0.5 U of Vent DNA polymerase. The template DNA added was 200 ng of total *P. putida* 06909 DNA. PCR amplification was performed with an automated thermocycler (EasyCycler Series; Ericomp, Inc., San Diego, Calif.) by

using the following program: an initial DNA template denaturation step consisting of 95°C for 5 min; 30 cycles consisting of denaturation at 95°C for 1 min, annealing at 52°C for 30 s, and extension at 72°C for 1 min; and a final extension step consisting of 72°C for 5 min. The 1-kb PCR product was cloned at the *Hin*cII site of pUC119 to generate pDAC41. The identity of the PCR product was confirmed by DNA sequencing. The PCR product was used for marker exchange mutagenesis. To disrupt the wild-type *pyrB* gene, a marker exchange plasmid was constructed as follows. The 400-bp internal *Hin*cII fragment of pDAC41 was deleted, and a 1.2-kb *Ava*II fragment carrying a kanamycin resistance cassette from pDSK509 (20) was filled with the Klenow DNA polymerase and ligated at the *Hin*cII site of pDAC41. The resulting plasmid was pDAC42. A 1.8-kb *Sph*I-*Eco*RI fragment carrying *pyrB* in pDAC42 with the kanamycin resistance cassette was filled with the Klenow DNA polymerase and ligated into a blunt-end *Eco*RI site of a broad-host-range plasmid, pRK415 (20), to generate plasmid pRIV11. The plasmid was introduced into *P. putida* 06909 by triparental mating in which pRK2013 was used as a helper plasmid. Marker exchange mutagenesis was carried out as described previously (23). Plasmid pRK415 is unstable in the absence of selection, and a cycloserine enrichment culture was used to enhance recovery of homologous recombinants. The growth rates of the mutants and the wild-type strain were compared by monitoring A_{600} in MG broth supplemented with 50 μg of uracil per ml.

Complementation of the *pyrB* mutant. To complement the *pyrB* mutation in *P. putida*, *pyrBC′* was amplified from wild-type *P. putida* 06909 with one specific primer, primer P3, and one degenerate primer, primer D3. Primer P3 (5′-dT A**GGAGAA**CCCCGCGATGAGCCGATCGACGCCAAG-3′) was designed based on the PCR product of *pyrB*, and primer D3 (5′-dTTACTCAGGCCCRT GRCAGACRTGNCCRTCNAC-3′) was designed based on the 3′ ends of *pyrC′* of *P. putida* PPN and *P. aeruginosa* PAO (in the primer P3 sequence the boldface type indicates a conserved Shine-Dalgarno sequence, and the underlined portion is the translation start codon of the *pyrB* gene). The *pyrC′* gene is a *pyrC* homolog downstream of *pyrB* that is required to form a functional ATCase in *P. putida* PPN, as previously described (42). The reaction mixture was the same as the reaction mixture used for *pyrB* amplification except that the concentration of each deoxynucleoside triphosphate was 400 μM and the concentration of $MgSO_4$ was 6 mM. DNA amplification was performed by using the following program: an initial DNA template denaturation step consisting of 95°C for 5 min; 24 cycles consisting of denaturation at 95°C for 1 min, annealing at 70°C for 30 s, and extension at 72°C for 2 min 20 s; and a final extension step consisting of 72°C for 5 min. The 2.4-kb amplified fragment was cloned into pUC119 at the *Hin*cII site as a blunt-end fragment to obtain pDAC43. The 2.4-kb *Hin*dIII-*Eco*RI fragment in pDAC43, carrying promoterless *pyrBC′*, was subcloned behind the *lac* promoter of pRK415 to generate pRIV13.

Bacterial growth on fungal cultures. To study the potential use of the *pyrB* gene as a selection marker for IVET, we compared growth of *P. putida* 06909u2 (*pyrB*) and growth of wild-type strain *P. putida* 06909 on V8C agar covered by fully grown *P. parasitica*. *P. parasitica* M191 was grown at 28°C on V8C agar plates until the plates were completely covered by the fungus. Wild-type *P. putida* and the *pyrB* mutant were grown in MG medium and MG medium supplemented with uracil and kanamycin, respectively, and then they were resuspended in sterile water. Each bacterial suspension was diluted with sterile water to obtain a concentration of 4×10^3 CFU/ml. A 1.5-ml portion of each dilution was applied to a fungal plate covered by *P. parasitica*. The plates were air dried briefly in a laminar flow hood and incubated at 28°C for 20 to 29 h. The bacteria were harvested from the fungal plates by adding 5 ml of sterile water to each plate, loosening the bacteria with a small glass rod, and removing the suspension with a pipette. The number of CFU per milliliter in each final suspension was determined by plating serial dilutions onto MG medium containing ampicillin for the wild-type strain, MG medium containing kanamycin and uracil for mutant strain 06909u2, and MG medium containing kanamycin and tetracycline for mutant strain 06909u2 carrying pRIV13.

Bacterial growth in the presence of *P. parasitica* was also studied by using small agar discs covered by the fungus. V8C agar discs containing actively growing *P. parasitica* were transferred to fresh V8C agar plates, and a bacterial inoculum was applied to the surface of each disc. Bacterial cultures were grown on MG medium and transferred with toothpicks. The plates were incubated overnight, and the fungal agar discs were dropped into 1-ml portions of sterile water to release the bacteria. The bacterial titer was determined by measuring the A_{600} of each final suspension. A fungal column assay was also used to determine whether the *pyrB* mutation affected the initial adhesion of *P. putida* 06909 to *P. parasitica* hyphae. Preparation of the fungal column and quantification of bacterial adhesion to fungal hyphae were performed as previously described (54).

