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Proline Catabolism by *Pseudomonas putida*: Cloning, Characterization, and Expression of the *put* Genes in the Presence of Root Exudates

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Pseudomonas putida **KT2442 is a root-colonizing strain which can use proline, one of the major components in root exudates, as its sole carbon and nitrogen source. A *P. putida* mutant unable to grow with proline as the sole carbon and nitrogen source was isolated after random mini-Tn*5*–Km mutagenesis. The mini-Tn*5* insertion was located at the *putA* gene, which is adjacent to and divergent from the *putP* gene. The *putA* gene codes for a protein of 1,315 amino acid residues which is homologous to the PutA protein of *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Rhodobacter capsulatus*, and several *Rhizobium* strains. The central part of *P. putida* PutA showed homology to the proline dehydrogenase of *Saccharomyces cerevisiae* and *Drosophila melanogaster*, whereas the C-terminal end was homologous to the pyrroline-5-carboxylate dehydrogenase of *S. cerevisiae* and a number of aldehyde dehydrogenases. This suggests that in *P. putida*, both enzymatic steps for proline conversion to glutamic acid are catalyzed by a single polypeptide. The *putP* gene was homologous to the *putP* genes of several prokaryotic microorganisms, and its gene product is an integral inner-membrane protein involved in the uptake of proline. The expression of both genes was induced by proline added in the culture medium and was regulated by PutA. In a *P. putida putA*-deficient background, expression of both *putA* and *putP* genes was maximal and proline independent. Corn root exudates collected during 7 days also strongly induced the *P. putida put* genes, as determined by using fusions of the *put* promoters to *'lacZ*. The induction ratio for the *putA* promoter (about 20-fold) was 6-fold higher than the induction ratio for the *putP* promoter.**

Pseudomonas putida KT2442 is an efficient root colonizer in a number of agriculturally important plants. In field assays, the root colonization of corn and broad bean by this *P. putida* strain ranged from about 10^5 to 10^7 CFU per g of soil, depending on the year and the season (38, 39). However, in soils without plants, the number of viable cells never surpassed 10^3 CFU per g of soil (39) and frequently remained at a level below 10^2 CFU per g of soil. Little is known about the nature of the nutrient source available for this strain during root colonization. Amino acids present in plant exudates may help satisfy the energy demands of rhizobacteria (25). Our group and others have identified the amino acids present in the root exudates of corn plants. Almost all of the 20 amino acids most frequently present in the proteins can be detected, with proline one of the most abundant (4, 8, 29, 41, 56; C. Ramos and L. Molina, unpublished results). These observations raise the possibility that, at least in the corn root rhizosphere, proline catabolism may play a relevant role in supporting root colonization. Nevertheless, information regarding proline catabolism by *Pseudomonas* strains is scarce (34, 35).

The first step for proline catabolism requires the entry of this amino acid into the cells (60). In enteric bacteria, proline is taken up by several transport systems that differ in their V_{max} and affinity for proline. The PutP protein represents the major proline uptake system in *Escherichia coli* and *Salmonella* spp., with a K_m of about 2 μM (61). The uptake of proline via PutP is coupled to the entry of sodium ions (7, 10, 26, 47, 60).

Proline is converted into glutamate in a two-step process carried out by proline dehydrogenase (PDH) (EC 1.5.99.8) and pyrroline-5-carboxylate dehydrogenase (P5CDH) (EC 1.5.1.12) (21, 33, 59). In eukaryotes, PDH and P5CDH are encoded by two different genes (30, 58), while in enteric bacteria (2, 31, 63), *Rhodobacter capsulatus* (27), *Rhizobium meliloti* (25), and *Bradyrhizobium japonicum* (53), both steps for proline utilization are catalyzed by a single polypeptide encoded by the *putA* gene. In addition to these enzymatic activities, the PutA protein, at least in enterobacteria, is involved in the transcriptional control of the *put* genes. It seems that PutA functions as a repressor, inhibiting expression from the divergent *put* genes (33, 44).

In the present study, we isolated a *P. putida* KT2442 mutant unable to use proline as its sole C and N source. The mutation was complemented by using a *P. putida* cosmid library, and we rescued and analyzed the complete nucleotide sequence of the *P. putida put* genes. We also show that *put* gene expression in this strain is inducible by proline present in root exudates.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *P. putida* KT2442 was described in an earlier publication (18). It can use benzoate as its sole C source and exhibits resistance to rifampin, chloramphenicol, and ampicillin. Strain S14D2 is a KT2242 mutant unable to use proline as its sole C and N source (Table 1). The *E. coli* strains used in this study are shown in Table 1.

Bacterial cells were grown in Luria-Bertani medium or minimal M9 medium with succinate (20 mM) and/or proline (20 mM) as a C source (1). When proline (20 mM) was used as the sole C and N source, M9 depleted of ammonium, called M8, was used. When necessary, ampicillin, chloramphenicol, kanamycin, rifampin, and tetracycline were added to final concentrations of 100, 30, 25, 10, and 10 μg/ml, respectively.

DNA techniques. Plasmid DNA was isolated by the alkaline lysis method with the QIAprep spin plasmid minipreps kit (Qiagen catalog no. 27104). Total DNA was isolated by modifying the method of Kado and Liu as described by Ramos-González et al. (46), except that the 30-min incubation step at 55°C was omitted. DNA digestions with restriction enzymes, ligations, and transformations were performed by standard procedures (48).

