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-Tn5-Km mutagenesis. The mini-Tn5 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 10⁵ to 10⁷ 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 10³ 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 μ M (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-Tn5 luxAB-Km | This study |
| E. coli | | |
| HB101 | Sm ^r recA | 5 |
| DH5aF' | recA | 62 |
| CC118Apir | Rif ^r ; λ <i>-pir</i> lysogen | 22 |
| RM2 | $\Delta(putA putP)$ | 20 |
| Plasmids | | |
| pCK220 | Ap ^r Km ^r mini-Tn5::/ <i>luxAB</i> | 52 |
| pCRR831 | Tc^{r} : chimeric cosmid of <i>P. putida</i> | C. Ramos and |
| 1 | library bearing the proline utiliza- tion operon | L. Molina |
| pMIS5 | $Tc^{r} P_{nut_{A}}:: 'lacZ oriRK2$ | This study |
| pMIS12 | $Tc^r P_{nuP}$::'lacZ oriRK2 | This study |
| pMP220 | Te ^r 'lacZ oriRK2 | 50 |
| pPC6 | Ap'; 6-kb <i>Aat</i> II- <i>Pvu</i> II fragment from the serovar Typhimurium chromo- some in pBR322 | 20 |
| pRK600 | $\operatorname{Cm}^{r} mob^{+} tra^{+}$ | 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-Tn5 *luxAB*-Km transposon. Triparental matings involving *P. putida* KT2442 as the recipient, *E. coli* CC118*xpir*(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 μ mol of Tris buffer (pH 7.0), 45 μ mol of proline, and 4.5 μ mol 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- μ m sterile nitrocellulose filter and stored at -20° C until use. Proline concentrations in these exudates ranged between 50 and 100 μ M.

Construction of P_{putA} ::*lacZ* and P_{putB} ::*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 pMISS, 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 NH₄Cl (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. \bigcirc , *P. putida* KT2442; \triangle , *P. putida* S14D2; \square , *P. putida* S14D11; \blacktriangle , *P. putida* S14D2 (pCRR831).

 TABLE 2. PDH activity of *P. putida* KT2442 and its mini-Tn5 transposon derivatives^a

| Stroin | PDH activit | у (%) |
|----------------|-------------|-------|
| Strain | -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 wildtype 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 Tcr 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 *PstI* and hybridized against the 4.2-kb *MluI* 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 *PstI* 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 *PstI* 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 HindIII 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 Gen-Bank 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-Tn5 '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-Tn5 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.

| | 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 | 90 | 100 |
|-------------------------------|--------------------------------------|--------------------------|---------------------------|--------------------------|----------------------------|----------------------------|--------------------------|-----------------------------------|----------------------------|------------------|
| P.putida T. coli | MATTTLGVKLDDPTRE | RLKAAAQSII | RTPHWLIK | AIFNYLEKLE | GGATLTELNG | HASNPADDAG | EVQA DHS | HOCFLEFARSI | LPQSVLRSAIT | FAAYR |
| E.COIL S.typhimurium | MGTTTMGVKLDDATRE | RIKMAASRII | ORTPHWLIK | AIFSYLDKLE | INSDILPELPA | LFVGAANESE | EPVAPQDEP | HQPFLEFAEQI | LPQSVSRAAI1 | TAAWR |
| K.aerogenes | MGTTTMGVKLDDATRE | RIKSAASRII | ORTPHWLIKQ | AIFNYLEKLE | NDETLPELPA | LLSGAANESE | DASEPTEEP | YQPFLEFAEQII | LPQSVRRAAIT | TAAWR |
| S.mellioti A.tumefaciens | ••••• | | ••••• | | ••••• | MADGASE | ADVNPQQTVI | NGIFQNFAPPVI | REQSPLERAIT | TAATR |
| R.capsulatus | •••••• | • • • • • • • • • • | • • • • • • • • • • | • • • • • • • • • • • | • • • • • • • • • • • | • • • • • • • • • • | •••• | • • • • • • • • • • • • | MTDLSALGE | RAKF |
| B.japonicum | | ••••• | • • • • • • • • • • | ••••• | •••• | | •••• | • • • • • • • • • • • • • | MPNIPPPF1 | TAPIA |
| consensus | | | | | | | B | F-DFAI | -PQS-LR-AII | FAAYR |
| - | 110 | 120 | 130 | 140 | 150 | 160 | 170 | 180 | 190 | 200 |
| P.putida E.coli | RPETEAVSMILEQARL | POPVAEQAH | LAASIAEKI | RNOKNASGRA | GIVQGLLQEF | SLSSQERKGV | MCLAEALLR | IPDKARRDAL II | NDKISTGNWQP NDKISNGNWQS | HIGR |
| S.typhimurium | RPETDAVSMIMEOARL | SPPVAEQAH | LAYQLAEKI | RNOKSASGRA | GMVQGLLQEF | SLSSOPGVAL | MCLAEALLR | IPDKATRDAL II | DKISNGNWQS | HIGR |
| K.aerogenes S.meliloti | RPETECLPPUVEAATO | SKEIRDAAAS | TARKLIEAI | RNOKTASGRA | VEGLVQEY | SUSSOEGVAL | MOLAERPVR | IPDTA-RDALHI | DKISNGNWQS RDKIADGNWKS | HLGR |
| A.tumefaciens | RPEEECLAPLIDAATV | TPEQAAAIRT | TATKLIEAL | RAKTKGTG | VEGLVQEY | SLSSHEGVAL | MCLAEALLR | IPDTATEDAL II | | HIGG |
| R.capsulatus B.japonicum | APEAEVLQAIVAQAAL PDDAEIAARULPASHL | SPPQEARINE | ARGADLVARI ATATRLIBAI | RAEAKPSL RKRDDRLGG. | VEDMLREF | 'ALSTREGVAL | MVLAEALLR | VPD/TAN IDALIIS VPDAR/JADQFIIS | SDKIAPSDWGK SDKLGEGDFIH | BETK |
| | PPP-P-V-ILPOAPI | | | | | SI.SSOFGVAT | MCLARALLR | | POKT CNW- | - |
| consensus | KPE-E-VDDEQARD | S-FVALAr | -AD-EKI | 1KK-A3G | | SUSSUE | INC URBRUUK | IFD-AIRDADI. | NDKI - GAN-S | SHIGK |
| P. putida | 210 SPSLFVNATWOLLT | 220 GKLVSTHNEI | 230 GLTSSLTRI | 240 IGKSEEPMIR | 250 KGVDMAMRLM | 260 CEODVICION | 270 AEMLANASRI | 280 FEAKGFRYSYD | 290 GGAALTEH | 300 200 KM |
| E.coli | SPSLFVNAATWGLLFT | GKLVSTHNEA | SLSRSLNRI | IGKSGEPLIR | KGVDMAMRLM | GEOFVTGET I | ABALANARKI | LEEKGFRYSYDI | II GEAALTAAD | AQAY |