Plasmid construction. Broad-host-range plasmid pRIV16, a derivative of pRK415, was constructed and used as a basic plasmid to deliver the IVET construct to *P. putida* 06909u2 (Fig. 1). A 3.6-kb *Bam*HI-*Hin*dIII fragment containing the promoterless *lacZ* gene was cut from pHRP309 and filled with the Klenow enzyme to generate blunt ends. This fragment was cloned into a blunt-end *Xho*I site of pHSK828, a derivative of pHSK728 (44). The *lux* operon of pHSK728 was replaced with an ice nucleation gene to create pHSK828 (unpublished data). A 2.4-kb *Hin*dIII-*Bam*HI fragment carrying the promoterless *pyrBC′* genes in pDAC43 was blunt ended with Klenow DNA polymerase and cloned into the *Sma*I site in front of the promoterless *lacZ* gene to generate pDAC54. Plasmid pDAC54 was first digested with *Bam*HI and then partially digested with *Hin*dIII to obtain a 6.1-kb fragment containing a promoterless transcriptional fusion between *pyrBC′* and *lacZ*. This fragment was cloned into the *Bam*HI-*Hin*dIII site of pRK415 to generate pRIV16. In addition, this fragment was filled with the Klenow enzyme and cloned at the blunt-end *Xba*I site of pRK415 to generate pRIV16C. Plasmid pRIV16C was used as a positive control to check the expression of the transcriptional fusion in *pyrB* mutant strain 06909u2, since the promoterless transcriptional fusion was located behind the *lac* promoter, which is constitutive in pseudomonads. The nucleotide sequences at the 5′ end of *pyrBC′*, including polylinker sites in pRIV16, were confirmed by DNA sequencing.

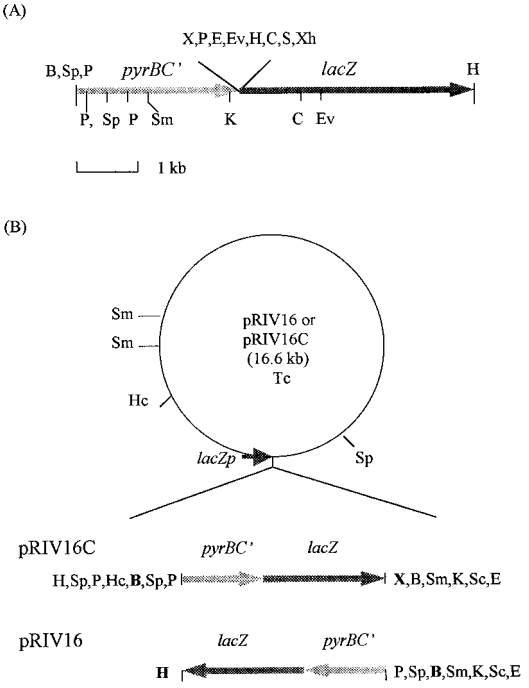
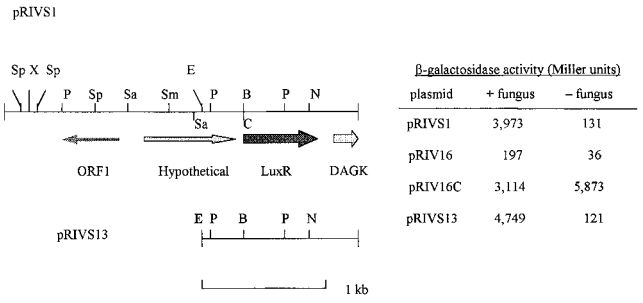


FIG. 1. Construction of IVET plasmids pRIV16 and pRIV16C. (A) Restriction map of a promoterless transcriptional fusion between *pyrBC′* and *lacZ*. (B) pRIV16 and pRIV16C carry *pyrBC′-lacZ* in the reverse orientation. Promoterless *pyrBC′-lacZ* digested with *Bam*HI and partially digested with *Hin*dIII was cloned into pRK415 to generate pRIV16 and was cloned into a *Xba*I site as a blunt-end ligation to generate pRIV16C. The restriction enzyme sites in the *pyrBC′-lacZ* genes are not shown for pRIV16 and pRIV16C. The *Sph*I (Sp) and *Pst*I (P) sites in the upstream region of *pyrBC′* were derived from pUC119 in the subcloning process. The polylinker sites between *pyrBC′* and *lacZ* were originally from pHSK728. The boldface *Bam*HI (B), *Xba*I (X), and *Hin*dIII (H) sites are cloning sites in pRK415. The boldface *Bam*HI (B) and *Xba*I (X) sites in pRIV16C were retained after blunt-end ligation. The enzyme sites located outside the boldface letters were derived from the polylinker of pRK415. Abbreviations: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; Hc, *Hin*cII; K, *Kpn*I; P, *Pst*I; S, *Sal*I; Sc, *Sac*I; Sm, *Sma*I; Sp, *Sph*I; X, *Xba*I; Xh, *Xho*I.