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TABLE 1. Bacteria and plasmids used

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
Strains		
P. putida		
KT2442	Rif^r Ap^r Cm^r; prototroph	18
S14D2	Rif^r Km^r *putA*::mini-Tn*5* *luxAB*-Km	This study
E. coli		
HB101	Sm^r *recA*	5
DH5αF′	*recA*	62
CC118λpir	Rif^r; λ-*pir* lysogen	22
RM2	Δ(*putA putP*)	20
Plasmids		
pCK220	Ap^r Km^r mini-Tn*5*::′*luxAB*	52
pCRR831	Tc^r; chimeric cosmid of *P. putida* library bearing the proline utilization operon	C. Ramos and L. Molina
pMIS5	Tc^r P_{putA}::′*lacZ ori*RK2	This study
pMIS12	Tc^r P_{putP}::′*lacZ ori*RK2	This study
pMP220	Tc^r ′*lacZ ori*RK2	50
pPC6	Ap^r; 6-kb *Aat*II-*Pvu*II fragment from the serovar Typhimurium chromosome in pBR322	20
pRK600	Cm^r *mob*$^+$ *tra*$^+$	22
pUC18/19	Ap^r; cloning vector	57

[a] Ap^r, Rif^r, Km^r, Cm^r, Sm^r, and Tc^r indicate resistance to ampicillin, rifampin, kanamycin, chloramphenicol, streptomycin, and tetracycline, respectively.

DNA in both strands was sequenced with the dideoxy sequencing method, using the ABI Prism dRhodamine terminator kit (reference no. 403042; Perkin-Elmer).

Southern hybridization and DNA labeling. DNA fragments were separated in agarose gels and transferred onto nylon membranes by capillary blotting as previously described (48). Specific probes for hybridization were recovered from agarose gels with an agarose gel DNA extraction kit (reference no. 1696505; Boehringer Mannheim). All probes were labeled with digoxigenin by Klenow random primer extension according to the recommended procedure (3). Blotted filters were prehybridized, hybridized, washed, and immunologically developed according to the supplier's instructions. High-stringency conditions (50% [vol/vol] formamide at 42°C) were used.

Mutagenesis of *P. putida* by the mini-Tn*5* *luxAB*-Km transposon. Triparental matings involving *P. putida* KT2442 as the recipient, *E. coli* CC118λ*pir*(pCK220) as the transposon donor strain (52), and *E. coli* HB101(pRK600) as the helper strain were carried out as described by de Lorenzo and Timmis (16). Transconjugants of *P. putida* were selected on M9 minimal medium plates with 5 mM benzoic acid as the sole C source and supplemented with kanamycin and rifampin. About 5,000 independent clones were tested for their ability to grow on M8 minimal medium with proline as the sole C and N source. Four mutants unable to produce colonies on minimal medium with proline were kept for further studies.

Complementation assays. The pCRR831 cosmid (Table 1) (C. Ramos and L. Molina, unpublished results) selected from a *P. putida* KT2442 gene bank (M. I. Ramos-González, unpublished data) was used for complementation studies. pCRR831 was transferred by conjugation by the filter-mating technique (16) to the *P. putida* S14D2 mutant unable to grow with proline as the sole C and N source. Filters with a mixture of donor [*E. coli* HB101(pCRR831)], recipient (*P. putida* S14D2), and helper [*E. coli* HB101(pRK600)] strains at a ratio of 1:5:1 were incubated for 4 h at room temperature on Luria-Bertani plates. The cells were suspended in 1 ml of M9 minimal medium, and 100 μl was plated on selective minimal medium (M9 minimal medium with 10 mM benzoic acid, 10 μg of rifampin per ml, and 10 μg of tetracycline per ml). The transconjugants obtained were tested for their ability to grow on proline as the sole C and N source.

Enzyme assay. *P. putida* cells were grown on succinate, proline, or succinate plus proline as the sole C source. Cells were harvested by centrifugation, resuspended in a Tris buffer (pH 7.0; 100 mM), and permeabilized with toluene by vortexing. PDH activity was measured at 30°C in a 7-ml reaction mixture that contained 100 μmol of Tris buffer (pH 7.0), 45 μmol of proline, and 4.5 μmol of *o*-aminobenzaldehyde. The Δ^1-pyrroline-5′-carboxylic acid (P5C) that formed reacted with *o*-aminobenzaldehyde to produce a complex that exhibited maximal absorbance at 443 nm (17). The absorbance was corrected with a blank consisting of the same reaction mixture with water instead of proline. PDH activity was expressed as the number of nanomoles of P5C formed per milligram of protein.

Protein concentration in the cell extracts was determined with the Bradford reagent (Bio-Rad reference no. 500.0006; Bio-Rad, Madrid, Spain) with bovine serum albumin as the standard.

Collection of corn root exudates. Seeds were germinated on a sterile petri dish with water-agar. Seedlings were transferred to a grid, and the hair root was submerged into a sterile solution of M9 medium without ammonium. After 7 days, the seeds were removed, and the solution was filtered through a 0.2-μm sterile nitrocellulose filter and stored at −20°C until use. Proline concentrations in these exudates ranged between 50 and 100 μM.

Construction of P_{putA}::*lacZ* and P_{putP}::*lacZ* fusions. The divergent *putA* and *putP* promoter region was amplified by PCR from total chromosomal DNA of *P. putida* KT2442 with primers 5′-TTACGAATTCCGATGTAGATCACGAA GG-3′ and 5′-TTACGGAATTCTGCTTTGAGTCGCTCACGG-3′, which are provided with a restriction site for *Eco*RI. Upon amplification, as recommended by Ausubel et al. (3), DNA was restricted with *Eco*RI and ligated to plasmid pMP220 digested with *Eco*RI, so that transcriptional fusions of the *putA* or *putP* promoters to a promoterless ′*lacZ* gene were generated. The nature of the fusion can be distinguished by PCR amplification with an oligonucleotide primer based on the *lacZ* sequence and on *putA*- or *putP*-based primers, which result in a 0.8-kb fragment. The plasmid bearing the P_{putA}::′*lacZ* fusion was named pMIS5, and the one bearing the P_{putP}::′*lacZ* fusion was called pMIS12. The fusions were further confirmed by sequencing the whole promoter region and the first 20 codons of the ′*lacZ* gene.