| S.typhimurium K.serogenes | SPSLFVNAATWOLLFT SPSLFVNAATWOLLFT | GRLVSTHNE | NLSRSLNRI | IGKSCEPLIR | KGVDMAMRIIM KGVDMAMRI M | GEOFVIGETI (CROFVIGETI | AQALANARKI ARALANARKI | LEEKGFRYSYD | MGDAALTAAD MGDAALTAAD | DAQAY |
| S.meliloti | SRSLFVNAATWGLVVT | GKLTSTVNDF | TLAARVTRL | ISRCGEPVIR | RGVDMAMRMM | (GEQFVIGET I | . EALKRSKEI | LEEKGFSYSYDI | IRERPTAA. | ABRY |
| A.tumefaciens R.capsulatus | GRSLFVNAATWGLVIT | GKLTSTVNDS GKVLDD.GAG | GLSAALTKI | IARAGERVIR MRRIGERVIR | RGVDMAMRMM AAVGOAMREM | GDOFVIGETI GROFVI GETI | GEAIKRSKPI RKALERARKI | LEEQGFQYSYDI REARGYT FSYDI | IIGEAATTARD | DAERY |
| B.japonicum | STAFIVNASAWAIGLS | ARVIQPGE | TPDGTIGRL | VKRLGABAVR | TATROAMRLM | GNHFVIGETI | e <u>o</u> alergkpi | RSGQKTRYSFD | II GEGARTAAI | DARRY |
| consensus | S-SLFVNAATWGLLLT | GKLVST-NE- | -LS-SL-RI | IGK-GEPLIF | RGVDMAMRLM | igeofvtget i | AEALARK | LEEKGFRYSYD | MLGEAALTAAI | DA-KY |
| | 310 | 320 | 330 | 340 | 350 | 360 | 370 | 380 | 390 | 400 |
| P.putida | LASVEOATHSIGKASH | GRGIYEGPGI | SIKLSALHE | RYSRAQYER | MBELNPRILLS | TLLAROYDI | GLNTDAEEA | ORLEL SLOLLE | RLCFEPSLAG | vi GũG |
| E.coli S turbimurium | MVSVOOATHAIGKASN | GRGIYEGPGI | STRLSALHE | RYSRAQYDR | MEELYPRIKS | TLLAROYDI | GINEDAEES GLAUDAEEN | DRLEISLDLLE | KLCFEPELAG | ଏି ପେର ∿ିପାସ |
| K.aerogenes | MVSV00ATHAIGKASN | GRGIYEGPGI | SIKLSALHE | RYSRAQYDR | MEELYPRLKS | TLLAROYDI | GINIDAEEA | DRUEISUDLLE | KLCFEPELAG | V GIG |
| S.meliloti | YRDVESATHATAKPR. | GRGIYEGPGI | SIKLSALHE | RYR. OAAR | MCELUPRVKA | TALLAKNYDI MULSEKVDI | GLNIDAEEA | DRUELSUDLLE | VLCLDGDLSG | ∿്©്G √്റിറ |
| R.capsulatus | RLAVAOAITAIGKAAT | RGSIAANPGI | SIKLSALHP | RYEVAQEAR | MAELVEVVRL | ARAAARAGI | ALHIDAEEO | DRLAL SLRVKA | AVIADPETAG | vi≥G ;G |
| B.japonicum | FDAMASANETI GRAAG | NHALPDRPG | SVKLSALHE | RFEAISRAR | MVELVPOLLD | AORGKAHDI | NFTVDABEA | DRLEDSLOVIA | ATLADP SLRG | WEGEG |
| consensus | Y-QAIHAIGKAS- | GRGIYEGPGI | SIKLSALHE | PEY-RAQ-DR | M-EL-PRLKS | 5L-LLARQYDI | iglni dae ea | Drlelsidlie | -LC-BPRLAG | ANGIG |
| | 410 | 420 | 430 | 440 | 450 | 460 | 470 | 480 | 490 | 500 |
| P.putida E.coli | FVIOAVORRCPYLINY FVIOAVORRCPLVIDY | FFDLAKRTPH LIDLATRSRF | REALIRLICKO | AYWDSEUKRA AYWDSEUKRA | QVEGNECYPV OMDGMECYPV | ATR KVATOVS ATR KVATOVS | WACARKLL | VPEATYPOFA VPNLLVPOFA | THNAHTLAAT | CHIAG COLAG |
| S.typhimurium | FVIOAYOKRCPLVIDY | LVDLASRSRF | RIMIRLYKO | AYWDSENKRA | QMECLEGYPV | TTREVT | MLACAKKLL | WENLIN POFA | HINAHTLAAIY | DALE |
| K.aerogenes S.meliloti | FVIOAVOKRCPFVIDY | LIDUATRSRF IIDLARRSGF | REMERLVKO | AYWD EKRA | OFFGUSSYPV | MTRKVITDVS FTRKEHTDVS | MERTOAAC | AVPNLIMPOFA RDRCGV.POFA | THINAHTLAATT | (QLAG) |
| A.tumefaciens | FVVOAVGRRCPFVLDY | IIDLAORAGE | RIMVRLVKG | AYWDENKRA | QVDGLEDFPV | TRKVHTDVS | VIACARKLL | ARDVVPPOFA | THINA OSMATIY | THLAG |
| R.capsulatus B.japonicum | AVVOAVGREAGAAIDA | LAAMARAAGH VDALARAHDH | IRINIRLAKO IKLANVRLAKO | AYWD' E KRA | ORREHPG PL | atskrgtdva Tyrkantdia | VICLAARLP | SIMDCI MPOFA MERBRI POFA | THINAUTVAAVI | JEN AA JEN AE |
| | | | | | | | - | | | |
| consensus | FULLATURACE-VIDI | | A DELLES | MI HUJEL AN | QIBDGHISG17 V | (11RF, 9-199) | JI UNCARAUII | AV | I MICHAEL | |
| P. nutida | 510 | 520 CEPICEOVVC | 530 KIADGKLNR | 540 PERVYAPVG | 550 | 560 RILLENGAN | 570 EVNRTADHS | 580 ISTORLVADRW | 590 PASTAWVPRKC | 600 SSIGL |
| E.coli | QNYYPGQYEFQCLHGM | GEPLYEQVIC | KVADAKLNE | IPCR/ YAPVG | HEALLAYLVR | RLLENGAN | FUNRIADTS | LPLOELVADPV | PAVERLAQQEC | SQTGL |
| S.typhimurium | ONYYPGOMEFOCLHGM | CEPLYBOVIC | KVADGKLNR KVADGKLNR | IZCRUYAPVG IZCRUYAPVG | HEALLAYLVR | RLLENGANGS | FVNRIADAT | LPLDELVADPV LPLDELVADPV | SAVEKLAQQEG | GOAGI |
| S.meliloti | KDFHVGKYEFQCLHGM | GEPLYEEVVO | RGKLDF | P.C.R.IYAPVG | HEALLAYLVR | RLLENGANSS | F VHRINDPK | VSIDELIADEV | BVVRAMP | . VVGA |
| A.tumefaciens | PDFKLGDYDFOCLHGM | CEPLYSEVVO | KKKLDR | PORUYAPVG | HEALLAYLVR | RLLENGAN | FVNRIADPA | VPVASLLEDPV | FVVKAYP | .VPGA |
| B.japonicum | GSSGFEFORLHGM | GRALYEQLAS | DHADI | AVRAYAPVG | HRILLAYLVR | RLLENGANSS | FVAQAADYR | VPVPALLQRPA | DALVRPO | A.A |
| CORGONSUS | | CRPLYROVV | | PCRTVAPVC | | RLIENGANP | FUNETAD-S | I.PTRI.VADPV | | G- |
| CONSENSUS | | | | | | | | | <u>200</u> | |
| P.putida | 610 PHPRIPLERDLYGTER | 620 AKLACIEMAN | 630 WEHRLGLLSC | 640 AMVAFAHKQV | 650 EAAPLLACAA | 660 RESAAAPVLI | 670 PADHRNVVG | 680 HVQEATVAKFD | 690 NAIHCALNPAJ | 700 PIWQA |
| E.coli | PHPKIPLPRDLYGHGR | DNSAGLDLAN | THRLASLSS | SALLNSALORV | QALPMLEOPV | AAGEMSPVIN | PAEPKDIVG | YVREATPREVE | QALESAVNNA | PIWFA |
| s.typnimurium K.aerogenes | PHPKIPLPRDLYGSGE | SNSAGLDLAN | NEHRLASLSS | SLLNSALHKV | vQALPMLEQPV VQALPMLEQPV | ALGENTPVII AEGEMQPVVN | PAEPKDIVG | WOREATESEVE | QALTSAINNA! | FVWFA PIWFA |
| 5.meliloti | KHDRIALPAVLFGDAR | TNSACFDLS | TETLASLT | ALRESAAME | TALPOFATGE | AAGETRTVL | PGDHRDVVG | SVTETRKRTHG | APCACRRRGA | GLG |
| A.tumefaciens R.capsulatus | KHDKIAANAGLFGPBR APRGLLAPADLFGAGR | ANSACLDLSI VNAQCFDLSI | NETALAALDN OPEVLARIEA | ARDV F LPDA | NAAAAPE APIVAGE | IAGGKTRPVLI PVSGTLRPVRI | PGDHNDVVG PAT.GAVVA | 1 VTEPTEADVE QVTEADAATVA | hamuraaasn. Laldaaqvws | wss |
| B.japonicum | AHPKDSAPLR | SVRACAAQF | RRRIRRAHE | ARPTADRROO | RDRRPQ | ADRRCNAGPO | PRSGGRSAR | GFRRLEPDARG | HTRGGAGAGR | ••••• |
| consensus | -HPKI-LP-DLYG-GR | -NSAGLDL-1 | WEHRLA-LS- | ALSAV | V-A-PP- | -A-GPVT | NPAE-KDIVG | -V-RATV- | | W-A |

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. aerogenses* (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.