Isolation of clones expressed on *P. parasitica*. A genomic library of *P. putida* 06909 was constructed in pRIV16 by using the standard recombinant DNA protocol (40). Briefly, total genomic DNA of *P. putida* 06909 was partially digested with *Sau*3AI and dephosphorylated with calf intestinal phosphatase to prevent self-ligation between small insert fragments. DNA fragments were fractionated in a sucrose density gradient (10 to 40% sucrose) for 24 h at 22,000 rpm. Fractions containing DNA fragments between 2 and 7 kb long were collected. The fragments were ligated into the unique *Bam*HI site of pRIV16 to generate the library. The recombinant clones were introduced into *pyrB* mutant strain *P. putida* 06909u2 by triparental mating on yeast extract-dextrose-calcium carbonate agar (53) supplemented with 50 μg of uracil per ml. The transconjugants carrying recombinant clones were applied to V8C agar plates covered by *P. parasitica* at levels of 50,000 to 70,000 CFU per plate. The fungal plates were air dried briefly in a laminar flow hood and incubated for 24 h at 28°C. The cells were harvested with 2 ml of sterile water and plated onto MG agar supplemented with uracil, tetracycline, and kanamycin. The cells that grew on MG agar were resuspended, diluted with sterile water, and reapplied to V8C agar fungal plates at levels of 50,000 to 70,000 CFU per plate to enrich the growing cells. This process was repeated at least three times to enrich for positive clones. Finally, cells were spread onto MG agar supplemented with uracil, tetracycline, kanamycin, and 80 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml. Ex-



β-galactosidase activity (Miller units)

plasmid	+ fungus	– fungus
pRIVS1	3,973	131
pRIV16	197	36
pRIV16C	3,114	5,873
pRIVS13	4,749	121

FIG. 2. Organization of the potential ORFs in pRIVS1 and various restriction enzyme sites. The promoterless *pyrBC′* and *lacZ* genes are located downstream of the DAGK gene as a transcriptional fusion in pRIVS1. ORF1 is a potential secreted protein. pRIVS13 restores induction of the clone during colonization of fungal hyphae at the level of pRIVS1. The levels of β-galactosidase activity in *P. putida* 06909u2 containing different plasmids were determined in the presence and in the absence of *P. parasitica*. Plasmids pRIV16 and pRIV16C were the negative and positive controls, respectively. The boldface *Eco*RI (E) site was used to subclone the 1.35-kb fragment to generate pRIVS13. Abbreviations: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; N, *Nco*I; P, *Pst*I; Sa, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; X, *Xba*I.

pression of the promoterless transcriptional fusion, *pyrBC′-lacZ* in pRIV16, was expected to be dependent on expression of the upstream library insert DNA. A *P. putida* 06909u2 transconjugant which carried putative clones expressed specifically during colonization of *P. parasitica* should have grown in the presence of *P. parasitica* or in media containing uracil. When the isolates were grown on V8C agar supplemented with uracil in the absence of *P. parasitica*, the colonies should have been white on X-Gal-containing medium due to a lack of expression of the upstream gene. A total of 30 white colonies were selected, and 11 plasmids were isolated and used for further characterization. The remaining 19 colonies turned blue at a late stage of bacterial growth and therefore were not considered as specific in their expression patterns. The plasmids isolated were analyzed by determining the restriction enzyme digestion patterns obtained with *Eco*RI, *Bam*HI, *Hin*dIII, *Sph*I, *Kpn*I, and *Xba*I. The plasmids were reintroduced into *pyrB* mutant strain 06909u2 by triparental mating. The transconjugants carrying selected plasmids were applied to fungal agar discs, as described above, to confirm that bacterial growth occurred in the presence of *P. parasitica*. Growth of the transconjugants in the absence of *P. parasitica* was also examined. *P. putida* 06909 genomic DNA fragments in induced clones pRIVS1, pRIVS2, pRIVS3, pRIVS4, and pRIVS5 were subcloned into pUC119 for sequencing. A 5.4-kb *Kpn*I fragment from pRIVS1, a 5.5-kb *Eco*RI fragment from pRIVS2, a 4.2-kb *Eco*RI fragment from pRIVS3, a 4.4-kb *Eco*RI fragment from pRIVS5, and a 2-kb *Sph*I-*Eco*RI fragment from pRIVS4 were subcloned into pUC119 that was digested with the same restriction enzymes. The subcloned fragments from pRIVS1, pRIVS2, pRIVS3, and pRIVS5 also contained the *pyrBC′* genes, because convenient restriction enzymes to subclone the inserts without *pyrBC′* were not found except for the pRIVS4 clone. The final constructs were designated pUIVS1, pUIVS2, pUIVS3, pUIVS4, and pUIVS5.

β-Galactosidase assays. *P. putida* 06909u2 transconjugants carrying selected plasmids were grown overnight in V8C broth supplemented with uracil and were subcultured in 5 ml of fresh V8C broth supplemented with uracil. The resulting subcultures were then grown for 6 h. The cells were harvested by centrifugation. Finally, the cells were resuspended in sterile water and assayed for β-galactosidase activity, as described by Miller (27), by using *o*-nitrophenyl-β-D-galactopyranoside as the substrate. The same transconjugants were also grown on V8C agar supplemented with 50 μg of uracil per ml or on V8C agar discs covered by *P. parasitica* for 20 to 24 h. The cells grown on a V8C agar plate containing uracil and appropriate antibiotics were resuspended in water and assayed for β-galactosidase activity by using the same procedure. The V8C agar fungal discs colonized by *P. putida* strains were dropped into 1 ml of sterile water and resuspended to release the bacterial cells grown on the fungal discs. The β-galactosidase activities of the cells obtained from fungal discs were determined by the same procedure. The controls included a fungal agar disc that was not inoculated with bacteria and a fungal agar disc that was inoculated with *P. putida* 06909u2 (pRIV13). Plasmid pRIV13 complemented the uracil auxotrophy of *P. putida* 06909u2, but this plasmid does not contain a *lacZ* gene.