β-Galactosidase activity was measured in *P. putida* KT2440 and in *P. putida* S14D2 bearing pMIS5 or pMIS12 and grown on M9 minimal medium with 20 mM succinic acid in the absence or the presence of 20 mM proline. Activity was determined according to Gallegos et al. (19), and activity was given in Miller units (36).

RESULTS

Growth of *P. putida* KT2442 on proline as the sole C and N source and isolation of mutants unable to metabolize proline. We first tested whether *P. putida* KT2442 was able to use proline as the sole source of C, N, or both nutrients. This strain was grown on M9 minimal medium with succinate as the sole C source. The culture was diluted 100-fold into M8 minimal medium with 20 mM proline and 10 mM NH_4Cl (proline as the sole C source), 20 mM succinate and 20 mM proline (proline as the sole N source), and 20 mM proline (proline as the C and N source). The strain grew exponentially with generation times of 1.70, 1.44, and 2.27 h when proline was used as the sole C, N, and C plus N source, respectively.

We then mated *P. putida* KT2442 with *E. coli* CC118λpir (pCK220) as described in Materials and Methods, and four mutants defective in proline utilization, called S14D2, S14D11, S15D3, and S16D2, were found.

To further confirm this initial selection, growth of the strains was tested in liquid M8 minimal medium with proline as the sole C and N source. Mutant S14D2 did not grow on minimal

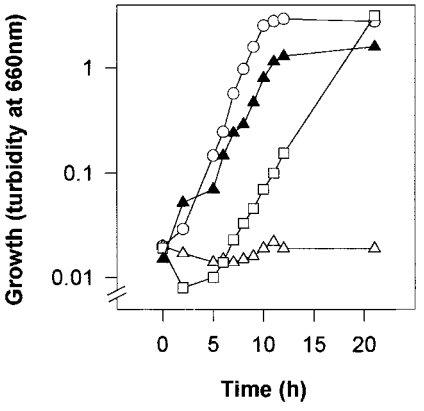

FIG. 1. Growth on minimal medium with proline as the sole C and N source of the wild-type *P. putida* KT2442 and its mutant strains deficient in proline utilization. Growth was monitored as an increase in turbidity of the culture. ○, *P. putida* KT2442; △, *P. putida* S14D2; □, *P. putida* S14D11; ▲, *P. putida* S14D2 (pCRR831).

TABLE 2. PDH activity of *P. putida* KT2442 and its mini-Tn*5* transposon derivatives[a]

Strain	PDH activity (%)	
	−Pro	+Pro
KT2442	5	100
S14D2	1	5
S14D2(pCCR831)	3	72

[a] Cells were grown on M9 minimal medium with succinate as the sole C source in the absence (−Pro) and the presence (+Pro) of 20 mM proline. PDH activity was determined as described in Materials and Methods. One hundred percent of activity corresponded to 190 nmol of P5C produced per milligrams of protein per minute.

medium after prolonged incubation (Fig. 1), whereas the other three mutants did grow, although they had a very long lag period before growth started. See Fig. 1 for mutant S14D11. We measured the PDH activity of the wild-type and the mutant strains growing on M9 with succinate or succinate plus proline. The results obtained are shown in Table 2. Neither the wild-type nor the mutant strains exhibited any statistically significant activity when grown on succinate alone, but the wild-type had high activity levels when it grew in the presence of proline. Mutants S14D11, S15D3, and S16D2 also had high levels of PDH activity when grown in the presence of proline (results not shown). In contrast, mutant S14D2 showed no activity when cells were grown on M9 with succinate and proline (Table 2). On the basis of these results, we considered S14D2 a true proline utilization-deficient strain, and it was retained for further studies. The other three mutants (S14D11, S15D3, and S16D2) were discarded.

Complementation of mutant S14D2 by pCRR831, cloning, and sequencing of the *put* genes. A *P. putida* KT2442 gene bank constructed in the broad-host-range pLAFR3 cosmid (M. I. Ramos-González, unpublished data) was used to complement *E. coli* RM2 (Table 1), a mutant unable to grow on proline because of a deletion of the *putA* and *putP* genes (20). A plasmid called pCRR831 was found to restore the ability to use proline as the sole C and N source to the *E. coli* mutant strain (C. Ramos and L. Molina, unpublished results). We transferred the Tc^r pCRR831 plasmid to *P. putida* S14D2 and selected Km^r Tc^r transconjugants able to grow on M8 minimal medium with proline as the sole C source. The frequency of appearance of transconjugants was 10^{-5} per recipient, and 100% of the transconjugants were able to grow on M8 liquid medium with proline as the sole C and N source. Figure 1 shows the growth of one random *P. putida* S14D2(pCRR831) clone, compared with the growth of the wild type and the mutant S14D2. This finding suggests that pCRR831 carries the proline degradation genes. To corroborate this finding, we determined the PDH activity of *P. putida* S14D2(pCRR831) growing on succinate or succinate plus proline. As expected, pCRR831 restored this activity in the mutant strain to levels similar to those found in the wild-type strain, when cells grew in the presence of proline (Table 2).