| P.putida E.coli S.typhimurium K.aerogenes S.meliloti A.tumefaciens R.capsulatus B.japonicum consensus | 710 TPPAERAAILERTA TPPAERAAILERTA TPPQERAAILORAA GRLAERAACLDRAA GRLAERAACLERAA APAATRAAVLORAA HLLESRSA TPERAAIL-RAA | 720 DLMEAEIHPI VLMESQMQQI VLMESQMPTI BLMQARMPTI DLMEAEMPAL DLYEENFGPI HF | 730 MGLLIREAGK IGILVREAGK MGILVREAGK LGLIIREAGK LGLIIREAGK FAALAQEAGK TALLQREGGK | 740 TFPNAIAEU TFSNAIAEV TFSNAIAEV SALNAIAEV SALNAIAEV TLGDAVSEL TLDDALSDV TNAIAEV | 750 REAVDFLRYY, REAVDFLHYY, REAVDFLHYY, REAIDFLRYY, REAIDFLRYY, REAVDFLRYY, REAVDFLRYY, REAVDFLRYY, | 760 VQALNDFSND GQVRDDFANE GQVRDDFDNE GQVRDDFDNE EQTRRTL.G AEGAADT AQGRKLFGSE AGQ-R-DFE | 770 A T T P N TAMPGPTGES | 780 HRPICPV HRPICPV HRPICPV ATPICPI ETPICPV RPPRCAV NALTMRGRCVF HRPLGPV | 790 VCISPWNFPL VCISPWNFPL VCISPWNFPL VCISPWNFPL VAISPWNFPL VAISPWNFPL VAISPWNFPL | 800 AIFIG AIFIG AIFIG AIFIG AIFIG AIFIG AIFIG |
|---|---|--|--|---|---|--|---|---|--|--|
| | 810 | 820 | 830 | 840 | 850 | 860 | 870 | 880 | 890 | 900 |
| P.putida E.coli S.typhimurium K.aerogenes S.meliloti A.tumefaciens R.capsulatus B.japonicum | OVAALAACNEVTA OTAAALAACNEVTA OTAAALAACNEVTA OTAAALAACNEVTA OTAAALAACNEVTA OVTAALAACNEVTA OVTAALAACNEVTA OVTAALAACNEVTA | KPAEOTPLIA KPAEOTPLIA KPAEOTSLIA KPAEOTPLIA KPAEETPLIA KPAEOTPLIA KPAEOTPLIA | AQAVRLLLEA AQGIAILLEA AQGIAILLEA AQGVAILLEA ABGVRILREA AQGVRLLHEA ALAVRLLHQA RGRSPAARGR | GIPEGVLQLI GVPPGVVQLI GVPPGVVQLI GVPPGVIQLI GIPASALQLI GVPQDAVQLI GVPETALQLI HPQERAVSRI | LPGRGETVGA LPGRGETVGA LPGRGETVGA LPGRGETVGA LPGDGR.VGA LPGDGK.TGA HRRPHRRGA | ELVGDERVKGV DLTGDDRVRGV DLTGDDRVRGV ALTSDERVRGV ALUSGALTAGV ALVAGALTAGV ALTRDPRVAGV DRASRHRRRL | MFTGSTEVAR MFTGSTEVAT MFTGSTEVAT MFTGSTEVAR MFTGSTEVAR VFTGSTETAQ HRLDRGRPQH | LLQRNVAGRLI LLQRNIASRLI LLQRNIASRLI LLQRNIASRLI LIQAQIAORLS LIQAQIAORLS LIQAQIAGRVI IIARAMAANLA QRALAARDG | NQGRPIPLIA AQGRPIPLIA AQGRPIPLIA PQGRPTPLIA SPAGRPVPLIA ANGQPVPLIA ANGQPVPLIA SPIVPLIA | ETGGQ ETGGM ETGGM ETGGQ ETGGQ ETGGL ETGGI |
| Compensus | VIANAL-AGN-VIA | KIABQIIDIA | W701-TU-FV | 9454Öm | Droko-1vok | HU-GD-KVKGV | MFIGSIEVA- | EDQKNIK-KD- | -QGKFIFDIF | EIGGM |
| P.putida E.coli S.typhimurium K.aerogenes S.meliloti A.tumefaciens R.capsulatus B.japonicum | 910 NAMIVDSSALTEOV NAMIVDSSALTEOV NAMIVDSSALTEOV NAMIVDSSALAEOV NAMIVDSSALAEOV NAMIVDSSALAEOV NAMIVDSSALAEOV NAMIVDSTALPEOA NAMIADATALPEOV | 920 VIDVVSSAFI VVDVLASAFI VDVLASAFI VIDVLASAFI VGDVITSAFI VADVLASAFI VRDVVASAFI ADDVVTSAFI | 930 SAGQRCSALR SAGQRCSALR SAGQRCSALR SAGQRCSALR SAGQRCSALR SAGQRCSALR SAGQRCSALR | 940 VLCLOEDSAI VLCLOEDSAI VLCLOEDVA VLCLOEDVA VLCLOEDVA ULCLOEDVA ULCLOEDVAI ULFVOEDVAI | 950 DRVIEMLKGAI DHTLEMLRGAI EHTLEMLRGAI DHTLTMLRGAI SPHPDDAEGR DRTLTMLRGAI PHLIGMLRGAI DRMIEMVAGA | 960 MAESRLGCPDR MAECRMGNPGR MAECRMGNPGR ARHCISAAPT LHELRIGRTDS MEELVSGDPAR ARELKIGDPSD | 970 L.AVDIGPVI L.TTDIGPVI L.TTDIGPVI L.TTDIGPVI VFSVDVGPVI L.STDVGPVI V.ATHVGPVI | 980 DAEAKAGTEKE DSEAKANTERE DSEAKANTERE TAEAKANTERE TSEAKONTEKE TAEAKGTIEKE DAEAKAGTETY DVEAKQRLDAE | 990 IIQCMREKGRF IIQTMRSKGRF IIQTMRAKGRF IIQAMRAKGRT IIERMRGLGRK IVDSMRALGHR LAANKARI IIARMKTEARL | 1000 VYQVA VYQAV VYQAV VYQAV VYQAV VYQAV VYQAV UEQIG LEQIS LHRST LHFAGP |
| consensus | NAMIVDSSAL-EQV | V-DVLASAFI | SAGORCSALR | VLCLQEDIA | DH-L-MLRGA | M-E-RMG-P-R | L-TTDIGPVI | D-EAKA-IERE | IIMRA-GR- | - VF Q |