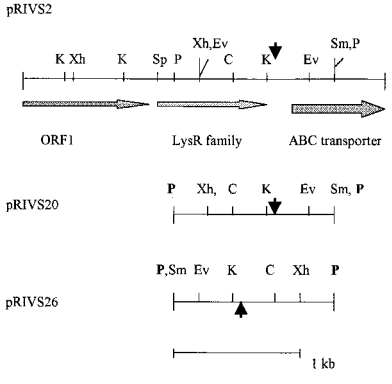
Defining potential inducible promoter areas. Potential fungus-induced clones were subcloned into pRIV16 to determine the location of promoters induced during colonization of *P. parasitica* M191 by *P. putida* 06909. Figures 2 and 3 show restriction maps of two clones that were completely sequenced and subclones with restriction enzyme sites. Sometimes fragments were first subcloned into pUC119 to have enough restriction sites to transfer into pRIV16, which had only a few unique restriction sites. The negative control plasmid used was pRIV16.

Nucleotide sequence accession numbers. The DNA sequences determined in this study have been deposited in the GenBank database under accession no. AF249735 (clone in pRIVS1), AF249736 (clone in pRIVS2), and AF249737 (clone in pRIVS3)].

RESULTS

Cloning of *pyrB* from *P. putida* 06909. ATCase is an enzyme that is essential for pyrimidine biosynthesis. We cloned a gene encoding the *P. putida* 06909 ATCase to determine whether it could be used as a selectable marker in the IVET strategy. An ATCase gene, *pyrB*, was amplified from *P. putida* 06909 by PCR by using a pair of degenerate primers designed on the basis of the amino acid sequence of PyrB from *P. putida* PPN and *P. aeruginosa* PAO. The PCR product, which was approximately 1 kb long, was cloned in pUC119 to generate pDAC41. The partial 5′ and 3′ nucleotide sequences of the cloned PCR product were very similar to the *pyrB* nucleotide sequences of *P. putida* PPN and *P. aeruginosa* PAO (data not shown). A restriction map of the PCR product of *pyrB* was determined by using restriction endonuclease cleavage patterns (data not shown).

Generation of *pyrB* mutant. To determine whether the *pyrB* gene would be a good selectable marker, it was necessary to create a mutation in the *pyrB* gene of *P. putida* 06909. *pyrB* mutants should be auxotrophic for pyrimidine. Therefore, a pyrimidine requirement for growth or survival of an auxotroph should be a good selection criterion in pyrimidine-deficient environments. To create a mutation in the *pyrB* gene of *P. putida* 06909, marker exchange mutagenesis was used. Plasmid pRIV11 carrying the marker exchange construct was introduced into wild-type strain *P. putida* 06909, and the wild-type *pyrB* gene was replaced with the marker exchange construct by homologous recombination. Two kanamycin-resistant but tetracycline-sensitive mutants were obtained, and they did not grow on minimal medium (MG medium) unless uracil was supplied. Growth of the two mutants in minimal medium (MG medium) supplemented with uracil was comparable to growth of the wild-type strain in the same medium (data not shown). This indicated that the mutational process did not impair bacterial growth except for the uracil requirement. The mutation was further confirmed by Southern blot hybridization with two



β-galactosidase activity (Miller units)

plasmids	+ fungus	– fungus
pRIVS2	2,248	124
pRIVS20	1,691	139
pRIVS26	220	46

FIG. 3. Organization of the potential ORFs in pRIVS2 and various restriction enzyme sites. The promoterless *pyrBC′* and *lacZ* genes are located downstream of the ABC transporter as a transcriptional fusion. ORF1 is similar to heavy-metal-transporting ATPases. pRIVS20 restores induction of pRIVS2 during colonization of fungal hyphae at the level of pRIVS2. The levels of β-galactosidase activity in *P. putida* 06909u2 containing different plasmids were determined in the presence and in the absence of *P. parasitica*. pRIVS26 carries the reverse-oriented *Pst*I fragment. The arrows indicate the positions of the transcriptional terminator-bearing palindromic sequences. Abbreviations: C, *Cla*I; Ev, *Eco*RV; K, *Kpn*I; P, *Pst*I; Sm, *Sma*I; Sp, *Sph*I; Xh, *Xho*I.

different probes (data not shown). When a *pyrB* probe was used, the expected size difference (800 bp) between the wild-type and mutant *pyrB* loci was observed. This difference was due to removal of the 400-bp internal *Hin*cII fragment and insertion of the 1.2-kb kanamycin resistance gene. In addition, the kanamycin resistance gene hybridized only with DNA isolated from the mutant strains. No differences between the two *pyrB* mutants were found. Therefore, only one of the *pyrB* mutant strains (*P. putida* 06909u2) was used in the experiments described below.