To locate the *put* genes in pCRR831, cosmid DNA was digested with *Pst*I and hybridized against the 4.2-kb *Mlu*I fragment of plasmid pPC6 (20), which carries the *putA putP* genes of *Salmonella enterica* serovar Typhimurium. The *P. putida put* genes were located within two *Pst*I fragments of 4.3 and 2.0 kb, which were subcloned in pUC19 to yield plasmids pLCR12 and pLCR4, respectively (Fig. 2). The DNA in both *Pst*I fragments was sequenced on both strands. The DNA sequences were compared with those deposited in the GenBank database, and the analysis revealed that the 4.3-kb DNA fragment bore the whole *putP* gene (1,479 bp), part of the *'putA'* gene (450 bp), and the intergenic region between *putP* and *putA* (355 bp). These genes were transcribed divergently. Plasmid pLCR4, bearing a 2-kb insert of the *P. putida* genome, also contained part of the *putA* gene; however, the translated DNA sequence did not exhibit a stop codon, nor did it account for the expected size of the PutA protein when compared with the PutA sequences deposited in GenBank. To complete the *putA* gene, a 12-kb *Hin*dIII fragment of pCRR831 was subcloned in pUC19 to yield pSLH4 (Fig. 2). DNA was sequenced with specific 20-mer primers, based on available *P. putida putA* sequences, until the complete *putA* gene sequence was obtained (3,948 bp). In all, the *putA* and *putP* genes and the intergenic region covered 5,757 bp. The DNA sequence is available from GenBank under accession no. AF153207. Downstream of both coding sequences, stem-loop transcription terminator sequences were found, which suggests that each gene makes a monocistronic mRNA.

The insertion of the mini-Tn*5* *'luxAB*-Km transposon in the genome of *P. putida* S14D2 was first located within the *putA* gene, based on hybridization assays. The region surrounding the mini-Tn*5* was PCR amplified and the insertion was specifically identified at nucleotide 1635 of the *putA* gene sequence.

Analysis of *putA* and *putP* gene products. The *putA* gene yielded the predicted PutA protein, which is 1,315 amino acids long and shows homology to PutA from different organisms such as *Klebsiella aerogenes* (71% identity) (54), *Salmonella* serovar Typhimurium (69% identity) (2), *E. coli* (69% identity) (31), *R. meliloti* (54% identity) (25), and *B. japonicum* (42% identity) (53). The highest homology was the domain involved in PDH activity (amino acids 337 to 588 in the *P. putida* PutA protein) (Fig. 3). Within this domain, a flavin adenine dinucleotide-binding pocket (residues 312 to 354) was identified. This domain exhibited homology with PDHs from *Saccharomyces cerevisiae* and *Drosophila melanogaster* and therefore seems to be involved in the conversion of proline to P5C, which equilibrates in solution with glutamic acid semialdehyde.

According to Ling et al. (31), amino acids 641 to 1074 are required for P5CDH activity. An NADPH pocket (residues 850 to 857) with the sequence FTGSTEVG was found within this region (31), which is highly similar to the corresponding PutA region in *E. coli* and *Salmonella* serovar Typhimurium (Fig. 3). This domain exhibited homology with aldehyde dehydrogenases, i.e., methylmalonate dehydrogenase, betaine dehydrogenase, and 2-hydroxymuconic acid semialdehyde dehydrogenase (9, 11, 13, 42, 45, 51). This finding suggests that the real substrate of this activity of PutA is glutamic acid semialdehyde.

A third region with high homology between PutA proteins but of unknown function is located between amino acids 78

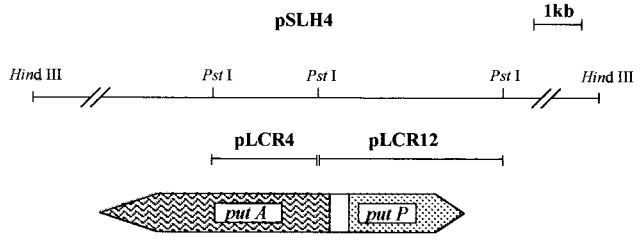

FIG. 2. Localization of *put* genes of *P. putida* KT2442 in vectors pLCR4, pLCR12, and pSLH4. The pLCR4 plasmid contains 2 kb of the *putA* gene, and pLCR12 contains 450 bp of the *putA* gene, all the *putP* genes, and the intervening regulatory region between the two genes that are transcribed divergently. A 12-kb insert in plasmid pSLH4 bears the complete proline utilization operon.