| P.putida E.coli S.typhimurium K.aerogenes S.meliloti A.tumefaciens R.capsulatus B.japonicum | 1010 IADAEIKRGT RENSEDAREWQSGT RENSEDAREWQTGT NENSEDAREWHGT IASETGVGT IAGETGRGT APEGGH AP.EGGH | 1020 FVAPTLIBLD FVAPTLIBLD FVAPTLIBLE FVPPTIBLE FVPPTIBLK FVAPALLQVG FVAPHIFELT | 1030 SFDELKREIF DFAELQKEVF NFAELEKEVF SFDELKKEVF KLSDLQREVF SLADLKEVF GIADLEREIF BAGQLTEEVF | 1040 SPVLHVVRYT SPVLHVVRYT SPVLHVVRYT SPVLHVVRYT SPVLHVIRFF SPVLHLATFF SPVLHLATFF | 1050 WRRNDQLIEG WRNDPBLIEG WRNDPAELIEG WRNEPDKLVEG RDDPDRLVEG RDDPDRLIDJ ABDIPAVIA RPENFERVLR | 1060 21NNSGYGLTI 21NASGYGLTI 21NASGYGLTI 21NASGYGLTI 21NATGYGLTF 21NATGYGLTF AINARGYGLTF AINARGYGLTI | 1070 WHIRIDE II. WHIRIDE II. WHIRIDE II. WHIRIDE II. UHIRIDE II. UHIRIDE II. UHIRIDE II. SUHIRIDE II. SUHIRIDE II. | 1080 AKVVETATPAT AQVTGSAHVGN AQVTGSAHVGN AQVTGSAKVGN AHVTSRIKAGN QHVLSRVAAGN ETVAETIRAGN ZAIIDRVQVGN | 1090 CR.HRNIVGA LYVNRNMVGA LYVNRNMVGA LYVNRNMVGA LYVNRNIIGA LYVNRNIIGA IYVNRNQIGA IYVNRNMIGA | 1100 VVGVQ VVGVQ VVGVQ VVGVQ VVGVQ VVGVQ VVGVQ |
| consensus | A-EGT | FV-PTLIELD | AEL-KEVF | GPVLHVVRY | -R-QLD-LIE | -INASGYGLTL | GVHTRIDETI | A-VS-H-GI | ILYVNRNMVG# | AVVGVQ |
| P.putida B.coli S.typhimurium K.aerogenes S.meliloti A.tumefaciens R.capsulatus B.japonicum | 1110 PFGCEGLSGTGPKA PFGCGLSGTGPKA PFGCEGLSGTGPKA PFGCFGLSGTGPKA PFGCFGLSGTGPKA PFGCFGLSGTGPKA | 1120 GGPIYLYRLI GGPIYLYRLI GGPIYLYRLI GGPIYLGRLV GGPIYLGRVI GGPIYLARFY GGPHYLARFY | 1130 .STRPADAIGR .ANRPPALAV .AHRPPNALNT .SSRPQDAVGV TTAPVPPQHS .QTAPKIDRVA .APEPVVAVGG .TEQTVTINTG | 1140 HFQQQDGEG' TLARQDAKYI TLTRQDARYI TFARQDAERI SV S K | 1150 TPDRTLHEQL PVDAQLKAAL PVDAQLKTTL PVDAQLKTLL HT | 1160 VKPLHGLKAWA TQPLNALREWA LAPLTALTQWA EKPLQALQQWA DPVLLDFAKWL DQAAVDLARWL | 1170 ENNQLADLAA ANRPELQA ADRPALQT AGRPELQA DGKGARAEVE DENGQSVAAE TEAA | 1180 LCSQFASQSQS LCTQYGELAQA LCRQFADLAQA LCQQYSEQAQS AARNAGSSSAI AARQAAALSGI TPILPEARET(.GRRQCCVAGW | 1190 SGIARLLPGPT GTQRLLPGPT GTQRLLPGPT GGLDLELPGPT GGLDLELPGPT JGGVSPGRSVA | 1200 VGERNS VGERNT VGERNT VGERNT VGERNU VGERNV VGELNR |
| consensus | PFGGEGLSGTGPKA | GGPLYL-RLL | PAL | T | | LLW- | | Q-G | GLPGP1 | IGERN- |
| P.putida E.coli S.typhimurium K.aerogenes S.meliloti A.tumefaciens R.capsulatus B.japonicum | 1210 YTILPRENVLCLAD WTLLPRERVLCIAD WTLLPRERVLCLAD LTIMPRERVLCVAD YTLHARGRILLVPA VALHPRGKVLLIPA LTRHQRGPILCLGP GLMLP | 1220 NETDLLAQFA DEQDALTQLA DEQDALTQLA NEQDALIQLA TESGLYHQLA TEQGLYRQLA GAEAASAQAA | 1230 AVLAVGSSAV AVLAVGSQVL AVLAVGSQAL AVLAVGCEVL AALATGNSVA AALATGNSVV AVVALGGQAV | 1240 WVDGEPGKAJ WPDDALHRQI WSDDAFHRDI WPDSALQRDI IDAASGLQAJ IDNASGLEK QASGAVSPKJ | 1250 LRARLPRELQ LVKALPSAVS LAKRLPAAVA LAKKLPREVS SLKNLPQTVG SIYGLPATVT ALETLT | 1260 AKVKLVADWNK ERIQLAKAENT ARVQFAKAETL ERIRFAKAEQL LRVSWSKDWAA SRITWADSWEK | 1270 DEVAFDAVIH TAQPFDAVIF MAQPFDAVIF PVQAFDAVIY DG.PFAGALV SA.PFAGALI PLAGVLW | 1280 HGDSDQLRGV(HGDSDQLRGV(HGDSDKLRTV(HGDSDQLRELC EGDAERIRAV EGDAERIVAIN WGAAEMGRAY | 1290 QQVAKRAGAI EAVAARDGTI EAVAAREGAI EQVAARDGAI KAIAALPGPI IKKIPALPGPI QALAVRPGPI | 1300 IVGVHG IVSVQG IVSVQG IVSVQG ILLVQA IVLVQA IVPLIT |
| consensus | WTLLPR-RVLCIA- | -EQLA | AVLAVGL | | LPV- | -RI | PF-AVI- | -GD-D-LRAL- | VAAR-G-1 | IV-VQG |
| P.putida B.coli S.typhimurium K.aerogenes S.meliloti A.tumefaciens R.capsulatus B.japonicum Consensus | 1310 PARGESNIL PARGESNIL PARGESNIL ASSGEIARNPDAYC ATTEALDRETOPYN ARPD | 1320 LERLVIERAV LERLVIERSL LERLYIERSL LERLVIEVSV LANVAHERHL LERL FR SL | 1330 SVNTAAAGGN SVNTAAAGGN SVNTAAAGGN SVNTAAAGGN CVDTTAAGGN | 1340 ASLMTIG ASLMTIG ASLMTIG ASLMAIG ASLMSIG AALLAG. ASLM-19 | | | | | | |
| -4110-0110 U.D | · | | | | | | | | | |

FIG. 3-Continued.

