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Analysis of *Pseudomonas putida* KT2440 Gene Expression in the Maize Rhizosphere: In Vitro Expression Technology Capture and Identification of Root-Activated Promoters

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Pseudomonas putida **KT2440, a paradigm organism in biodegradation and a good competitive colonizer of the maize rhizosphere, was the subject of studies undertaken to establish the genetic determinants important for its rhizospheric lifestyle. By using in vivo expression technology (IVET) to positively select single cell survival, we identified 28 *rap* genes (*r*oot-*a*ctivated *p*romoters) preferentially expressed in the maize rhizosphere. The IVET system had two components: a mutant affected in aspartate-β-semialdehyde dehydrogenase (*asd*), which was unable to survive in the rhizosphere, and plasmid pOR1, which carries a promoterless *asd* gene. pOR1-borne transcriptional fusions of the *rap* promoters to the essential gene *asd*, which were integrated into the chromosome at the original position of the corresponding *rap* gene, were active and allowed growth of the *asd* strain in the rhizosphere. The fact that five of the *rap* genes identified in the course of this work had been formerly characterized as being related to root colonization reinforced the IVET approach. Up to nine *rap* genes encoded proteins either of unknown function or that had been assigned an unspecific role based on conservation of the protein family domains. Rhizosphere-induced fusions included genes with probable functions in the cell envelope, chemotaxis and motility, transport, secretion, DNA metabolism and defense mechanism, regulation, energy metabolism, stress, detoxification, and protein synthesis.**

The rhizosphere is a densely populated area in which plant roots interact with soilborne microorganisms, including bacteria, fungi, and invertebrates, feeding on an abundant source of organic material (43). Many colonization traits and genes have been identified by random mutagenesis of good competitive root-colonizing bacteria (mainly *Pseudomonas fluorescens* and *Pseudomonas chlororaphis*) and through screening for gain or loss of competitive root tip colonization ability (see reference 28 for a recent review). Often these genes have been found to be unimportant for growth in the laboratory, and their role in bacterial fitness has required competition studies involving wild-type and mutant strains.

Information on gene expression in the rhizosphere is, however, limited and partial. Previous results obtained by our group have shown that utilization of the imino acid proline by *Pseudomonas putida* KT2440, which involves uptake and catabolism, was induced by maize root exudates (50). In addition, an aminotransferase involved in the catabolism of lysine was also identified in a screen to select for maize root exudate-induced genes by using a promoter probe transposon (15).

The most extensive study so far based on positive selection of bacterial traits as a consequence of their contribution to root colonization is that of Rainey (38). In that work, the in vitro expression technology (IVET) approach was used to select *P. fluorescens* genes activated during sugar cane root colonization. The study was partial, since only 10% of the genome was analyzed.

Our work represents an attempt to expand our knowledge of bacterial gene expression in a complex environment, such as the rhizosphere, using another model system. We faced the identification of *P. putida* genes (*rap*, for *r*oot-*a*ctivated *p*romoters) induced during maize root colonization by this bacterium by taking advantage of the fact that the complete genome sequence is available for *P. putida* KT2440.

P. putida KT2440 is being used by our research group as a model organism in studies of plant-microbe interaction to establish the molecular bases of the initial seed adhesion and subsequent root colonization. KT2440 is a TOL plasmid-cured derivative of the natural isolate *P. putida* mt-2, which was isolated in 1960 from a planted field in Japan and whose historical itinerary has been reviewed by Nakazawa (35). Not surprisingly, *P. putida* KT2440, besides its potential for removing xenobiotics, exhibits high fitness in the colonization of the rhizosphere of a large number of plants (32).

Our approach was to use a positive selection system based on IVET. This strategy has been previously used to identify *Pseudomonas* in vivo-induced genes under different conditions (17), including a recent study of *P. fluorescens* genes induced in soil (47). The system was originally developed to select *Salmonella enterica* serovar Typhimurium virulence genes that were induced during host infection (29). For IVET implementation in *P. putida*, we used a derivative of the pIVPRO plasmid containing a promoterless gene expression reporter cassette (*asd*) (20), which is useful in generating transcriptional fusions, and as a host to select appropriate fusions, we used an *asd* null mutant unable to survive in the rhizosphere (42), which allowed selection on the basis of their in vivo induction. The *asd*

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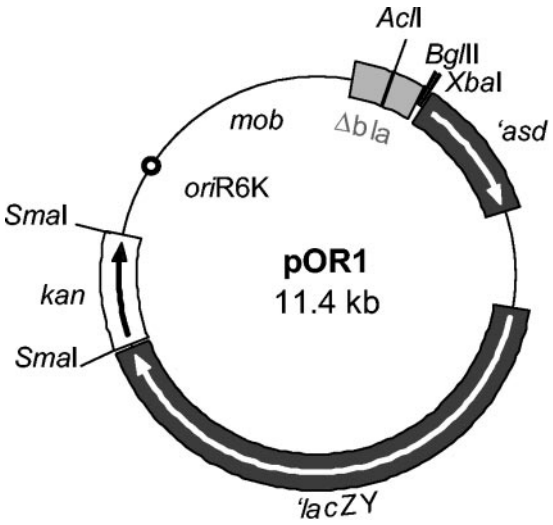

FIG. 1. Map of pOR1 plasmid (IVET vector) used to identify *P. putida* root-activated promoters. Modifications to the original plasmid pIVPRO (20) are described in the text. This plasmid has the replication origin *ori*R6K and thus requires protein II for stable maintenance. The plasmid is therefore a suicide in *Pseudomonas* and is mobilizable as *tra* functions are supplied in *trans*.

gene product is involved in the biosynthesis of aspartate-β-semialdehyde dehydrogenase, a key intermediate in the biosynthesis of diaminopimelic acid (DAP), which is required for integrity of the cell wall, and of amino acids such as lysine, methionine, and threonine (21).