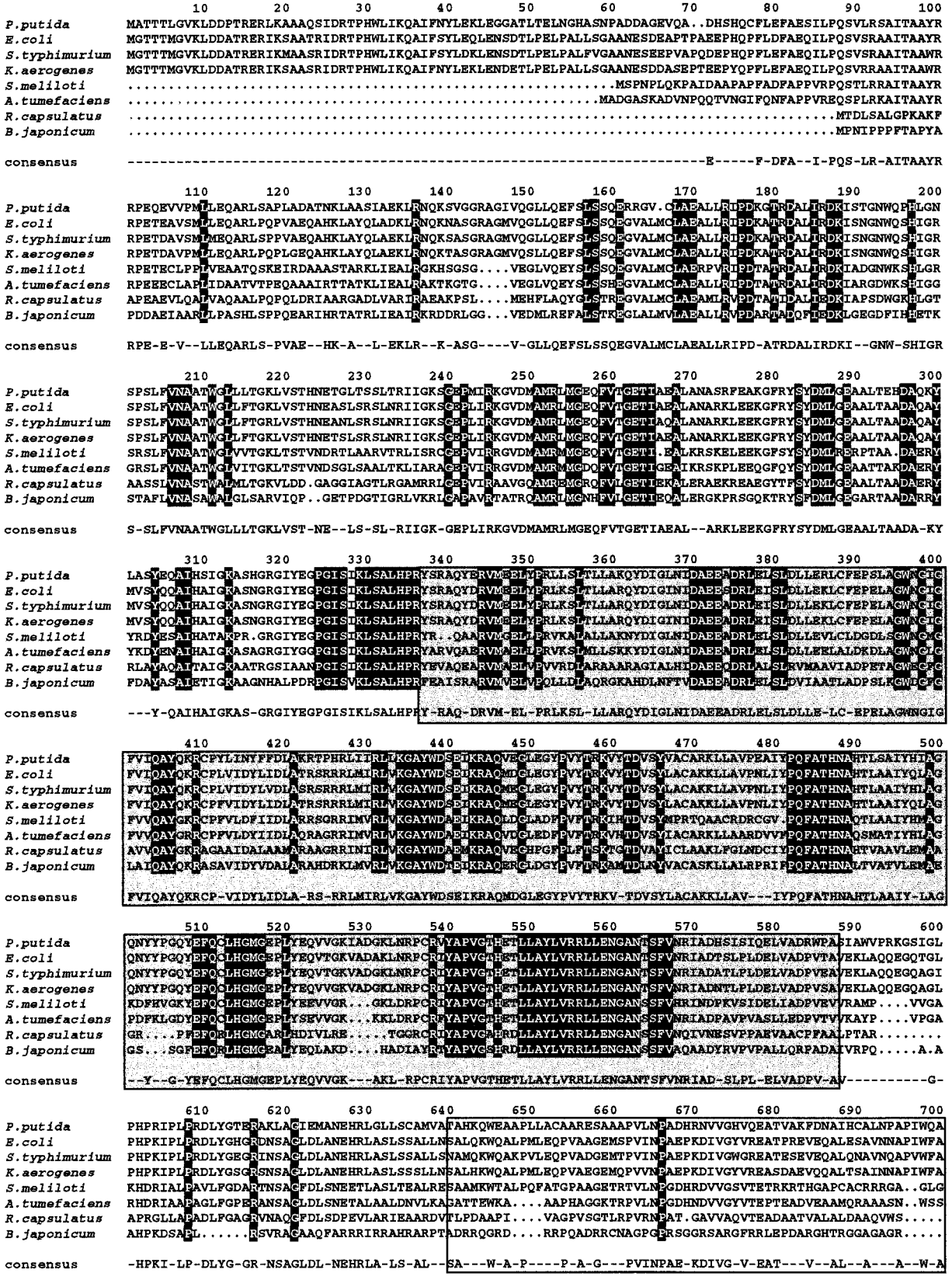

FIG. 3. Sequence alignment of PutA proteins of prokaryotic origin. The strains and sources of the protein sequences were as follows: *P. putida* (this study); *E. coli* (31); *Salmonella* serovar Typhimurium (2); *K. aerogenes* (54); *Agrobacterium tumefaciens* (14); *R. capsulatus* (27); *S. meliloti* (25); *B. japonicum* (53). The ALIGN program was used. If the residue is identical in all the aligned proteins, it appears printed on a black background. If the residue is identical in 50% of the aligned proteins, it appears on a gray background. The amino acid chosen for the consensus was present at the given position in at least 50% of the aligned sequences. The PDH domain, residues 337 to 588, is shown in a grey box, and the P5CDH domain, residues 641 to 1074, is also boxed.