Complementation of *pyrB*. The *pyrB* gene with a complete Shine-Dalgarno sequence, driven by the *lac* promoter, did not complement mutants *P. putida* 06909u1 and *P. putida* 06909u2 in *trans*. A similar observation was made by Schurr et al. (42), who proposed that another open reading frame (ORF) (*pyrC′*) downstream of *pyrB* was required for complementation of the *pyrB* mutation in *trans*. Even though PyrC′ is similar to PyrC at the amino acid level, the PyrC′ polypeptide does not exhibit the dihydroorotase activity that is associated with PyrC. The PyrC′ polypeptide appears to be required for ATCase folding. Since six polypeptides of PyrB and six polypeptides of PyrC′ form a dodecameric functional ATCase in *P. putida* PPN, these polypeptides may have to be cotranslated to fold correctly (42). Therefore, we amplified the 2.4-kb *pyrBC′* fragment with specific primer P3, which was based on the 5′ nucleotide sequence of the *pyrB* PCR product, and degenerate primer D3, which was based on the 3′ end of *pyrC′* from *P. putida* PPN. Primer P3 has a conserved Shine-Dalgarno sequence, GGAGAA. The 2.4-kb *pyrBC′* PCR product was cloned into pRK415 to generate pRIV13. *pyrB* mutant strain *P. putida* 06909u2 carrying pRIV13 grew in minimal medium. This result confirmed that the *pyrBC′* gene was required for complementation of pyrimidine auxotrophy in *P. putida* 06909u2. The promoterless *pyrBC′* gene was therefore used as a selection marker in our IVET construction.

Bacterial growth on fungal plates. A fungal plate assay was developed to determine whether the pyrimidine requirement provided a good selection criterion for clones expressed during colonization of *P. parasitica* by *P. putida* 06909. *P. parasitica* was grown so that it completely covered a V8C agar plate. Since V8C agar did not contain sufficient pyrimidine to support the growth of *P. putida* 06909u2 (a *pyrB* mutant strain), the only way for the mutants to grow on this fungal plate was to acquire pyrimidine from *P. parasitica*. Therefore, if pyrimidine was not available from *P. parasitica*, the pyrimidine requirement would provide a good criterion to select between wild-type strain *P. putida* 06909 and *pyrB* mutant strain *P. putida* 06909u2.

Bacterial growth on the fungal plate revealed that there was significant selection between the wild type and the *pyrB* mutant (Fig. 4). *P. putida* 06909u2 did not grow on the fungal plate after 24 h, while the wild-type strain grew rapidly. There was a more-than-4-log difference between the sizes of the wild-type and *pyrB* mutant bacterial populations (Fig. 4A). The size of the *pyrB* mutant population did not increase above the size of the initial population applied (approximately 3×10^3 to 6×10^3 CFU per plate). In addition, the *pyrB* mutant carrying *pyrBC′* behind the *lac* promoter (pRIV13) could grow and complement pyrimidine auxotrophy on the fungal plate (Fig. 4A). The mixed-application experiment in which both the wild type and the mutant (50:50) were used resulted in a similar selection (Fig. 4B). The results obtained after mixed application of the two strains indicated that there was no cross-feeding on fungal hyphae by the strains during the fungal colonization process. Additional experiments in which we used different ratios of the wild-type strain and the *pyrB* mutant strain on fungal plates revealed that there was a significant selection for the wild-type bacteria (data not shown). We concluded that the *pyrB* gene was a good selection marker with which to study colonization of *P. parasitica* by *P. putida* 06909.

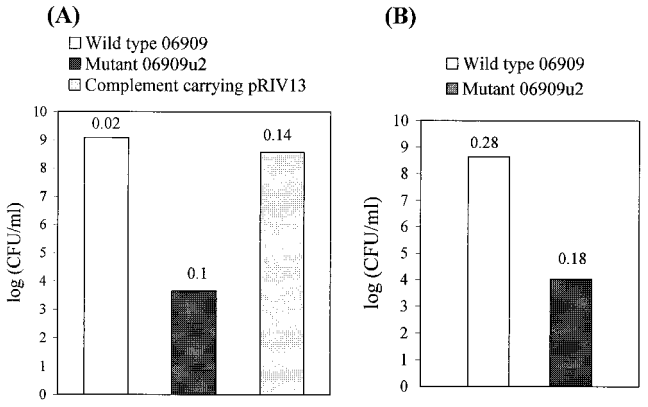


FIG. 4. Bacterial growth on *P. parasitica*-covered V8C agar plates. (A) Individual application of the wild type, the *pyrB* mutant, and the *pyrB* mutant carrying pRIV13. (B) Mixed application of both the wild type and the *pyrB* mutant. The *P. putida* 06909 inoculum contained 4×10^3 CFU/ml. The numbers above the bars are the standard errors based on three replications.

Adhesion of the wild-type strain and the *pyrB* mutant strain to fungal hyphae was determined by using a fungal column assay (54). The fungal column assay showed that the initial adhesion to the fungal hyphae was not affected by the *pyrB* mutation (data not shown).

Selection of clones expressed on *P. parasitica*. Putative clones that were specifically expressed during colonization of fungal hyphae were selected as described in Materials and Methods. A total of 30 clones were selected. Only 11 of these clones were used for further characterization because the other 19 clones exhibited increases in gene expression in culture media when they were cultured for a prolonged period (data not shown). It was hard to determine whether these 19 clones were expressed during colonization of *P. parasitica* or induced at the late bacterial growth stage. However, we could not rule out the possible involvement of these genes in colonization of fungal hyphae. The 11 clones which we studied were analyzed by comparing restriction enzyme digestion patterns obtained with different restriction enzymes. Some of the clones were identical, and five unique clones were characterized further.

In addition to the clones that were induced during colonization of *P. parasitica*, we identified one plasmid (pRIVA1) that was constitutively expressed. pRIVA1 was isolated from a blue colony of *P. putida* on V8C agar supplemented with X-Gal. This plasmid carried an unknown insert in front of the promoterless *pyrBC′-lacZ* genes. Plasmid pRIVA1 was used as a positive control.