MATERIALS AND METHODS

Bacterial strains, culture conditions, and solutions. *P. putida* KT2440, a derivative of the *P. putida* soil isolate mt-2, has been described previously (35). In the capture of rhizosphere-activated promoters, an *asd*::*xylE* null mutant of KT2440 was used, as it is unable to survive in the rhizosphere because of its dependence on DAP, Lys, Thr, and Met (42). Plasmids with an *ori*R6K replication origin were maintained in *Escherichia coli* DH5αλ*pir* (25), and *E. coli* S17-1λ*pir* (48) was used for conjugation. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) medium (44). *P. putida* strains were grown at 30°C in either LB or minimal medium, which was basal M9 medium (44) supplemented with Fe-citrate (6 μg/liter), $MgSO_4$ (1 mM), and trace metals as described before (1) and with glucose (25 mM) or sodium citrate (10 mM) as the carbon source, unless otherwise specified. When appropriate, the following antibiotics were added to the media at the given concentrations (in μg/ml): ampicillin, 100; chloramphenicol, 30; kanamycin, 25 and 50 for *E. coli* and *Pseudomonas* strains, respectively; and rifampin, 10. Diaminopimelic acid was added at a final concentration of 0.5 mg/ml. The L amino acids Lys, Met, and Thr were supplied at 40 μg/ml.

DNA techniques. Plasmid DNA was isolated with a QIAGEN miniprep kit. Preparation of chromosomal DNA, digestion with restriction enzymes, dephosphorylation, ligation, and electrophoresis were carried out using standard methods (4, 44). DNA fragments were recovered from agarose gels with a QIAGEN gel extraction kit.

Construction of pOR1. pIVPRO promoter probe vector (20) was optimized for in vivo expression technology in *P. putida* as follows: (i) a kanamycin cassette contained in the SmaI fragment of plasmid p34S-Km3 (12) was cloned at the unique SmaI site of pIVPRO and (ii) in the resulting plasmid, a partial deletion of the *bla* gene was performed by removing the 0.4-kb AclI fragment internal to this gene. This deletion was carried out to avoid recombination between plasmid and chromosome through the *ampC* homologous gene present in the chromosome of *P. putida* KT2440, which encodes PP2876. As a result, plasmid pOR1 (11.4 kb) was obtained. Figure 1 shows that this plasmid carries a promoterless *asd* gene followed by the promoterless *lacZY* as reporter genes. Plasmid pOR1 is the IVET vector used to construct a promoter library of KT2440.

Construction of libraries. *P. putida* KT2440 genomic DNA was partially digested with Sau3AI, size selected by agarose gel electrophoresis (1 to 4 kb), and cloned into the BglII site 5′ of the promoterless *asd-lacZY* encoded by pOR1 (Fig. 1). The pool of fusions was electrotransformed in *E. coli* S17-1λ*pir*, and about 12,000 clones were obtained. Eighty percent of the GenBank clones exhibited inserts in the plasmids as analyzed by double digestion with XbaI and AclI. The pOR1-KT2440 GenBank was then transferred en masse by bacterial conjugation to *P. putida* Δ*asd* using as selective medium for the transconjugants LB supplied with DAP, kanamycin, and chloramphenicol, which counterselected *E. coli*. The frequency of exconjugants containing integrated fusions was 10^{-4} to 10^{-5} per recipient cell.

Identification of the sequences captured by the *rap* fusions. DNA sequences from the *rap* isolates were determined by arbitrary PCR (8). A first round of amplification was done by using the chromosomal DNA of the mutants as a template, with an arbitrary primer (ARB1; 5′-GGCACGCGTCGACTAGTAC NNNNNNNNNGATAT-3′) and an internal primer of the *′asd* gene borne by pOR1 (Asdext; 5′-TCGCTGTATGAGTACGGAACCC-3′). The first round was as follows: 3 min at 95°C; six cycles of 1 min at 95°C, 1 min at 30°C, and 1 min at 72°C; 30 cycles of 30 s at 95°C, 30 s at 50°C, and 1 min at 72°C; and an extension period of 7 min at 72°C. A second round of amplification was done using as the template 5 μl of the first-round reaction as follows: 3 min at 95°C; 30 cycles of 30 s at 95°C, 30 s at 60°C, and 1 min at 72°C; and 7 min at 72°C. Primers used for the second round were those corresponding to the conserved region of ARB1 (ARB2; 5′-GGCACGCGTCGACTAGTAC-3′) and a second internal primer of the *′asd* gene closer to the 5′ end (Asdint; 5′-CGATCAGA CCTACACGCTTCATC-3′). Reaction mixtures were electrophoresed, and the most intense bands were isolated and sequenced. Sequencing was done on an ABI PRISM 310 automated sequencer using oligonucleotide Asdint as a primer. The ~50-bp distance between this primer and the BglII site used for cloning provided an internal control to ensure that the sequence obtained corresponded to the junction between the promoterless *asd* gene and the flanking chromosome in the cointegrate.

Sequences were analyzed and compared with the GenBank database by using BLAST programs (3). Sequence data for the *P. putida*, *Pseudomonas syringae* pv. tomato strain DC3000, and *Pseudomonas aeruginosa* genomes were obtained from The Institute for Genomic Research (www.tigr.org) and the *Pseudomonas* Genome Project (www.pseudomonas.com).

Surface sterilization, germination of seeds, and root colonization assays. Corn seeds were surface sterilized by rinsing with sterile deionized water and washing twice for 10 min with 70% (vol/vol) ethanol and once with 20% (vol/vol) bleach, followed by thorough rinsing with sterile deionized water. Surface-sterilized seeds were germinated on a petri dish in the presence of penicillin G (500 μg/ml) (37) at 30°C for 2 days for root colonization assays or for 3 days if seedlings were to be transferred to a tube and grown hydroponically in the specified solution. For root colonization assays, overnight cultures grown in LB were diluted in M9 salts to a turbidity at 660 nm of about 1, and seeds were inoculated with an appropriate dilution of bacterial suspensions (5 μl of the suspension per ml of M9). After incubation for 1 h without shaking at 30°C, the seeds were washed and planted in pots containing vermiculite or used to determine the number of bacteria attached to the seed. To analyze gene expression in the maize rhizosphere, Sterilin tubes (50 ml) containing sterile sea sand (40 g) were used, and plant nutrient solution (PNS) was added (10% [vol/wt]). PNS was $Ca(NO_3)_2$ (5 mM), KNO_3 (5 mM), $MgSO_4$ (5 mM), KH_2PO_4 (1 mM) (pH 5) supplemented with Fe-EDTA (100 μM) and micronutrients of MS medium as described previously (34). Controls without plant seeds were run similarly as required. Plants were maintained in a controlled chamber at 24°C and 55 to 65% humidity with a daily light period of 16 h for 1 to 2 weeks. To recover bacteria from the rhizosphere, plants were removed and roots were cut, weighed, and placed in sterile 50-ml screw-cap tubes containing 20 ml M9 and 4 g glass beads (diameter, 3 mm). The tubes were vortexed for 2 min, and the number of CFU per gram of root was determined for each plant by plating serial dilutions on selective media consisting of either LB medium supplied with DAP and antibiotics or M9 minimal medium supplied with citrate, DAP, Lys, Met, Thr, and antibiotics. The same process was used with inoculated seeds to determine the number of attached bacteria, i.e., the initial inoculum on the seeds, except that 2 ml of M9 and 10 to 12 glass beads were used. To recover bacterial cells for β-galactosidase assays, glass beads were not used.