| P. putida P. fluorescens E. coli S. typhimurium R. typhi H. influenzae B. subtilis H. pylori consensus | 10 20 30 40 50 60 70 80 90 100 MGNPLTITFVIYIAAMVLTGFAAYRATNNLSDYILGGRSLGSVVTALSAGASDMSGWLLMGLPGAIYFAGLSEAWIA IGLTVGAYLNWLFVAGRL MSVSNPTLITFVIYIAAMVLTGHAYRSTNNLSDYILGGRSLGSVVTALSAGASDMSGWLLMGLPGAIYMSGLSESWIA IGLTVGAYLNWLFVAGRL MAISTPMLVTFCVYIFGMILIGFIAWRSTKNPDDYILGGRSLGPVTALSAGASDMSGWLLMGLPGAVFLSGISESWIA IGLTIGAWINWKLVAGRL MAISTPMLVTFCVYIFGMILIGFIAWRSTKNPDDYILGGRSLGPFVTALSAGASDMSGWLLMGLPGAVFLSGISESWIA IGLTIGAWINWKLVAGRL MAISTPMLVTFCVYIFGMILIGFIAWRSTKNPDDYILGGRSLGPFVTALSAGASDMSGWLLMGLPGAVFLSGISESWIA IGLTIGAWINWKLVAGRL MAISTPMLVTFCVYIFGMILIGFIAWRSTKNPDDYILGGRSLGPFVTALSAGASDMSGWLLMGLPGAVFLSGISESWIA IGLTIGAWINWKLVAGRL MAISTPMLVTFCVYIFGMILIGFIAWRSTKNPDDYILGGRSLGPFVTALSAGASDMSGWLLMGLPGAVFLSGISESWIA IGLTIGAWINWKLVAGRL MAISTPMLVTFCVYIFGMILIGFIAWRSTKNPDDYILGGRSLGPFVTALSAGASDMSGWLLMGLPGAVFLSGISESWIA IGLTIGAWINWKLVAGRL MAISTPMLVTFCVYIFGMILIGFIAWRSTKNPDDYILGGRSLGPFVTALSAGASDMSGWLLMGLPGAVFLSGISESWIA IGLTIGAWINWKLVAGRL MAISTPMLVTFCVYIFGMILIGFIAWRSTKNPDDYILGGRSLGPFVTALSAGASDMSGWLLMGLPGAVFLSGISESWIA IGLTIGAWINWKLVAGRL MAISTPMLVTFCVYIFGMILIGFIAWRSTKNPDDYILGGRSLGPFVTALSAGASDMSGWLLMGLPGAVFLSGISESWIA IGLTIGAWINWKLVAGRL MAISTPMLVTFCVYIFGMILIGFIAWRSTKNPDDYILGGRSLGPFVTALSAGASDMSGWLLMGLPGAVFLSGISESWIA IGLTIGAVFNWLLVAGRL |
|--|---|
| COMPANYAS | |
| P. putida P. fluorescens E. coli S. typhimurium R. typhi H. influenzae B. subtilis H. pylori | 110 120 130 140 150 160 170 180 190 200 RVOREHNGDALELPDY SSREEDNSGLURIISAIVELVETTIKCASGLUAGARENESTEGNSVETAUWAGAAATIAVEFVGG FLAVSWETTVOATLEIFA RVOREHNGDALELPDY SSREEDNSGLURIISAIVELVETTIKCASGLUAGARENESTEGNSVETAUWAGAAATIAVEFVGG FLAVSWETTVOATLEIFA RVEREYNNALELPDY STOREDKSREURIISALVELUETTIKCASGLUAGARENESTEGNSVETAUWAGAAATIAVEFVGG FLAVSWETTVOATLEIFA RVEREYNNALELPDY STOREDKSREURIISALVELEFTIKCASGLUAGARENESTEGNSVETAUWAGAAATIAVEFVGG FLAVSWETTVOATLEIFA RVEREYNNALELPDY STOREDKSREURIISALVELEFTIKCASGLUAGARENESTEGNSVETAUWAGAAATIAVEFVGG FLAVSWETTVOATLEIFA RVEREYNNALELPDY STOREDKSREURIISALVELEFTIKCASGLUAGARENESTEGNSVETAUWAGAAATIAVEFVGG FLAVSWETTVOASENIFA RVEREYNNALELPDY STOREDKSREURIISALVELEFTIKCASGLUAGARENESTEGNSVETAUWAGAAATIAVEFIGG FLAVSWETTVOASENIFA RVEREYNNALELPDY STOREDKSREURIISALVELEFTIKCASGLUAGARENESTEGNSVETAUWAGAAATIAVEFIGG FLAVSWETTVOASENIFA RVEREYNNALELPDY STOREDKSREURIISALVELEFTIKCASGLUAGARENESTEGNSVETAUWAGAAATIAVEFIGG FLAVSWETTVOASENIFA RVEREYNNALELPDY STOREDKSREURIISALVELEFTIKCASGLUAGARENESTEGNSVETAUWAGAAATIAVEFIGG FLAVSWETTVOASENIFA RVEREYNNALELPDY STOREDKSREVERIISALVELEFTIKCASGLUAGARENGENSVETAUWAGAAATIAVEFIGG FLAVSWETTVOASENIFA RVEREYNNALELPDY STOREDKSREVERITISALVELEFTIKSSENSGLUAGARENGENSVETAUWAGAAATIAVEFIGG FLAVSWETTVOASENIFA RVEREYNNALELPDY STORE SERVERENTIKKUN SALTIAVEFTIKSSENSGLUAGARENGENSVETAUWAGAAATIAVEFTIKSSENTETVOASENTEN RVEREYNNALELPTY STARVEGGENESSENSGLUKENSENSTENSVETSTAUWAGAAATIAVEFTIGG FLAVSWETTVOASENTEN RVEREYNNALELPTY STARVEGGENESSENSENSENTEN STARVEGGENESTIGUN STARVEGGENESSENSTERENTEN RVEREYNNALELEFTIKSSENSENSENTEN STARVEGGENESSENSENSENTEN STARVEGGENESSENSENSTERENSENSTERENTEN RVEREYNNALELEFTIKSSENSENSENSENSENSENSENSENSENSENSENSENSEN |
| consensus | RVQTEHNGNALTLPDYFSSRFED-SGLLKIISAIVILVFFTIYCASGIVAGARLFESTFGMSYETALWAGAARTIAYTFIGGFLAVSWTDTVQASLMIFA |
| P. putida P. fluorescens E. coli S. typhimurium R. typhi H. influenzae B. subtilis H. pylori | 210 220 230 240 250 260 270 260 270 280 290 300 ILTEVIVLISTGGPDQTFAAIEAVNRRNFDMLKGATFIGIISLMGWGLGYFRFHHLARFNAADSVNSIAKARRISMTXWILCLAGTCAVGFCGI ILTEVIVLISVGGFGDSLEVIKQKSIENVDMLKGLNFVAIISLMGWGLGYFGOPHILARFNAADSHNSIVHARRISMTWWILCLAGAVAVGFFGI ILTEVIVIISVGGFGDSLEVIKQKSIENVDMLKGLNFVAIISLMGWGLGYFGOPHILARFNAADSHNSIVHARRISMTWWILCLAGAVAVGFFGI ILTEVIVIISVGGFSDSLEVIKQKSIENVDMLKGLNFVAIISLMGWGLGYFGOPHILARFNAADSHNSIVHARRISMTWWILCLAGAVAVGFFGI ILTEVIVISVGGFSDSLEVIKQKSIENVDMLKGLNFVAIISLMGWGLGYFGOPHILARFNAADSHNSIVHARRISMTWWILCLAGAVAVGFFGI ILTEVIVISVGGFSDSLEVIKQKSIENVDMLKGLNFVAIISLMGWGLGYFGOPHILARFNAADSHNSIVHARRISMTWWILCLAGAVAVGFFGI ILTEVIVISVGGFSDSLEVIKQKSIENVDMLKGLNFVAIISLMGWGLGYFGOPHILARFNAADSHNSIVHARRISMTWWILCLAGAVAVGFFGI ILTEVIVIVISVGGFSDSLEVIKQKSIENVDMLKGLNFVAIISLMGWGLGYFGOPHILARFNAADSHNSIVHARRISMTWWILCLAGAVAVGFFGI ILTEVIVISISVGFSDSLEVIKQKSIENVDMLKGLNFVAIISLMGWGLGYFGOPHILARFNAADSHNSIVHARRISMTWWILCLAGAVAVGFFGI ILTEVIVISISVGFSDSLEVIKQKSIENVDMLKGLNFVAIISLMGWGLGYFGOPHILARFNAADSHNSIVHARNISMTWWILCLAGAVAVGFFGI ILTEVIVISISVGFSDSLEVIKQKSIENVDMLKGLNFVAIISLMGWGLGYFGOPHILARFNAADSHNSIVHARNISMTWWILCLAGAVAVGFFGI ILTEVIVISISVISTISVISTISSILGVGKGGYFGOPHILARFNAADSVKSLIKARRISMGWVLCLAGAIGIGLFAI VIVFIVAFTHVGGVAPFFHEIDAVNPHLLDIFKGASVISIISVIAWGLGYFGOPHILARFNAADSVKSLIKARRISMGWVLCLAGAIGIGLFAI |