To confirm that the clones selected were specifically expressed during colonization of *P. parasitica*, we measured the bacterial growth and β-galactosidase activity of the *P. putida* 06909u2 *pyrB* mutant carrying each of the selected clones (Table 2). The *pyrB* mutant carrying pRIV16C or pRIVA1 grew in the absence of the fungus due to constitutive expression of downstream *pyrBC′*. In contrast, the *pyrB* mutant carrying one of five selected clones (pRIVS1, pRIVS2, pRIVS3, pRIVS4, or pRIVS5) did not grow in the absence of the fungus but grew quickly in the presence of *P. parasitica*. The *pyrB* mutant carrying pRIV16, a negative control, did not grow in the presence of the fungus or in the absence of the fungus unless uracil was present in the medium.

TABLE 2. Bacterial growth and β-galactosidase activity of *P. putida* 06909u2 containing different plasmids

Introduced plasmid or prepn	Bacterial growth on V8C agar		β-Galactosidase activity (Miller units)		
	Without fungus[a]	With fungus[b]	V8CU broth[c]	V8CU agar[d]	V8C agar fungal disc
pRIV13	+	+	ND[e]	ND	ND
pRIV16	−	−	49	26	ND
pRIV16C	+	+	1,570	4,375	2,166
pRIVA1	+	+	3,009	933	3,005
pRIVS1	−	+	237	189	3,297
pRIVS2	−	+	201	142	2,597
pRIVS3	−	+		371	3,957
pRIVS4	−	+	166	144	731
pRIVS5	−	+	306	141	1,834
Fungal disc			ND	ND	ND

[a] Bacterial growth on a V8C agar plate. −, no visible bacterial colonies after 2 days; +, visible bacterial growth after 1 day.

[b] Bacterial growth on a fungal disc as determined by turbidity. −, A_{600} less than 0.075; +, A_{600} more than 0.15.

[c] V8CU broth, V8C broth supplemented with uracil (50 μg/ml).

[d] V8CU agar, V8C agar supplemented with uracil.

[e] ND, not detected.

Gene expression was quantified by measuring β-galactosidase activity in the bacterial suspensions. Expression of pRIVS1, pRIVS2, pRIVS3, pRIVS4, and pRIVS5 increased at least 5- to 20-fold during colonization of fungal hyphae (Table 2). The enhanced level of *lacZ* expression was equivalent to the level of expression by the *lac* promoter (pRIV16C) or the other constitutive promoter (pRIVA1). In contrast, expression of pRIV16 was not detected in the presence of the fungus. A control V8C agar disc covered by *P. parasitica* was also dropped into 1 ml of sterile water to monitor the β-galactosidase activity in the fungal mycelium alone. The suspension did not become turbid even when it was vortexed vigorously, and there was no β-galactosidase activity in the suspension, indicating that the fungal mycelium itself did not exhibit detectable β-galactosidase-like enzyme activity.

Another control experiment was conducted by applying the *pyrB* mutant strain carrying pRIV13, which expressed *pyrBC′* from the *lac* promoter and lacked the *lacZ* reporter gene, to a fungal disc. The bacteria grew actively on the fungal disc, but the bacterial suspension released from the fungal hyphae did not exhibit β-galactosidase activity. Therefore, we concluded that the clones selected, clones pRIVS1, pRIVS2, pRIVS3, pRIVS4, and pRIVS5, were specifically induced in the presence of the fungus.

DNA sequence analysis. Two of the clones selected were completely sequenced, and three of them were partially sequenced at the 3′ ends of the inserts. Two of the clones were not significantly similar to any genes in the GenBank database, and three of them exhibited high levels of similarity to previously described bacterial genes.

We determined putative ORFs and the organization of these putative ORFs in a 2.9-kb DNA fragment spanning most of the insert in front of *pyrBC′* in pUIVS1 (Fig. 2). The 3′ end of the genomic DNA insert of pUIVS1 fused to promoterless *pyrBC′* was a truncated ORF encoding only 63 amino acids. This truncated ORF was similar to the corresponding 5′ regions of genes encoding the diacylglycerol kinases (DAGK) of *Escherichia coli* (52% amino acid sequence identity; GenBank accession no. K00127) (22), *Haemophilus influenzae* (43% identity; GenBank accession no. U32718) (49), and *Pseudomonas denitrificans* (44% identity; GenBank accession no. M62868) (5). Intact *E. coli* DAGK is 122 amino acids long, and *Pseudomonas denitrificans* DAGK has 137 residues. Thus, the amino acid sequence of the truncated DAGK of *P. putida* 06909 corresponds to one-half of the DAGK coding region. The membrane-spanning domains of *E. coli* DAGK (47) were conserved in *P. putida* 06909 DAGK (data not shown).

Upstream of the DAGK homolog, a LuxR family response regulator homolog was found. The deduced 216-amino-acid sequence exhibited high levels of similarity to the sequences of a large number of response regulators from various bacteria. High levels of similarity were found with AgmR of *P. aeruginosa* (42% amino acid sequence identity; GenBank accession no. M60805) (43), which is involved in glycerol metabolism; FlcA of *Azospirillum brasilense* (38% identity; GenBank accession no. Y12363) (35), which is involved in colonization of the wheat rhizosphere and flocculation of bacteria; FlhR of *Paracoccus denitrificans* (GenBank accession no. AJ223460) (unpublished data), which is responsible for methanol, methylamine, and formaldehyde oxidation; and NarL of *E. coli* (34% identity; GenBank accession no. X14884) (38). An alignment of the sequence of the response regulator with the sequences of AgmR of *P. aeruginosa* and FlcA of *A. brasilense* showed that these proteins exhibit high levels of identity in a conserved area corresponding to residues 168 to 191 for a DNA-binding, helix-turn-helix motif (3, 17).