β-Galactosidase activity assay. Specific β-galactosidase activity from bacterial suspensions growing on either liquid cultures or seedling hydroponic cultures was determined spectrophotometrically (31). Given that samples showed color and contained low numbers of cells, activities of the *rap* fusion strains as recovered from the maize rhizosphere were determined with 4-methylumbelliferyl-β-D-galactoside as the substrate and detection of the fluorescent product methyl-

umbellipherone with a spectrofluorometer (excitation wavelength, 365 nm; emission wavelength, 450 nm).

RESULTS

Development of IVET strategy for *P. putida*. For the selection of root-activated promoters with IVET, we took advantage of the *asd* null mutant developed previously in our laboratory. This mutant did not survive in the maize rhizosphere because of its inability to synthesize three amino acids (Lys, Met, and Thr) and DAP; however, when the *asd* gene was supplied in *trans* in a plasmid, colonization ability was restored to the wild-type level (42). Plasmid pOR1 was constructed as described in Materials and Methods to generate transcriptional fusions of chromosomal DNA to the promoterless cassette *asd-lacZ*. Relevant characteristics of this plasmid are shown in Fig. 1. There is a unique BglII site for cloning upstream from the 5′ end of *′asd*. The *mob* functions confer mobilization properties to the plasmid so that it can be transferred by bacterial conjugation to *P. putida* Δ*asd*. The pOR1 plasmid is unstable in *Pseudomonas* (due to the replication origin *ori*R6K), so that selection for kanamycin resistance forces integration of its chimeric derivatives in the chromosome by homologous recombination through the DNA inserts cloned in BglII at their corresponding loci. Thus, the so-generated IVET fusions strains, as merodiploids, mostly present intact copies of the targeted loci, while their promoters, in their native chromosomal context, are responsible for the expression of the essential *′asd* gene.

Screening of the *P. putida* cointegrate library in planta to select *rap* genes. We developed the IVET strategy for *P. putida*, as reported above, to identify specific bacterial functions expressed during root colonization. The screen was based on that used for *P. fluorescens* (38). Cointegrate strains containing fusions were screened for survival in the rhizosphere in pools of about 500 clones. Initially, four pools were screened by inoculating 12 seeds with 10^6 bacteria. Because bacterial adhesion was 0.5% of the inoculum on average, the number of cells attached to the seed was $\sim 5 \times 10^3$. Bacterial pools were recovered from the rhizosphere at different times, and survival was analyzed. Maximal CFU/g of root were obtained after 1 week (10^7 to 10^8), and therefore subsequent screening runs were carried out for 1 to 2 weeks. *P. putida* Δ*asd* was kept as a negative control for survival in the rhizosphere (Fig. 2A). Up to 6,000 clones were analyzed. Bacteria from the rhizosphere were removed as described in Materials and Methods. Up to 10,000 clones containing fusions of putative rhizosphere-activated promoters were selected on M9-citrate agar supplied with DAP, Lys, Met, Thr, and kanamycin. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was also added to monitor inactivity of the *rap* promoters in vitro. Fusion strains carrying constitutive promoters, detected for their ability to grow in M9-citrate agar without the addition of DAP, Lys, Met, and Thr, were discarded. Seventy-eight *rap* fusions were selected for their auxotrophy or diminished growth on citrate-supplied M9 minimal medium. Because active promoters upstream to *′asd* were required for the survival of these fusions in the rhizosphere, lack of growth (or residual growth) of the fusions in vitro suggested that expression from the promoters was preferential in the rhizosphere.