| consensus | $\tt liltpvivlisvggvgQsfaaiea-nrrnvdmlkgltfigiislmgwglgyfgQphilarfmaadsvnsiv-arrismtwmilclagavavgffgiislmgwglgyfgQphilarfmaadsvnsiv-arrismtwmilclagavavgffgiislmgwglgyfgQphilarfmaadsvnsiv-arrismtwmilclagavavgffgiislmgwglgyfgQphilarfmaadsvnsiv-arrismtwmilclagavavgffgiislmgwglgyfgQphilarfmaadsvnsiv-arrismtwmilclagavavgffgiislmgwglgyfgQphilarfmaadsvnsiv-arrismtwmilclagavavgffgiislmgwglgyfgQphilarfmaadsvnsiv-arrismtwmilclagavavgffgiislmgwglgyfgQphilarfmaadsvnsiv-arrismtwmilclagavavgffgiislmgwglgyfgiislmgwglgyfgQphilarfmaadsvnsiv-arrismtwmilclagavavgffgiislmgwglgyfgQphilarfmaadsvnsiv-arrismtwmilclagavavgffgiislmgwglgyfgiislmg$ |
| P. putida P. fluorescens E. coli S. typhimurium R. typhi H. influenzae B. subtilis H. pylori consensus | 310 320 330 340 350 350 360 370 380 390 400 AM FSAHPELAGPVSENHRKVFTELAK IJENEWVACVLSATLAAVMITISCOLIVCSSALTENPIKAFLRKNSQVELVWVCRLMVLAVALIAIAMANP AM FSAHPEVAGPVTENPERVFTELAQIJENEWVACVLSATLAAVMITISCOLIVCSSALTENPIKAFLRKGSQRELVWVCRLMVLVALIAIAMANP AM FNDHPALAGAVNQNAERVFTELAQIJENEWIACILSATLAAVMSTISCOLIVCSSALTENPIKAFLRKGSQRELVWVCRVMVLVVALIAIAMANP AM FNDHPALAGAVNQNAERVFTELAQIJENEWIACILSATLAAVMSTISCOLIVCSSALTENPIKAFLRKASQKELVWVCRVMVLVVALIAIALAANP AM FNDHPALAGAVNQNAERVFTELAQIJENEWIACILSATLAAVMSTISCOLIVCSSALTENPIKAFLRKHASQKELVWVCRVMVLVVALIAIALAANP AM FNDHPALAGAVNQNSERVFTELAQIJENEWIACILSATLAAVMSTISCOLIVCSSALTENPIKAFLRKHASQKELVWVCRVMVLVVALIAIALAANP AM FNDHPALAGAVNQNSERVFTELAQIJENEWIACILSATLAAVMSTISCOLIVCSSALTENPIKAFLRKHASQKELVWCRVMVLVVALIAIALAANP AM FNDHPALAGAVNQNSERVFTELAQIJENEWIACILSATLAAVMSTISCOLIVCSSALTENPIKAFLRKHASQKELVWCRVMVLVALIAIALAANP AM FNDHPALAGAVNQNSERVFTELAQIJENEWIACILSATLAAVMSTISCOLIVCSSATTENPIKAFLRKASQQELVWVCRVMVLVALIAIAANP AM HKFGVAVKDPENIFIIFSKIJEHELITERLISATLAAVMSTISCOLIVCSSATTENPIKAFLRNASSKELVWCRVMVCRVMVLVALIAIAANP AM HKFDLSLEDPEKIFTVMSQLENEWITETILSATLAAVMSTISCOLIVCSSATTAEDIMASFFRNASSKELVWCRVMVLVALIAAVMSTISCOLIVCSSATTAEDIMASFFRANSSKELVWCRVMVLVALIAIAMANP |
| COMPENSED | |
| P. putida P. fluorescens E. coli S. typhimurium R. typhi H. influenzae B. subtilis H. pylori | 410 420 430 440 450 460 470 480 490 500 ENRVIGLVAVANAGFGAAFGPVVIISVIÄKGITRNGALAGIVVGALTVILWKNFDTLGLVEIIPG5IPASLAIVLVSKLG.SPSQTMVKR5EAAD ENRVIGLVSVANAGFGAAFGPVVIISVIÄKATTRNGALAGIIVGATTVILWKNFBLGLVEIIPG5IPASLAIVFVSKAG.APTLGAVER5DAAE ENRVIGLVSVANAGFGAAFGPVVIFSVMSRNTRNGALAGNIIGALTVILWKNFGULGLVEIIPG5IFASLAIVFVSKAG.APTLGAVER5DAAE ENRVIGLVSVANAGFGAAFGPVVIFSVMSRNTRNGALAGNIIGALTVILWKNFGULGLVEIIPG5IFGSIGIVVFSLLGKAPSAAMQKR5AEAD DNRVIGLVSVANAGFGAAFGPVVIFSVMSRNTRNGALAGNIIGALTVILWKNFGULGLVEIIPG5IFGSIGIVVFSLLGKAPSAAMQKR5AEAD DNRVIGLVSVANAGFGAAFGPVVIFSVMSRNTRNGALAGNIIGAVTVIVWKQYGWLGLVEIIPG5IFGSIGIVVFSLLGKAPSAAMQKR5AEAD DNRVIGLVSVANAGFGAAFGPVVIFSVMSRNTRNGALAGNIIGAVTVIVWKQYGWLGLVEIIPG5IFGSLGIVIFSLLGKAPSAAMQKR5AEAD NSKVIKLVEFANAGFGSAFGPVVIFSVMSRNTRNGALAGNIVGAVTVFAWKEVVPADTDWFKVFSNIFG5AFASLAIIVISLLSNKPEQDILNTDAKAE NSKVIKLVEFANAGFGSAFGPVVIFSLPWKNTSSGAMAGMLVGAVTVFAWKEVVPADTDWFKVFSNIFG5AFASLAIIVISLLSNKPEQDILNTDAKAE NSTIDDVGYAWAGFGSAFGPAIJLSLYWKNNWKGALAANIVGAATVLIWITTGLAKSTGVEIIPGFLSMIAGIVGMITKRPAKASYRLGVME NASILSIVSYAWAGFGASFGSVIFSLF9LFWSNTRIGATAGMLSGASTVILWKFFKSFLDIVFILOFFLSMIAGIVSVAVAFSLFS.SVRSGKEAAFTML |
| consensus | ENRVLGLVSYAWAGFGAAFGPVVLFSVLW-RMTRNGALAGMVVGALTVILWKNFD-LGLYEIIPGFIFASIAIVLVSLLG-SPSQGMVERFEEAD |
| P. putida P. fluorescens E. coli S. typhimurium R. typhi H. influenzae B. subtilis H. pylori consensus | 510 AAYHADKX KDYNLNK AHYHSAPPSRLQES AHYHSAPPSRLQES A KAYKEAK KLLKRKK KEIESLKH |

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_{nutP} ::'lacZ | Succinate | 700 ± 50 |
| pan | Succinate plus proline | $2,800 \pm 100$ |
| $P_{nutA}::'lacZ$ | Succinate | 350 ± 30 |
| pull | Succinate plus proline | $6{,}950\pm100$ |
| Mutant S14D2 | | |
| P_{nutP} ::'lacZ | Succinate | $2,700 \pm 200$ |
| pair | Succinate plus proline | $2,600 \pm 150$ |
| $P_{putA}::'lacZ$ | Succinate | $7,900 \pm 200$ |
| paul | Succinate | $8,600 \pm 250$ |

^{*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_{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

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 20 + 10 |
| P_{putP} ::'lacZ | Corn exudate | 20 ± 10 270 ± 50 |
| | M8 | 60 ± 10 |

^{*a*} *P. putida* KT2442 cells bearing pMIS5 (P_{putA} ::'*lacZ*) or pMIS12 (P_{putB} ::'*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.