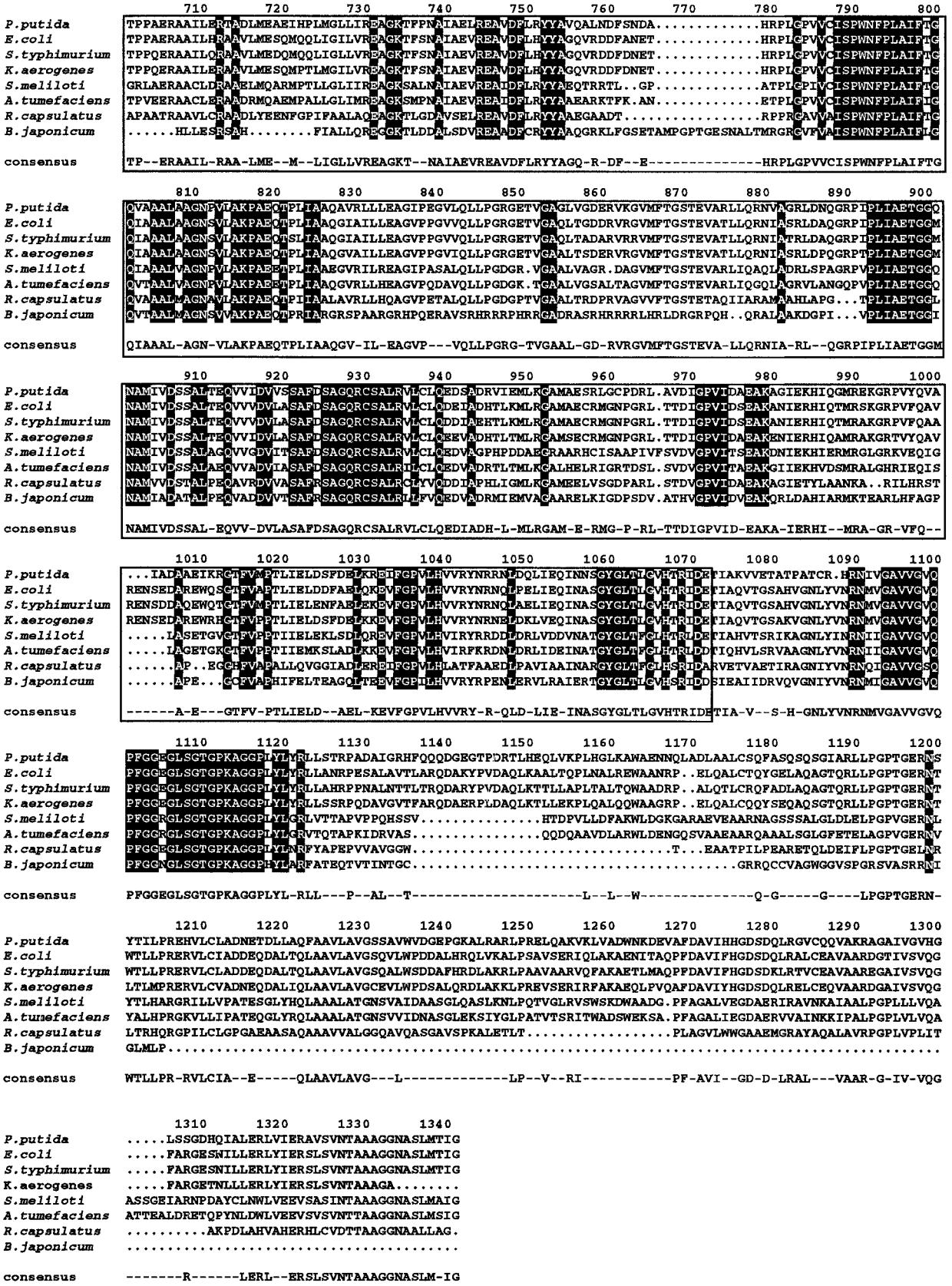

FIG. 3—*Continued.*

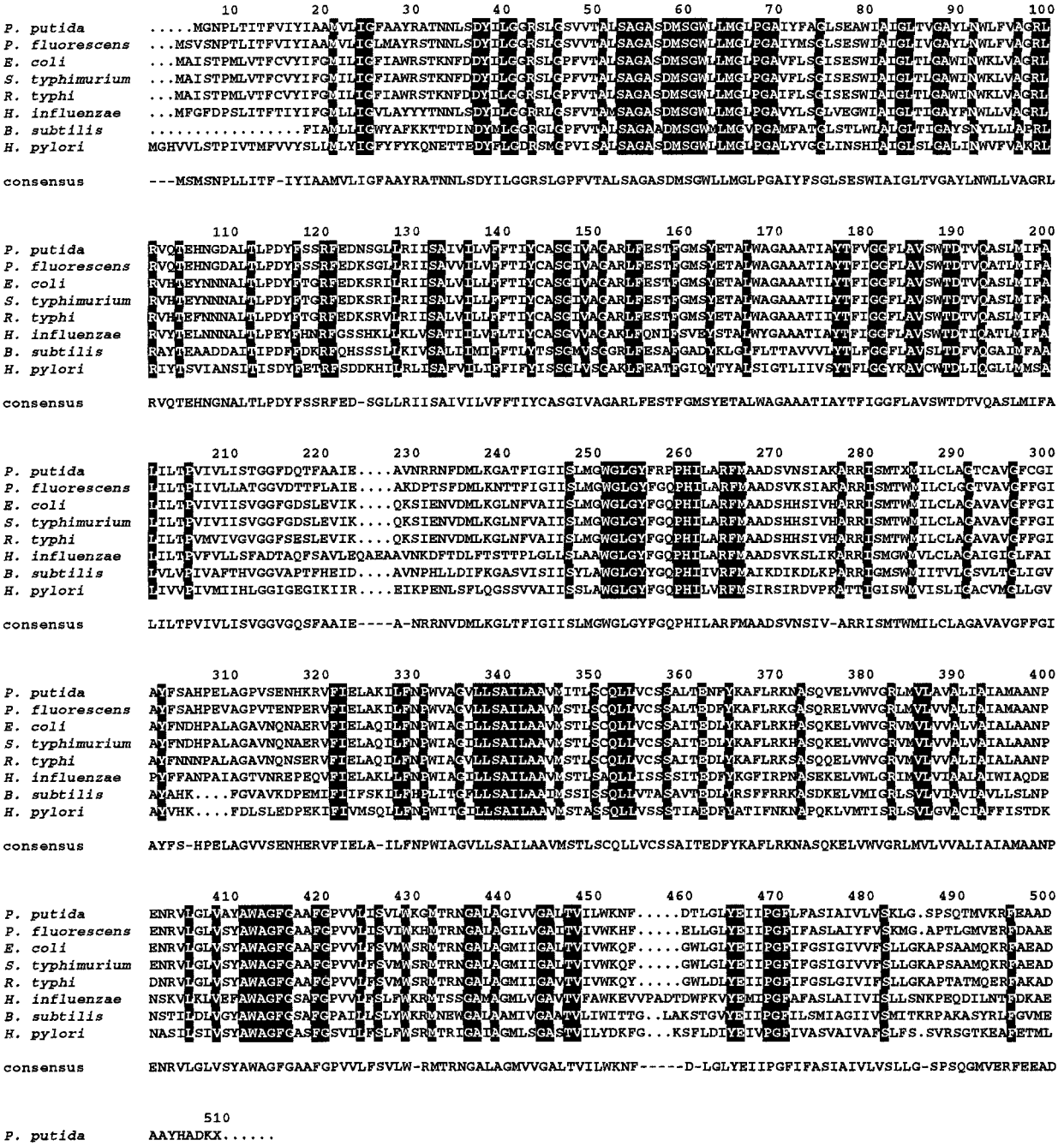

FIG. 4. Sequence alignment of PutP proteins. Strains and sources of the sequences were as follows: *P. putida* (this study); *Bacillus subtilis* (64); *P. fluorescens* (23); *E. coli* (40); *Salmonella* serovar Typhimurium (37); *Rickettsia typhi* (40); and *Haemophilus influenzae* and *Helicobacter pylori* (55). The ALIGN program was used. Other details are as in the legend for Fig. 3.

and 190. In *E. coli*, the PutA protein is able to associate with the cell membranes. Three hydrophobic segments between residues 158 and 167, 767 and 817, and 1205 to 1220 may be important for such interactions. These segments are present in the *P. putida* PutA protein. In general, the interdomains were less conserved (Fig. 3).