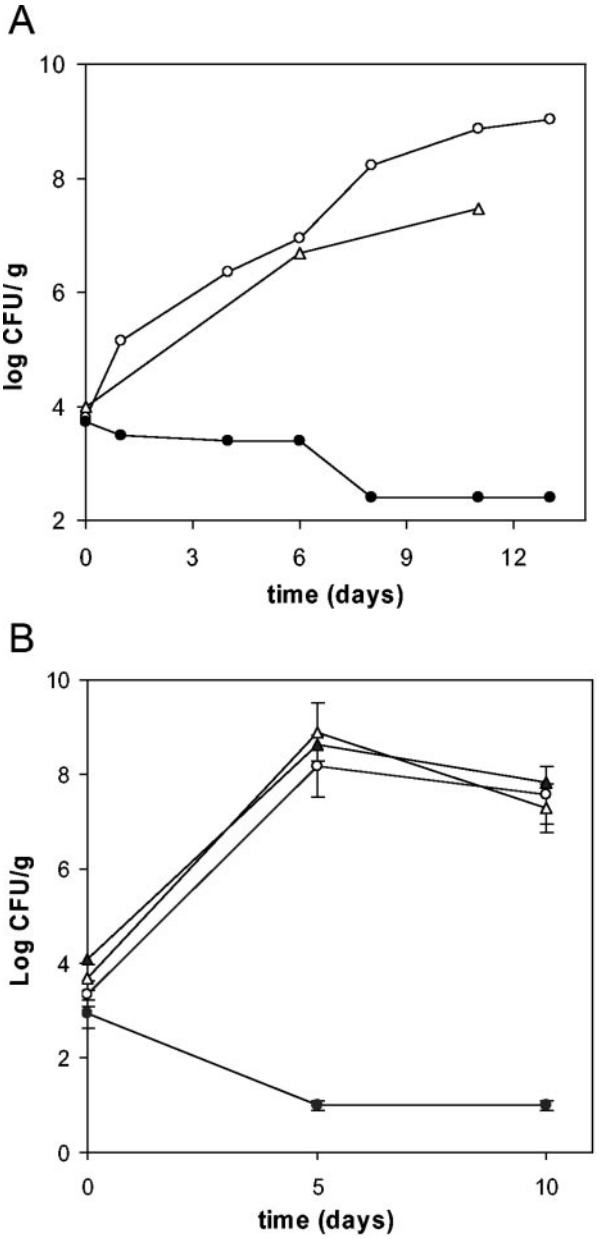

FIG. 2. Effect of *rap* promoter activity on colonization ability. (A) Survival of chromosomal IVET cointegrates pool in the rhizosphere. ○, KT2440R; ●, Δ*asd*; △, a pool of approximately 500 clones of *P. putida* IVET cointegrates. (B) Survival of *rap* fusion strains. ○, KT2440R; ●, Δ*asd*; △, $rap_{1\text{-}2}$ fusion; and ▲, $rap_{1\text{-}4}$ fusion. Maize seeds were inoculated and plants grown as described in Materials and Methods. Bacterial cells were recovered on plates at the indicated times. At time zero, CFU was per g of seed; afterwards, CFU is plotted per g of rhizosphere including root. Means and standard deviations for three independent experiments are shown.

Sequence analysis of *rap* fusions. To identify the genes whose promoters were responsible for the survival of the *asd* mutant in the rhizosphere, arbitrary PCR was performed with each fusion as described in Materials and Methods. This technique allowed us to amplify fragments containing 300 to 750 bp of DNA flanking the promoterless *asd* gene, which was responsible for the expression of this gene in the fusion strains. Twen-

TABLE 1. Function of the *P. putida rap* genes identified during maize root colonization

Function and/or role	Gene and/or *rap* fusion	Locus
Cell envelope (GDP-mannose 6-dehydrogenase)	*algD* ($rap_{2\text{-}45}$)	PP1288
Chemotaxis and motility (flagellar assembly protein)	*fliO* ($rap_{1\text{-}8}$)	PP4356
Transport		
ABC-type sulfate transport system, periplasmic component	$rap_{2\text{-}16}$	PP4305
Gamma-aminobutyrate (GABA) transporter permease	$rap_{2\text{-}21}$	PP2543
Sodium/proline symporter	*putP* ($rap_{2\text{-}40}$)	PP4946
Secretion		
Protein chaperone	*secB* ($rap_{1\text{-}2}$)	PP5053
Preprotein translocase subunit	*yidO* ($rap_{2\text{-}26}$)	PP0006
DNA metabolism and defense mechanism		
Type 1 restriction-modification system, M subunit	*hsdM* ($rap_{1\text{-}19}$)	PP4741
Single-stranded DNA-specific exonuclease	*recJ* ($rap_{2\text{-}28}$)	PP1477
DNA topoisomerase IV, A subunit	*parC* ($rap_{2\text{-}37}$)	PP4912
Energy metabolism		
Pyruvate dehydrogenase, decarboxylase component	*aceE* ($rap_{2\text{-}2}$)	PP0339
Isocitrate lyase putative (glyoxylate bypass)	*aceA* ($rap_{2\text{-}14}$)	PP4116
6-Phosphogluconate dehydrogenase	*gnd* ($rap_{2\text{-}15}$)	PP4043
2,3-Biphosphoglycerate-independent phosphoglycerate mutase	*pgm* ($rap_{2\text{-}77}$)	PP5056
Regulatory functions		
SAM-dependent methyltransferase transcriptional regulator	$rap_{1\text{-}4}$	PP4966
Transcriptional regulator, AsnC family	$rap_{1\text{-}12}$	PP4424
Transcriptional regulator, AraC family	$rap_{2\text{-}44}$	PP2070
Sensor histidine kinase	*colS* ($rap_{2\text{-}63}$)	PP0902
Stress (general stress protein Ctc [ribosomal 5S rRNA E-loop binding protein Ctc/L25/TL5])	$rap_{1\text{-}9}$	PP0721
Detoxification (lactoylglutathione lyase [methylglyoxal metabolism])	*gloA* ($rap_{2\text{-}53}$)	PP3766
Protein synthesis (glutamyl-tRNA synthetase tRNA-Ala, tRNA-Glu)	*gltX* ($rap_{2\text{-}1}$)	PP1977
Unknown		
Seed adhesion, antisense	*mus-5* ($rap_{2\text{-}74}$)	PP4615
Hypothetical protein	$rap_{2\text{-}7}$	PP5390
Hypothetical putative lipoprotein of unknown function	$rap_{2\text{-}18}$	Between PP3854 and PP3855[a]
Conserved hypothetical protein alpha/beta hydrolase fold superfamily, antisense	$rap_{2\text{-}19}$	PP4634
Amino oxidase, putative	$rap_{2\text{-}23}$	PP0383
Conserved hypothetical protein (predicted SAM-dependent methyltransferases)	$rap_{2\text{-}39}$	PP4306
Hypothetical protein	$rap_{2\text{-}73}$	PP2298

[a] The junction was at a position between the two indicated proteins. A putative 348-bp open reading frame was detected (4.378.820–4.379.167). See the text for details.

ty-eight independent fusions were identified after sequencing the DNA adjacent to the *'asd* gene with the primer Asdint. To confirm the legitimacy of these fusions, they were recovered from the genome by conjugative cloning as described by Rainey and coworkers (39) and sequenced with the primer pOR1fw (CATGAGCGGATACATATTTGAATG), which allowed us to determine the flanking DNA at the 3′ end of the fusion.

The *rap* genes are listed and organized by function in Table 1. Genes whose translation products play a role in energy generation, DNA metabolism, transport and secretion, stress and detoxification, motility, and synthesis of components of the cell envelope were identified. Nine genes (32%) of unknown function were identified, including genes predicted to have a regulatory function.