The 3-kb insert of pUIVS2 was subcloned into pUC119 as two *Sph*I fragments. The 2-kb *Sph*I fragment was completely sequenced, and it consisted of one complete ORF and one truncated ORF (Fig. 3). The truncated ORF was located at the fusion with the promoterless *pyrBC′*. The deduced amino acid sequence encoded by the truncated ORF was very similar to the sequences of many ABC transporters from various bacteria. The 282-amino-acid sequence encoded by this truncated ORF was very similar to the sequences of sulfate transporters from *Synechococcus* sp. (53% amino acid sequence identity; GenBank accession no. J04512) (12), *Synechocystis* sp. (52% identity; GenBank accession no. D90916) (18), and *E. coli* (48% identity; GenBank accession no. M32101) (46). It was also very similar to the sequences of the ferric transporter of *E. coli* (44% identity; GenBank accession no. P37009) (51), the putrescine transporter of *E. coli* (49% identity; GenBank accession no. M93239) (36), and the maltose transporter of *Pseudomonas fluorescens* (42% identity; GenBank accession no. U39468) (4). Other transporters, such as the transporters for polyamines, sugars, peptides, etc., from various bacteria were also very similar to the putative ABC transporter from *P. putida* 06909 (data not shown).

A second complete ORF was located upstream of the ABC transporter. Since there is 229 bp between the stop codon of this ORF and the start codon of the ABC transporter, it is likely that a potential promoter is located in this space. The deduced 289-amino-acid sequence encoded by the ORF was similar to the sequences of the ribulose bisphosphate carboxylase operon transcriptional regulators of various photosynthetic bacteria, such as *Synechocystis* sp. (31% amino acid sequence identity; GenBank accession no. D90910) (18; see reference 45 for a review). The ORF-encoded protein was also similar to the LysR family response regulators, such as OxyR from *E. coli*, which regulates the oxidative stress response, a specific colony morphology switch, bacterial aggregation, and piliation (8, 52). The highly conserved DNA-binding motif, a helix-turn-helix, is located in the N-terminal region of this response regulator and aligns with the DNA-binding motifs of other LysR family response regulators (3) (data not shown). Our DNA sequence comparison of the clone in pRIVS2 and the incomplete *P. aeruginosa* genomic DNA sequence showed that the similar sequences were scattered through different

contigs. This indicated that the gene organization in this locus in *P. putida* 06909 is different from the gene organization in *P. aeruginosa* (data not shown).

One of the clones (pRIVS3) was partially sequenced, and it exhibited high levels of similarity to a few outer membrane porin proteins. The deduced amino acid sequence encoded by the truncated ORF was similar to the sequence of outer membrane porin OprD2 of *P. aeruginosa*, which forms the imipenem-permeable porin (56), and was also similar to the sequence of PhaK of *P. putida*, which transports phenylacetic acid (33).

Potential promoter areas in pUIVS1 and pUIVS2. Potential promoters induced during colonization of *P. parasitica* were subcloned into pRIV16. The 1.35-kb portion of the pUIVS1 *Eco*RI fragment spanning the truncated *dgk* homolog and the *luxR* family response regulator (nucleotides 1547 to 2915) retained inducible promoter activity (Fig. 2). In the presence of *P. parasitica*, the level of β-galactosidase activity of this subclone was the same as the level of activity observed with the original pRIVS1 clone. Thus, the inducible promoter could be located in front of the LuxR family response regulator gene or between the response regulator gene and *dgk*.

The 1.4-kb *Pst*I fragment of pUIVS2 exhibited inducible promoter activity when the fragment was oriented correctly with respect to the promoterless *pyrBC′-lacZ* genes (pRIVS20). No inducible promoter activity was observed when the fragment was placed in the opposite orientation (pRIVS26) (Fig. 3). DNA sequence analysis of the genomic DNA fragment in pUIVS2 revealed a strong palindromic sequence with a 33-bp stem and a 24-bp loop between the stop codon for the LysR family response regulator and the start codon for the ABC transporter (Fig. 3). It is likely that the palindromic sequence is a transcriptional terminator of the LysR family response regulator gene. Therefore, we concluded that the inducible promoter is probably located in front of the ABC transporter gene and that the ABC transporter may play a role in colonization of *P. parasitica* by *P. putida* 06909.

DISCUSSION

P. parasitica hyphal colonization and subsequent inhibition of fungal growth by *P. putida* 06909 are important for the biocontrol activity of *P. putida* (54). The colonization of hyphae by *P. putida* is a biologically interesting phenomenon. However, little is known about bacterial colonization of fungal hyphae. In this study, we developed an IVET system based on a pyrimidine requirement for growth of a mutant bacterial strain in vivo to genetically investigate bacterial colonization of *P. parasitica*. It has been shown previously that the thymidylate synthase gene is a good alternative selection marker that can replace antibiotic resistance genes in *Rhizobium meliloti* and *Lactococcus lactis* (32, 39). Our data suggested that the pyrimidine requirement for bacterial growth was a good selection marker during colonization of fungal hyphae by *P. putida*. The fungal hyphae did not produce sufficient pyrimidine to support growth of the *pyrB* mutant strain. The pyrimidine requirement also provided a good selection criterion for studying bacterial gene expression in the citrus rhizosphere, especially on actively growing citrus root tips (21).