<u>_____putP</u>

| | 10 | 20 | 30 | 40 | 5 C |
|--|--|---|---|--|--|
| E.coli | CATCTAAAGTCT | CAAAAAATTAT | T.ATCGGCA | ATGTCGAAA | CTTGCCCI |
| S.typhimurium | CATCAAGGTTCT | CAAATTTAT | TTATTGGCA | CTACCGTAG | CGTGCCG1 |
| K.aerogenes | CATGCAAAGTCT | CAGA ATTTT | TT.ATCGACA | GCGTCCAAC | TTCGTCGT |
| P.putida | CATGC | G. | | (| CTACCO |
| | | | | | |
| | 60 | 70 | 0.0 | 0.0 | 100 |
| T and | 00 mamonooa | 00000000000 | 00 | 90 | 100 |
| E.COII | TA. TATCIGUCA | | GTAACAGAG | TTTATCTTT | L.ACCAGC |
| S.typhimurium | T. TTTGTACCC | IGC CG G AAC GTO | GCAAGGGTG | TT.A TG T TT | ACCAGG |
| K.aerogenes | T TTTTTCCAC | PAC CG G AAC GGG | GTAATGGCG | CA.T TG T TT | AATTGC |
| P.putida | TTGTTTTTATCA | F.G CGCAAC C | ACACCG | TTGG TG G TT G | GCACCCAG |
| | | | | | |
| | 110 | 120 | 130 | 140 | 150 |
| E.coli | GCGACCGTATCC' | ROCCOGRAAGCOC | TCCTTATTC | ACA ATCOAT | TAACACA |
| S.tvohimurium | GCGACCGTATCC | CCCCCCAAACCCC | TCOTTATIC | ACANTCONT | PTAACACA |
| K aerogenes | GCGACCGTATCC | CACCCAACCUT | | ACADACCAR | |
| P nutida | CCCC CANECC | DACCOMMOCIL | CO CO CO | ACAAACGAI. | I INACACA |
| ripucida | GCGGC . GAATGC | L'ATGUAA CAA | | AAAGAAGGT | GAAC . CA |
| | | | | | |
| | 160 | 170 | 180 | 190 | 200 |
| E.CO11 | CCA TT TACATTA | ATTT.TAGT | GCTCAGCGA | CACT ATTT TI | CATCAGG |
| S.typhimurium | CCATTTACATCA | ATTT.GATT | GCAGAGGGA | TACT ATTT TI | CTCCAGG |
| K.aerogenes | GCATTTACACGA | AAACCGACGGC | GCAAAACGT | CA ATTTGI | . TAGAGG |
| P.putida | TTT TT CTTTTGC | AGTTGCAC | . CTGCCCGT | GC ATTTTC | CGACAAA |
| - | | | | | |
| | 210 | 220 | 220 | 240 | 250 |
| E coli | TTCC ACTCTC | | COOMMOON | 240 | 250 |
| C trochimumium | TIGC. ACICICI | CACATTTTTG | CGGTTGCA. | | TTTCAAA |
| s.cypninuriun | TIGCC. ACTUTC | CACATITTT. | CGGTTGCA. | · · · · · cc · · · · | TTTTAAA |
| K.aerogenes | TTGCACTCTC | CACGTT TTT AA | AGGTTGCA. | CC | AAAC A AA |
| P.putida | GCCCACT . TCI | TGCTCCTTT | C GG GA GCA A. | AAAA CC GCTT | TTTCAGA |
| | | | | | |
| | | | | | |
| | 260 | 270 | 0.00 | 0.00 | |
| | | 270 | 28U | 290 | 300 |
| E.coli | AATGTTAACTGC | GCAGAGAAA | 280 AAG | 290 T C TG, AGTTA | 300 տր դորդ որդ |
| E.coli S.tvphimurium | AATGTTAACTGCC AGTGTTAACTACC | GCAGAGAAA | 280 AAG | 290 FCTG.AGTTA FCC AGATC | 300 TT TTT TT CT TTT TCT |
| E.coli S.typhimurium K.aerogenes | AATGTTAACTGCC AGTGTTAACTACC AGTGTTAACTACCAC | CCAGAGAAA | 280 AAG' ATAAA' | 290 FCTG.AGTTA FCC.AGATG | 300 TT TTT TT GT TTT CT |
| E.coli S.typhimurium K.aerogenes P.putida | AATGTTAACTGCC AGTGTTAACTACC AGTGTTAACTCAC | CCAGAGAAA CCAGAGAAA CCATACAAATA | AAG ATAAA .CCCTATGAG | 290 ICTG.AGTTA ICC.AGATG CCCGGGGGTTA | 300 TT TTT TT GT TTT CT AA TTT CT |
| E.coli S.typhimurium K.aerogenes P.putida | AATGTTAACTGCC AGTGTTAACTACC AGTGTTAACTAAC ATAAATC | CCCAGAGAAA CCCAGAGAAA CCCAGAGAAAA CCCATACAAATA | 280 AAG ATAAA .CCCTATGAG | 290 ICTG.AGTTA ICC.AGATG CCCGGGGTTA CCC | 300 ATT TTT TT GT TTT CT AA TTT CT TTT |
| E.coli S.typhimurium K.aerogenes P.putida | AATGTTAACTGCC AGTGTTAACTACCACC AGTGTTAACTCACCACCACCACCACCACCACCACCACCACCACC | CCAGAGAAA CCAGAGAAA CCATACAAATA CCACCGAAA | AAG ATAAA .CCCTATGAG | 290 ICTG.AGTTA ICC.AGATG CCCGGGGGTTA CCCC | 300 TT TTT TT GT TTT CT AA TTT CT TTT |
| E.coli S.typhimurium K.aerogenes P.putida | AATGTTAACTGCC AGTGTTAACTACC AGTGTTAACTCAC ATAAATC | ZOCAGAGAAA CCCAGAGAAA CCCAGAGAAA CCCACCGAAA | AAG ATAAA .CCCTATGAG | 290 FCTG.AGTTA FCC.AGATG CCCGGGGGTTA CCCC | 300 TT TTT TT GT TTT CT AA TTT CT TTT |
| E.coli S.typhimurium K.aerogenes P.putida | AATGTTAACTGCC AGTGTTAACTACC AGTGTTAACTCAC ATAAATC 310 | CCCACACAAA CCCACACAAA CCCACACAAAATA CCCACCCGAAA | AAG' ATAAA' .CCCTATGAG GCG(| 290 rctg.agtta rcc.agato cccggggtta cccc 340 | 300 TTTTTTT GTTTTCT AATTTCT TTT 350 |
| E.coli S.typhimurium K.aerogenes P.putida E.coli | AATGTTAACTGCC AGTGTTAACTACC AGTGTTAACTCAC ATAAATC 310 .CCCTGTCATA | GCACCGAAA GCATACAAATA GCACCGAAA GCACCGAAA 320 .TCGATT.TCT | A.AG ATAAA .CCCTATGAG GCG GCG | 290 TCTG.AGTTA TCC.AGATC CCCGGGGTTA CCCC 340 ATTTCATT | 300 TTTTTTT GGTTTTCT MAATTTCT TTT 350 CATTTTT |
| E.coli S.typhimurium K.aerogenes P.putida E.coli S.typhimurium | AATGTTAACTGCC AGTGTTAACTACC AGTGTTAACTACC ATAAATC 310 .CCCTGTCATACGTCAGACA | CCACAGAGAAA CCACAGAGAAA CCCATACAAATA CCCACCGAAA 320 TCGATT.TCT ACTCCCTTATCT | A.AG ATAAA .CCCTATGAG GCG GCG TTTATTAAC TTTTTTAAC | 290 PCTG.AGTTA PCC.AGATG CCCGGGGTTA CCCC <u>340</u> A.TTTCATT A.ATTCATT | 300 TT TTT TT GT TTT CT AA TTT CT TTT <u>350</u> CATT T TT CATT T TT |