Activity of *rap* promoters versus root colonization. Because the DAP auxotrophic mutant *P. putida* Δ*asd* was unable to colonize the rhizosphere (Fig. 2), *rap* genes must be induced in the rhizosphere to promote *asd* transcription and thus growth of the strains bearing the corresponding fusions on their chromosome. The survival of a representative pool of 500 clones of the IVET cointegrates library is shown in Fig. 2A. All *rap* fusion strains were analyzed individually for their colonization capacity. Figure 2B shows an example of the colonization ability of two *rap* fusion strains. After 10 days, the number of bacteria per g of rhizosphere was not notably different from *P. putida* KT2440R, a rifampin-resistant derivative of KT2440 used as a positive control strain (16). Slightly different behavior was observed in four *rap* fusions, $rap_{2\text{-}44}$, $rap_{2\text{-}45}$, $rap_{2\text{-}73}$, and $rap_{2\text{-}74}$, which exhibited 1 to 1.5 log lower survival on average (not shown). In the absence of induction, colonization by the Δ*asd* mutant is not possible, so induction must take place from the *rap* fusion for colonization of the rhizosphere.

Expression from the *rap* promoters in conditions of hydroponically growing seedlings. Induction by plant exudates of the *rap* promoters was initially evaluated by incubating the *rap* fusion strains in M9 in the presence of 3-day-old seedlings for 72 h. Bacteria were also cultivated in M9-citrate (2.5 mM) supplied with DAP, Lys, Met, and Thr up to an optical density at 600 nm of about 0.3, which was on average the growth supported by the maize seedlings under these conditions. β-Galactosidase activity was determined by spectrophotometric assay (Fig. 3). All of the fusions exhibited induction in the presence of the seedling in comparison to the same cells incubated in citrate-supplied minimal M9 medium. The highest induction rate was normally observed in strains which exhib-

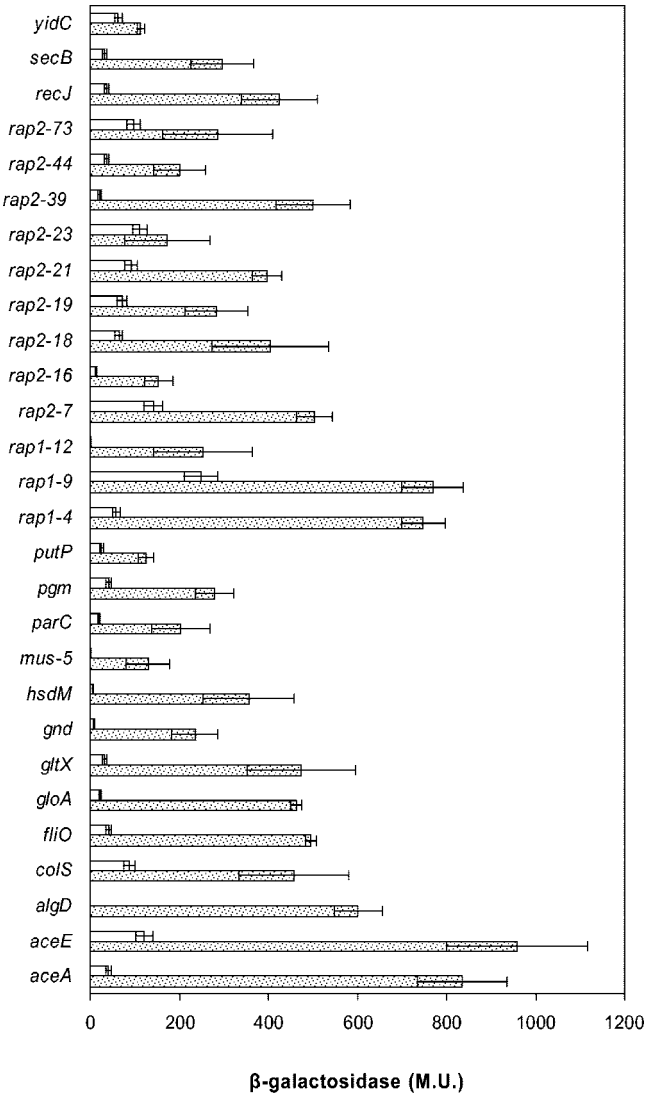

FIG. 3. β-Galactosidase activity (Miller units) of *rap* fusions during seedling colonization in hydroponic culture. Dotted bars show values from cells in suspension in the presence of maize seedling. Open bars are values in 2.5 mM citrate-supplied M9 minimal medium with all requirements to support the growth of the *rap* fusions, as described in Materials and Methods. Inoculum size was 2.5×10^6 to 5×10^6 CFU/ml. Means and standard deviations from three independent experiments are shown.