The *P. putida* PutP protein is 493 amino acids long and exhibits 85% similarity with PutP from *Pseudomonas fluore-*

TABLE 3. Expression from the *put* promoters in *P. putida*[a]

Strain and fusion	Growth conditions	β-Galactosidase activity
Wild type		
P_{putP}::'*lacZ*	Succinate	700 ± 50
	Succinate plus proline	2,800 ± 100
P_{putA}::'*lacZ*	Succinate	350 ± 30
	Succinate plus proline	6,950 ± 100
Mutant S14D2		
P_{putP}::'*lacZ*	Succinate	2,700 ± 200
	Succinate plus proline	2,600 ± 150
P_{putA}::'*lacZ*	Succinate	7,900 ± 200
	Succinate	8,600 ± 250

[a] *P. putida* KT2442 and *P. putida* S14D2 bearing pMIS5 (P_{putA}::'*lacZ*) or pMIS12 (P_{putP}::'*lacZ*) were grown on M9 minimal medium with succinate or succinate plus proline. β-Galactosidase activity (in Miller units) was determined in permeabilized whole cells according to the method of Gallegos et al. (19). Data are the average of four independent assays.

scens, 76% with *Salmonella* serovar Typhimurium, and 78% with *E. coli*. The Scamprosite program predicted 12 transmembrane segments for the *P. putida* PutP protein, and multiple alignments revealed extended homology with PutP from other sources that corresponded to transmembrane segments (Fig. 4), whereas cytoplasmic and periplasmic loops were less well conserved. In addition, PutP presents homology to transport systems that are involved in the uptake of chemicals related to sodium entry, i.e., *E. coli* porter systems for inositol, phenylacetic acid, and pantothenate (7, 15, 49, 55, 58).

Expression from the *putA* and *putP* gene promoters. To determine the expression of the *put* genes, the divergent *put* promoter region was fused in a broad-host-range vector to *'lacZ* as described in Materials and Methods to generate transcriptional fusions yielding pMIS5 and pMIS12. These plasmids were transferred to the wild-type *P. putida* KT2442 and to its mutant *P. putida* S14D2. β-Galactosidase (LacZ) activity in *P. putida* KT2442 with one of these plasmids was measured in cells growing on minimal medium with succinate and succinate plus proline under highly aerated conditions. In wild-type cells growing on succinate, basal activity from P_{putP} (700 Miller units) was twofold higher than for P_{putA} (350 Miller units) (Table 3). In the presence of proline, the increase in activity was 4- and 20-fold for the P_{putP} fusion and the P_{putA} fusion, respectively (Table 3). These results suggest that the genes for proline catabolism are inducible.

Expression of the *putA* and *putP* genes was also measured in the S14D2 mutant strain bearing pMIS5 or pMIS12 in cells growing on M9 minimal medium with succinate or with proline. In both the absence and the presence of proline, high levels of expression were found, about 2,700 Miller units for the P_{putP}::'*lacZ* fusion and about 8,000 Miller units for the P_{putA} fusion. These results suggest that the PutA protein is involved in the control of expression from the *putA* and *putP* gene promoters.

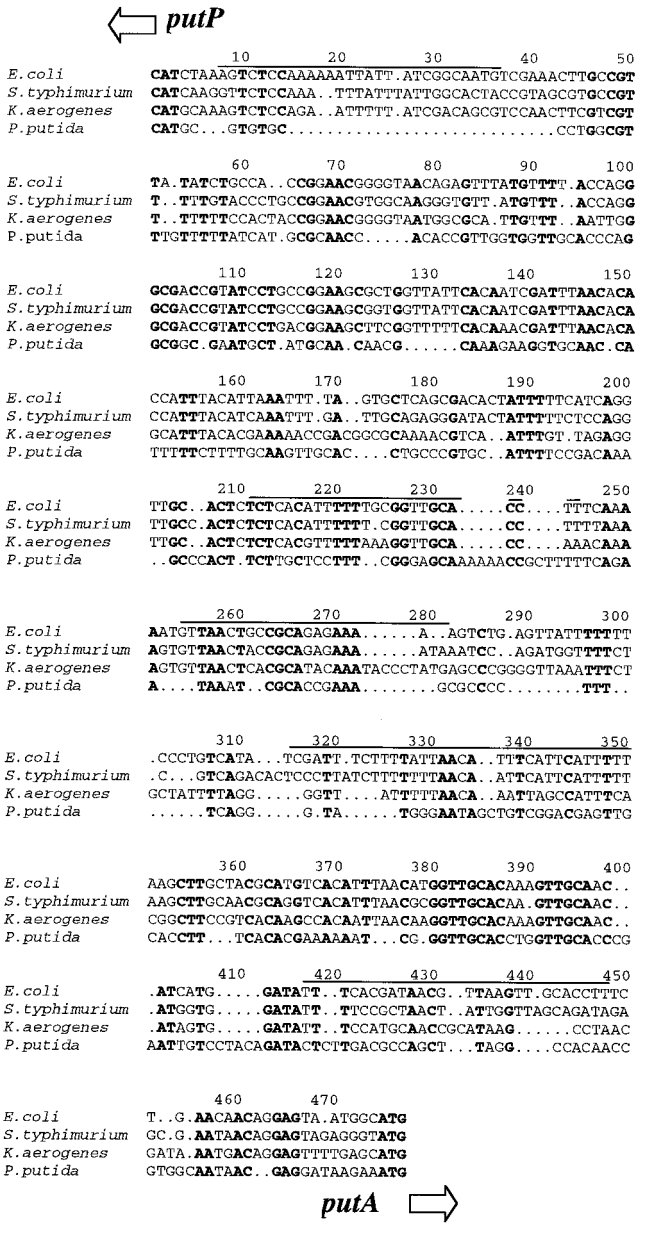

FIG. 5. Alignment of the *putA* and *putP* intergenic regions of enteric bacteria and *P. putida*. The alignment includes the region between the start codons of *putA* and *putP*. Gaps were introduced to allow maximal scoring in the alignment with identical positions being shown in boldface. The overlined bases indicate putative PutA binding sites.