We selected five clones that were specifically induced in the presence of the fungus *P. parasitica*. Truncated ORFs were found to be fused to the promoterless *pyrBC′* genes at the 3′ end of each these clones. These truncated ORFs, or upstream genes, should be induced during colonization of *P. parasitica* by *P. putida* 06909. The gene fused to the promoterless *pyrBC′* genes in pRIVS1 was a *dgk* homolog encoding DAGK. The function of DAGK in *E. coli* is to recycle diacylglycerol generated during membrane-derived oligosaccharide biosynthesis (16). The same enzyme is involved in recycling of bacterial diacylglycerol generated during cyclic β-1,2-glucan biosynthesis in *Rhizobium meliloti* (29). It has been suggested that the glucans of members of the *Rhizobiaceae* may function during plant infection and adaptation to osmotic stress (7, 28, 37). We do not know if the DAGK of *P. putida* 06909 is linked to specific cell surface carbohydrate biosynthesis to recycle diacylglycerol. Since the DNA sequence of the *dgk* gene that we obtained was only 190 bp long, the complete ORF of the *dgk* should be cloned to conduct mutational studies to test the direct involvement of the gene in colonization of *P. parasitica*.

A LuxR family response regulator was found upstream of *dgk*. Since subclone pRIVS13, which carried *luxR-dgk*, restored the inducible promoter activity of pRIVS1, the inducible promoter expressed during colonization of fungal hyphae should be either in front of this LuxR family response regulator or in front of *dgk*. Since 1% glycerol in the culture medium slightly induced expression of pRIVS1, the chemical nature of the inducer of pRIVS1 may be similar to glycerol. It will be interesting to investigate whether the LuxR family response regulator regulates expression of the DAGK gene. No sensor kinase-like DNA sequence was found in the vicinity of the LuxR family response regulator. However, this finding does not rule out the possibility that the LuxR family response regulator is a response regulator of a two-component regulatory system. Further definition of the inducible promoter area is required to determine if the response regulator-like gene is induced or if only *dgk* is induced during colonization of the fungus by *P. putida* 06909.

Another clone in pRIVS2 had an ABC transporter gene at the fusion with *pyrBC′*, and its promoter was induced during colonization of *P. parasitica*. The truncated ABC transporter gene encodes a 282-amino-acid protein that includes most of the conserved domains in different ABC transporters. The sizes of ABC transporters are variable but usually are more than 300 amino acids. The C-terminal portions of the ABC transporters are not highly conserved compared with the N-terminal conserved regions (15). The ABC transporters import or export many different molecules by using ATP, and each transporter is highly specific for its substrate (31). Therefore, the ABC transporter induced during colonization of *P. parasitica* may be involved in uptake of a specific compound from *P. parasitica* or in secretion of a compound onto *P. parasitica* to inhibit its growth. Since the ABC transporter was similar to transporters for sulfate, ferric ions, polyamine, maltose, etc., we added several compounds to V8C medium individually to examine ABC transporter induction specificity. None of the substrates induced ABC transporter gene expression. High levels of similarity to the ABC transporter were observed for sulfate transporters from various bacteria that are induced during sulfate depletion (11, 12). However, the ABC transporter was not induced under sulfate-deficient conditions. We did not observe any induction of this ABC transporter on various culture media, including minimal medium and rich medium, except in the presence of *P. parasitica*. Each locus induced during colonization of *P. parasitica* may cooperate to inhibit fungal growth in natural environments. Further work is necessary to define the role of the ABC transporter either in specific chemical uptake from *P. parasitica* or in specific chemical export to *P. parasitica*.

The inducible promoter area of pRIVS2 has a potential transcriptional terminator and three direct repeats (7 to 9 bp) (data not shown) between the LysR family response regulator and ABC transporter genes. It is likely that the transcriptional

terminator blocks transcription of the LysR type of response regulator found in front of the ABC transporter. It is not clear if the LysR family response regulator is induced during colonization of *P. parasitica* by *P. putida* 06909. Since the promoter area between the LysR family response regulator and the ABC transporter alone restores original pRIVS2 induction activity, the LysR family response regulator may be the last ORF of a separately transcribed operon.

One of the partially sequenced clones encodes a putative outer membrane porin protein at its 3′ end, and it is likely that the porin gene is induced during colonization of *P. parasitica*. It may be involved in uptake of specific chemicals from the fungus; together with the ABC transporter, this suggests that *P. putida* 06909 may acquire multiple compounds from *P. parasitica*.

Adding various chemicals as possible inducers did not induce pRIVS1 or pRIVS2. Only 1% glycerol in V8C agar resulted in a slight induction of pRIVS1 carrying *dgk*. Therefore, the nature of the inducing substances during colonization of *P. parasitica* by *P. putida* 06909 is not known. Further work to define the roles of other genes selected by using the IVET strategy should help us understand the nature of colonization of *P. parasitica* by *P. putida* 06909. It would be interesting to determine whether the genes are induced by the chemicals produced by *P. parasitica* or by the presence of *P. parasitica* hyphae. In the future we will confirm the potential involvement of selected clones during fungal colonization by *P. putida* 06909 and biological control on citrus by analyzing mutations at specific loci of selected clones in the wild-type bacterial chromosome.

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