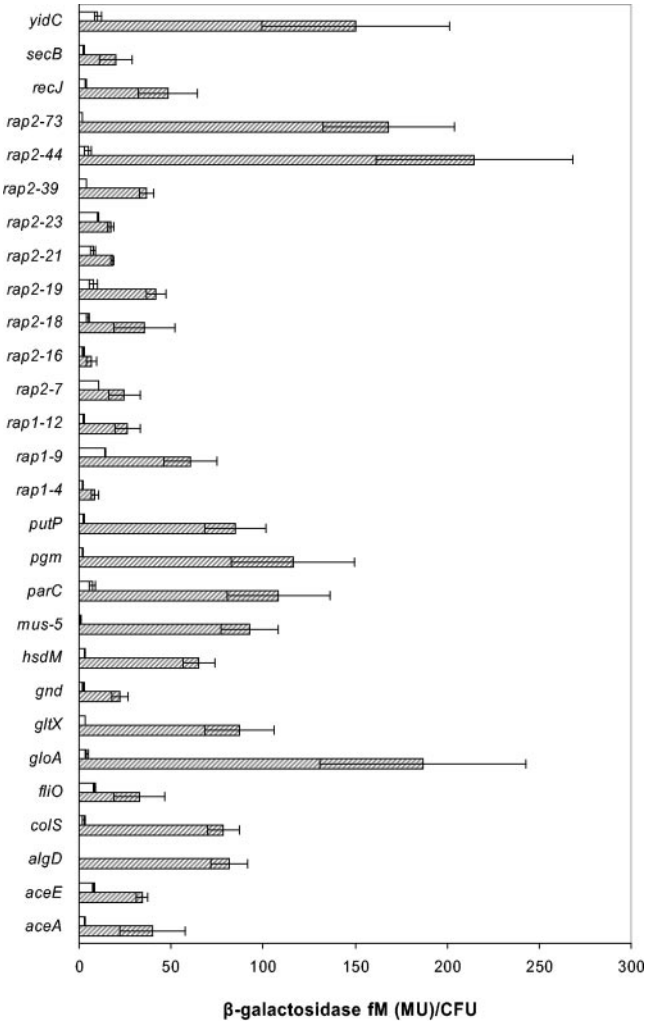

FIG. 4. β-Galactosidase activity of *rap* fusions in the rhizosphere. Hatched bars show values from cells recovered from the maize rhizosphere supplied with PNS. Open bars are values from control cells in sand supplied with PNS, citrate, and all requirements to support the growth of the *rap* fusions as described in Materials and Methods. Inoculum size for controls was 1×10^4 to 2.5×10^4, similar to the number of cells attached to the seeds. Means and standard deviations from three independent experiments are shown. Units are given as the concentration of 4-methyl-umbelliferone (femtomolar) per CFU.

ited lower (or null) basal activity under laboratory conditions in the absence of plant exudates. Two fusions were remarkable: those identified as *algD* (up to 100-fold) and *hsdM* (higher than 50-fold). The *algD* upstream regulatory region was identified as active in the presence of the corn seedling. *algD* is the first gene in the *algD-8-44-KEGXLIJFA* operon, a cluster of 12 genes responsible for the biosynthesis of the exopolysaccharide alginate in *Pseudomonas* strains and other bacteria that synthesize alginate. *hsdM* encodes a methylase of the type I restriction-modification system. Another interesting fusion was $rap_{2\text{-}74}$ (*ddcA*), which corresponded to *mus-5* (in its antisense strand), reported to have a role in adhesion (16). Two metabolic genes, *aceA* and *aceE*, showed high expression levels. The $rap_{1\text{-}9}$ and $rap_{1\text{-}4}$ fusions were also highly expressed, although the former had shown considerable basal expression.

Analysis of *rap* gene expression in the rhizosphere. To confirm that *rap* promoters were active preferentially during root colonization, we evaluated their activity in cells that had been colonizing the maize rhizosphere for 1 week. As a control, cells were incubated in the absence of plants on sea sand supplied with all of the requirements of *rap* strains and citrate (10 mM) as the sole carbon source. Data for gene expression measured as β-galactosidase activity are shown in Fig. 4. For all of the *rap* fusions assayed, gene expression measured as β-galactosidase activity per cell was higher in cells recovered from the rhizosphere than in control cells. One of the genes with the highest expression was *yidC*. This gene encodes a 60-kDa inner membrane protein responsible for the insertion of membrane pro-

teins into the lipid bilayer. The basal level of expression observed for this gene was consistent with YidC essentiality in *E. coli* (46). Other fusions that were expressed highly during colonization were $rap_{2\text{-}73}$ and $rap_{2\text{-}44}$. The former encodes a hypothetical protein located upstream to the trigger factor, which—like the ribosomal chaperone trigger factor—is also involved in protein fate. The latter is an AraC family transcriptional regulator of unknown function. $Rap_{2\text{-}73}$ homologues are present in *P. syringae* and *P. fluorescens*. Intriguingly, neither of these fusions was highly expressed during hydroponic growth. Several fusions exhibited high rates of induction during colonization as a consequence of their low basal levels of gene expression. Among them, the most silent *rap* promoter was the *algD* promoter. Also silent were the control cells of $rap_{2\text{-}74}$ and *hsdM* fusions, which also exhibited high induction during hydroponic growth. Three other fusions were also highly induced during colonization. The $rap_{2\text{-}63}$ fusion, which involves the promoter of the two-component system *colRS*, was previously reported to play a role in colonization (11), the $rap_{2\text{-}40}$ fusion substantiated the induction of the proline transport system PutP in the rhizosphere (50), and the $rap_{2\text{-}77}$ fusion revealed that the phosphoglycerate mutase encoded by *pgm* was induced in vivo.

DISCUSSION

In this report we identify *P. putida* genes that are induced during maize root colonization as a way to explore the biology of this saprophyte in the rhizosphere. In view of its isolation origin, a crop field (35), the rhizosphere must constitute a familiar niche for this bacterium.

The IVET method utilizes a variant of the promoter trap vector pIVPRO, which we optimized and named pOR1. The capture of an active promoter in pOR1 commanded transcription of the *asd* gene and therefore conferred survival in the rhizosphere to the *asd* mutant carrying the root-activated fusions. These derivatives of pOR1 were maintained stably in *Pseudomonas* only as a consequence of their integration in the chromosome through homologous recombination by the promoter region. Although the IVET fusion strains, as merodiploids, mostly present intact copies of the targeted loci, exceptions causing the loss of the host gene(s) cannot be discarded, in particular for those fusions involving operon internal promoters. In the hypothetical case of gene inactivation, there would be a possibility of change in promoter activity of autoregulated genes, which could lead to their initial isolation. Even so, this fact would not affect the conclusions reached for *rap* genes in this study, since controls in the absence of plants are included. Nevertheless, it is impossible to select inactivation involving loci essential for survival in the rhizosphere with the IVET strategy.

The *bla* gene was removed from the ancestor pIVPRO because this plasmid itself, before capturing promoters, used to form cointegrates by recombination with a 98% identical *ampC* gene in the chromosome of KT2440.

With our selection strategy, 28 transcriptionally active units of *P. putida* that were induced in the rhizosphere of maize seedlings were isolated. About 17% of the genome was represented in the IVET library. A similar system based on the *dap* gene was used with *P. fluorescens* to identify genes induced during sugar cane root colonization (38). In that species, about 10% of the genome was analyzed. Interestingly, no gene overlap was observed between *P. fluorescens* and *P. putida*. Further analyses will be needed to discern whether the reason for this was the partial nature of the genome analysis in both cases or species-dependent gene expression specificity. It cannot be ruled out that different genes were identified as a consequence of the different host strains used for the IVET fusions, i.e., *asd* in this work versus *dap* in *P. fluorescens*.