TABLE 4. Induction of the *put* promoters in the presence of corn root exudates[a]

Fusion	Incubation conditions	β-Galactosidase activity
P_{putA}::'*lacZ*	Corn exudate	425 ± 50
	M8	20 ± 10
P_{putP}::'*lacZ*	Corn exudate	270 ± 50
	M8	60 ± 10

[a] *P. putida* KT2442 cells bearing pMIS5 (P_{putA}::'*lacZ*) or pMIS12 (P_{putP}::'*lacZ*) grown on M9 minimal medium with succinate were harvested and suspended in M8 minimal medium (M8) or in the same medium enriched for 7 days with corn root exudates (corn exudate) and incubated at room temperature without agitation. β-Galactosidase activity was determined as described in the footnote to Table 3.

Induction of the P_{put} promoters by corn root exudates. *P. putida* KT2442 bearing plasmid pMIS5 or pMIS12 was grown on minimal medium with succinate as the sole C source until the mid-exponential growth phase was reached. Cells were then either harvested and suspended in M8 minimal medium without a C source or suspended in 7-day-old root exudates. The suspensions were incubated at room temperature without agitation for 30 min to follow induction from the *put* promoters. The level of β-galactosidase activity from P_{putA} and P_{putP} when cells were incubated in the presence of corn root exu-

dates was around 20- and 4-fold higher than the basal level (Table 4). This suggests that proline present in root exudates was able to promote expression of the *P. putida put* catabolic genes.

DISCUSSION

Recent studies have focused their attention on the possible role of amino acids as carbon substrates to support growth of microorganisms in the rhizosphere of plants (24, 28, 63, 65). Proline has been found to be a major compound in the corn root exudates; therefore, this amino acid could be an important energy source for bacteria during the first stages of colonization of the roots of plants. How deficiency in the utilization of proline or other amino acids affects rhizosphere colonization has not yet been studied in detail, although an *R. meliloti* mutant altered in proline catabolism exhibited reduced ability to colonize the alfalfa root (25).

In this work we have approached the study of proline utilization in *P. putida*, for which we isolated mutants unable to use proline as their C or N source. *P. putida* S14D2 was considered a true proline utilization-deficient mutant because it did not grow with proline, in contrast with other mutants isolated in this study that showed retarded growth on proline. We found that in the S14D2 mutant strain, the mini-Tn*5* transposon was inserted in the chromosome within a gene involved in proline catabolism (*putA*). Analysis of the *P. putida putA* gene product revealed a domain structure similar to that of enteric bacteria such as *R. capsulatus* and *B. japonicum* in which the two steps for proline degradation to glutamate are catalyzed by a single bifunctional dehydrogenase enzyme (2, 25, 27, 31, 51, 53). Analysis of the *P. putida* PutP protein suggests that it is an integral inner-membrane protein that belongs to the family of Na^+ substrate symporters (15, 49, 58, 60). We showed that the *putA* gene is adjacent to the *putP* gene and that these genes are transcribed divergently, as is the case for enteric bacteria.

In *P. putida*, the *putA* and *putP* genes seem to be regulated at the transcriptional level, with proline—either supplied in culture medium or in root exudates—acting as an inducer, as the expression from the *putA* and *putP* gene promoters increased by about 20- and 4-fold, respectively, in the presence of proline. In a *putA* mutant background, high levels of expression from these genes occurred, suggesting that the *P. putida* PutA protein acts as a repressor of *putA* and *putP* gene expression, as also described for enteric bacteria (11, 44). The fact that proline metabolism in the soil bacterium *P. putida* is regulated by a mechanism similar in principle to that of enteric bacteria is rather surprising in the light of the differences in the ecological habitats of these organisms. These similarities in the regulation of the *put* genes in enteric bacteria and in *Pseudomonas* prompted us to compare the intergenic regions between *putA* and *putP* in these microorganisms. Figure 5 shows an alignment of the intergenic region between *putA* and *putP* of *Salmonella* serovar Typhimurium, *E. coli*, *K. aerogenes* and *P. putida*, from which it can be seen that this region is 63 to 65 bp longer in enteric bacteria than in *P. putida*, with a very large gap (28 nt) being observed near the ATG start codon of the *putP* gene. In all four microorganisms, *putA* and *putP* genes are transcribed divergently, although differences in the location of promoters are known, with overlapping promoters in *Salmonella* serovar Typhimurium and well-separated transcription starts in *K. aerogenes* and *P. putida* (12, 44; S. Vílchez and J. L. Ramos, unpublished results). In *Salmonella* serovar Typhimurium, the intergenic *putA-putP* DNA is intrinsically curved and it has been found that up to five segments (marked in Fig. 5 by a line above the sequence) could be bound by purified PutA protein. In enteric bacteria, it has been suggested that the integration host factor plays a role in the expression from *putA* and *putP*, and two sites (positions 1 to 13 and 330 to 344) (Fig. 5) in the *Salmonella* serovar Typhimurium promoter region were found (6, 43, 44). Those sites are not well conserved in the corresponding aligned sequence in *P. putida*, and at present, we cannot predict whether or not integration host factor plays a role in the transcription of the *put* genes in the soil bacterium *P. putida*.

Therefore, we can conclude that although the pattern of gene control of the *putA* and *putP* genes is similar in enteric bacteria and in the soil-borne *P. putida* KT2440, the molecular mechanisms of control may be very distinct.

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