Our approach was validated by the fact that our positive selection scheme based on pOR1 isolated five fusions that identified bacterial functions previously highlighted in *Pseudomonas*-plant interactions. These fusions were $rap_{2\text{-}40}$, which identified *putP*, encoding a proline permease (50), $rap_{2\text{-}74}$, corresponding to *mus-5*, which is involved in seed adhesion (16), $rap_{2\text{-}63}$, identifying *colS*, which encodes the sensor histidine kinase element of the double component system ColR/ColS (11), $rap_{1\text{-}2}$, which identified *secB*, encoding a protein chaperone (26), and $rap_{1\text{-}8}$, corresponding to *fliO*, which is involved in the synthesis of the flagella export apparatus (14). The putative roles of these genes in colonization are described in detail below as appropriate in the context of this discussion.

In the system reported here, survival and β-galactosidase activity were both affected by the promoter activity and so contributed together to the level of gene expression. Thus, lower and higher values of β-galactosidase activity, as plotted in Fig. 3 and 4, did not necessarily correlate with lower and higher cell survival.

The expression level of *rap* genes was measured under two different bacterial lifestyles: planktonic during plant hydroponic growth (Fig. 3) and as a result of rhizosphere colonization (Fig. 4). All *rap* genes were induced under both conditions in comparison to the control situation. However, differences in gene expression were observed in the two data sets, and further work will be required to unveil the basis for these differences.

The $rap_{2\text{-}45}$ fusion isolated the *algD* promoter, a possible indication of alginate biosynthesis in the rhizosphere, thus establishing an important parallelism with the biofilm lifestyle (6). It was recently reported that *algA*, the last gene in the *algD-A* operon, is controlled by water stress in *P. putida* (49). Nelson and coworkers (36) suggested that the absence of the regulatory gene *mucC* from the *algT-mucC* operon might explain the *P. putida* nonmucoid morphotype under standard culturing conditions. However, the same divergence in the *algT-mucC* operon has been described for *P. syringae*, a bacterium that does synthesize alginate (23). Hence, alginate-defective mutants of *P. syringae* are compromised in their ability to colonize plant tissue (51). As in *P. syringae*, alginate biosynthesis regulation in *P. putida* might respond to environmental signals. Further research is required to document the synthesis of alginate by KT2440 and a role for alginate in colonization.

The IVET strategy identified genes involved in nutrient acquisition. This is the role of *putP* (which encodes proline permease), a gene known to be induced in the presence of maize exudates (50). This finding was not surprising, since proline can be used by *P. putida* as the sole carbon and nitrogen source and maize exudates are rich in this imino acid (C. Ramos, personal communication). A permease for the nonprotein amino acid gamma-aminobutyric acid (GABA) was also identified in this screening. GABA is produced from putrescine in *E. coli*, and

putrescine has been found in tomato root exudate (28). Thus, the induction of GABA transporter permease in the rhizosphere is not unexpected, since *Pseudomonas* strains are able to use GABA as the sole nutrient source. In connection with amino acid utilization, a mutant in the gene identified with the $rap_{2\text{-}23}$ fusion (which codes for a putative amino oxidase) is not able to use L-lysine as the sole nitrogen and carbon source (O. Revelles, personal communication), whereas wild-type KT2440 is able to grow on this amino acid. This gene was erroneously annotated as a putative tryptophan-2-monooxygenase (36). Genes involved in the catabolism of Lys have been shown to be induced by maize exudates, and this is an indication of the presence of Lys itself or any other Lys-related intermediary metabolites in the plant exudates (15).

Specific studies of bacteria of the genus *Pseudomonas* were recently compiled (40). However, many aspects of the metabolism and the general physiology of these bacteria still remain unknown. Knowledge about the entry of carbon sources, such as sugars and carboxylic acids, into the central metabolic pathways is limited and incomplete, and the same applies to the biosynthesis and catabolism of amino acids. Nevertheless, important aspects of energy metabolism have been revealed in this work. The induction of *gnd* (which codes for 6-phosphogluconate dehydrogenase) indicates that the pentose-phosphate pathway, an alternative to the Embden-Meyerhoff pathway, is active in the rhizosphere. Ribulose-5-phosphate, an important precursor for the synthesis of purine nucleotides and histidine (27), is generated via the so-called pentose shunt. Evidence that the Embden-Meyerhoff pathway was also induced comes from the isolation of the $rap_{2\text{-}77}$ fusion. Interestingly, the cofactor-independent phosphoglycerate mutase appeared to be essential for growth and pathogenicity of *P. syringae* in its host tomato plant (33). Pyruvate dehydrogenase, an enzyme with a major role in central metabolism, was also induced in the rhizosphere. The *aceE* gene, which encodes the pyruvate dehydrogenase E1 component, was identified as being induced in vivo in *E. coli* during septicemic infection in a murine model (24). In addition, isocitrate lyase, an enzyme of the glyoxylate bypass pathway, was probably induced in response to the presence of acetate and/or fatty acids, which have been identified as major components of the plant exudates (28).

Genes with a known role in protein trafficking have been shown to be induced in vivo. One example is *secB*, which encodes the component of the general secretory pathway SecB. This gene was previously identified as being essential for competitive root tip colonization in *P. fluorescens*, but it is not required for competitive growth in laboratory culture media (26). In *E. coli*, the protein chaperone SecB facilitates the targeting of periplasmic and outer membrane proteins, whereas insertase YidC is used for the insertion of membrane proteins (45). The gene encoding the inner membrane protein YidC was also identified in our screening.

Of special interest is the $rap_{2\text{-}74}$ fusion, which isolated a transcriptionally active region, apparently commanding the expression of putative antisense *ddcA* (*mus-5*). The *mus-5* gene was first identified by our group as playing a role in corn seed adhesion. It was recently reported that *ddcA* encodes a putative membrane polypeptide and expression of this gene is directly dependent on cell density and seed exudates (16). However, the detection of antisense expression in vivo during root colonization reported here might be a consequence of readthrough transcription from the convergent gene coding for PP4614, since no rho-independent termination sequence determinant was detected. Further studies will be needed to determine whether this messenger is an indication of *ddcA* silencing after the adhesion stage. Putative genes carried in the strand opposite to that of known genes have been also identified using IVET to recognize soil-induced genes in *P. fluorescens* (47).

Regulatory genes were also isolated in our IVET screening. *colS* was identified as highly induced during colonization (Fig. 4). A *P. fluorescens* mutant with a mutation in the sensor kinase member of the ColR/ColS two-component system was seriously impaired in competitive root colonization in several plants (11). The $rap_{1\text{-}4}$ fusion also identified a regulatory gene. The 3′ end of this probable transcriptional regulator of the ArsR family encoded by $rap_{1\text{-}4}$ is located 18 base pairs upstream from *metK*. In *E. coli*, the *metK* gene, which codes for *S*-adenosylmethionine synthetase (SAM), has been found to be induced in vivo during the biofilm lifestyle (41) and septicemic infection (24). In this bacterium, SAM is a corepressor of *met* genes, the donor of most methyl groups (9), and the source of the propylamino group of spermidine (19).

In each situation of the two assayed in vivo, a regulatory gene of unknown function was highly expressed and induced in comparison to the control ($rap_{1\text{-}12}$ in hydroponic culture and $rap_{2\text{-}44}$ in root colonization). This finding suggests a regulatory role for $Rap_{1\text{-}12}$ in exudate nutrient utilization. In support of this hypothesis is the finding that $rap_{1\text{-}12}$ is located convergent to genes that encode an amino acid ABC transporter.

Flagella also seem to be involved in seed adhesion (10). Our work identified a promoter of the operon comprising *fliO* as induced in vivo. *P. fluorescens* flagellar mutants are impaired in the ability to colonize developing roots (14), and a role for the flagella in chemotactic motility toward exudate components has been reported (13).

Our results identified the *parC* gene, which encodes DNA topoisomerase subunit A, as being induced in vivo. Interestingly, *parE* (which encodes subunit B) was found to be induced in vivo in *E. coli* during septicemia infection (24).

Strain KT2440 has been reported to be a restriction-minus derivative (*hsdR1*) of *P. putida* mt-2 (18). In this work, we identified the *hsdM* gene as being induced during colonization. *hsdM* exhibits translational coupling with *hsdS*, and both genes encode a functional modification methylase. All three *hsdRMS* genes encode a type IA restriction-modification system commonly found in *Enterobacteriaceae*. These enzymes are believed to play a role not only as a defense mechanism for bacterial cells against foreign DNA but also in a specialized recombination system thought to control the flow of genes between bacterial strains (5). It does not seem exceptional that the expression of genes that code for methyltransferases and endonucleases is tightly regulated, since it is essential that the methyltransferase completely modifies the cellular genome at all times to protect it from the lethal action of the endonuclease. This regulation must be especially important when restriction-modification genes first enter a cell, on conjugative plasmids, for example, but it might also be important during changes in the physiology of the cell (e.g., entry into the sta-

tionary phase, starvation associated with a deficiency of methyl donors, and other stresses).

The *gloA* gene, with a probable role in detoxification, was identified. Lactoylglutathione lyase (glyoxalase I) encoded by *gloA* catalyzes the isomerization of hemithioacetal to *S*-lactoylglutathione. Hemithioacetal is formed by the spontaneous reaction between the cytotoxic methylglyoxal and glutathione. The synthesis of methylglyoxal has been related to glucose flux exceeding the potential for growth (22) and growth on some carbon sources in the presence of cyclic AMP (2).

Fusion $rap_{1\text{-}9}$ identified the ribosomal protein L25, which belongs to the ribosomal L25p family. This family includes Ctc from *Bacillus subtilis*, which is induced by stress (7).

More than 30% of the *rap* fusions were to genes of unknown function. Fusion $rap_{2\text{-}19}$ was to a gene that encodes a hypothetical protein: a predicted hydrolase of the alpha/beta-fold family of unknown function. No similar protein was identified in other *Pseudomonas* species. However, a conserved hypothetical protein showing 55% identity and 65% similarity was found in *Burkholderia* (BPSL2346). Fusion $rap_{2\text{-}7}$ identified a hypothetical protein that exhibited translational coupling to a putative protein-*S*-isoprenyl cysteine methyltransferase. Homologues were not present in other species of *Pseudomonas*, although a homologous protein was found in the cyanobacteria *Nostoc* sp. (55% identity, 66% similarity). Fusion $rap_{2\text{-}18}$ identified what corresponded to an intergenic region in the *P. putida* KT2440 annotation by Nelson et al. (36). However, using the blastX program (BLAST 2.2.10) we identified in this region an unknown hypothetical protein conserved in *P. aeruginosa* (PA3399) (47% identity and 62% similarity), between PP3854 and PP3855. $Rap_{2\text{-}39}$ is a predicted SAM-dependent methyltransferase highly conserved among *Pseudomonas* species. Fusions $rap_{2\text{-}16}$ and $rap_{2\text{-}39}$ were to convergent genes with no loop structure sequence observed in the intergenic region. The $rap_{2\text{-}16}$ fusion identified the periplasmic component of an ABC-type sulfate transport system, and ABC transport system components have been suggested to act as adhesins (30). Fusion $rap_{2\text{-}73}$ identified a hypothetical protein, which exhibited homology to a hypothetical protein found only in *P. syringae* and *P. fluorescens*, two bacteria that also colonize plant roots.

Our work with in vivo expression technology allowed us to identify 28 *rap* genes, which have the potential to elucidate the colonization process and adaptation of *P. putida* to the rhizosphere environment. Given the biodegradative potential of KT2440, identifying promoters of preferential induction in the rhizosphere may open new avenues in rhizoremediation. The strictest *rap* promoters, such as those identified by fusions $rap_{2\text{-}45}$ (*algD*) and $rap_{1\text{-}19}$ (*hsdM*), might represent attractive genetic tools for exploiting the potential of this strain for biocontrol or rhizoremediation purposes.

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