| E.coli S.typhimurium K.aerogenes P.putida E.coli S.typhimurium K.aerogenes | AATGTTAACTGCC AGTGTTAACTACC AGTGTTAACTCAC AGTGTTAACTCAC ATAAATC 310 .CCCTGTCATA CGTCAGACZ GCTATTTTAGG | 220 CCACAGAAA CCACAGAAAA CCATACAAATA CCACCCGAAA | A.AG' ATAAA' GCCTATGAG GCG' GCG' | 290 TCTG.AGTTA TCC.AGATG CCCGGGGTTA CCCC 340 ATTTCATT AAATTAGC | 300 ATTTTTT GATTTCT AATTTCT TTT 350 CATTTTT CATTTTT CATTTTCA |
| E.coli S.typhimurium K.aerogenes P.putida E.coli S.typhimurium K.aerogenes P.putida | AATGTTAACTGCC AGTGTTAACTACC AGTGTTAACTACC AGTGTTAACTACC AGTGTTAACTACC 310 .CCCTGTCATA GCCATGTCATAA GTCAGACC GCTATTTAGG | 220 CCACAGGAAA CCCAGAGAAA CCCATACAAATA CCACCGAAA 320 TCGATT.TCT CCTCCTTATCT GTT G.TA | 280 A.AG GCG GCG GCG TTTATTAAC TTTTTAAC ATTTTTAAC GCGAAT | 290 TCTG.AGATG TCC.AGATG CCCGGGGTTA CCCC 340 ATTTCATT A.AATTCATT A.AATTCAG AGCTGTCGGA | 300 TTTTTTT GTTTTCT AATTTCT TTT 350 CATTTTT CATTTTTCA CGATTTTCA CGAGTTG |
| E.coli S.typhimurium K.aerogenes P.putida E.coli S.typhimurium K.aerogenes P.putida | AATGTTAACTACC AGTGTTAACTACC AGTGTTAACTACC ATAAAT 310 TAAAT GCCATGTCATA GCCATGTCATA TCACG | 220 CCAGAGGAAA CCAGAGAAAA CCATACAAATA CCATACAAATA CCCATCGAAA 320 TCGATT.TCT CCCCTTATCT GTA | 280 A.AG ATAAA CCCTATGAG GCG | 290 TCTG.AGTTA TCC.AGATC CCCGGGGTTA CCCC <u>340</u> ATTTCATI A.ATTCATI A.AATTAGG AGCTGTCGGA | 300 TTTTTTT GATTTTCT AATTCT AATTTCT TTT 350 CATTTTT CATTTTT CATTTTCA .CGAGTTG |
| E.coli S.typhimurium K.aerogenes P.putida E.coli S.typhimurium K.aerogenes P.putida | AATGTTAACTGCC AGTGTTAACTACC AGTGTTAACTACC ATAAAT.C 310 .CCCTGTCATA. .CGTCAGACC GCTATTTAGG. | 200 CCAGAGAAA CCAGAGAAA CCAGAGAAA CCACAGAAA CCACCGAAA 320 | 280 | 290 TCTG.AGTTA PCC.AGATC CCCGGGGTTA CCCC 340 A.TTTCATT A.ATTCATT A.ATTCATT A.ATTAGC AGCTGTCGGA | 300 TTTTTTT GTTTTTCT NAATTTCT TTT 350 CATTTTT CATTTTT CATTTTCA .CGAGTTG |
| E.coli S.typhimurium K.aerogenes P.putida E.coli S.typhimurium K.aerogenes P.putida | AATGTTAACTGCC AGTGTTAACTACC AGTGTTAACTACC ATAAATC 310 .CCCTGTCATA .CGTCAGACC GCTATTTTAGG TCAGG 360 | CCAGACARA CCAGACARA CCATACARATA CCATACARATA CCACCGARA 320 TCGATT.TCT CCCCCTTATCT GTT 370 | 280 A.AG GCCTATGAG GCG TTTATTAAC ATTTTTAAC ATTTTTAAC TGGGAAT | 290 TCTG.AGTTA TCC.AGTTA CCCGGGGGTTA CCCGGGGGTTA CCCC 340 ATTTCATT AATTCATT AAATTAGC AGCTGTCGGA | 300 TTTTTTT GTTTTCT AATTTCT AATTTCT TTT 350 CATTTTT CATTTTT CATTTTTCA .CGAGTTG 400 |
| E.coli S.typhimurium K.aerogenes P.putida E.coli S.typhimurium K.aerogenes P.putida E.coli | AATGTTAACTGCC AGTGTTAACTACC ACTGTTAACTACC ATAAAT.C 310 .CCCTGTCATA CGTCAGACZ GCTATTTAGG TCAGG. 360 | 200 CCAGAGAAA CCAGAGAAA CCATACAAATA CCACCGAAT CCACCGAAT CCCCCCTTATCT G.TA 370 | 280 | 290 TCTG.AGTTA PCC.AGTTA CCCGGGGGTA CCCGGGGGTA CCCC 340 ATTTCATT AATTCATT AATTAGC AGCTGTCGGA 390 | 300 TTTTTTT GTTTTTCT MAATTCT MAATTCT MAATTCT MAATTTCT 350 CATTTTT CATTTTT CATTTTCA CGACTTG 400 |
| E.coli S.typhimurium K.aerogenes P.putida E.coli S.typhimurium K.aerogenes P.putida E.coli | AATGTTAACTACC AGTGTTAACTACC AGTGTTAACTACA ATAAAT.C 310 .CCCTGTCATA GCTATTTTAGC. TCAGG. 360 AAGCTTCCTACCA | 200 CCCACACARA CCCACACARA CCCACCGARA 320 | 280 | 290 TCTG.AGTTA TCC.AGATC CCCGGGGTTA CCCC .340 ATTTCATI AATCATI AATTAGC AGCTGTCGGA 390 CCCCAAAGTT | ATTTTT AATTTCT AATTTCT AATTTCT AATTTCT ATTTCA CATTTTT CATTTTCA CGAGTTG 400 GCAAC |
| E.coli S.typhimurium K.aerogenes P.putida E.coli S.typhimurium K.aerogenes P.putida E.coli S.typhimurium | AATOTTAACTACC AGTOTTAACTACC AGTOTTAACTACC AGTOTTAACTACC AGTOTTAAAT 310 TAAAT GCCATGTCATA GCCATGTCATACG 360 AAGCTGCCTACGC AAGCTTGCAACGC | 200 200 200 200 200 200 200 200 | 280 | 290 TCTG.AGTTA TCC.AGATC CCCGGGGTTA CCCC.AGATC CCCC.AGATC 340 A.ATTACATT A.ATTACATT A.ATTACC AGCTGTCGGA 390 GCACAAGTT GCACA.GTT | JUG TTTTTT GTTTTTT GTTTTTT AATTTCT TTT 350 CATTTTT CATTTTT CATTTTT CATTTTCA CGAGTTG 400 GCAAC GCAAC |
| E.coli S.typhimurium K.aerogenes P.putida E.coli S.typhimurium K.aerogenes P.putida E.coli S.typhimurium K.aerogenes | AATGTTAACTGCC AGTGTTAACTACC AGTGTTAACTACC ACTGTTAACTACC 310 .CCCGGTCATA CGTCAGACZ GCTATTTTAGG 360 AAGCTGCTACCTACCACG CGGCTTCCGTCACC | CCAGAGAAA CCAGAGAAA CCAGAGAAA CCAGAGAAAAAA CCAGGAAAAAAAAAA | 280 | 290 TCTG.AGTTA TCC.AGATA CCCGGGGGTTA CCCC 340 ATTTCATT AATTCATT AATTCATA A.CATCATA ACCTGTCGGA 390 GCACAA.GTT GCACAA.GTT GCACAA.GTT | ATTTTTT GTTTTCT AATTTCT AATTTCT CATTTTC CATTTTC CATTTTC CATTTTC CATTTTCA CGAGTTG 400 GCAAC GCCAC |
| E.coli S.typhimurium K.aerogenes P.putida E.coli S.typhimurium K.aerogenes P.putida E.coli S.typhimurium K.aerogenem K.aerogenem F.putida | AATOTTAACTGCC AGTOTTAACTACC AGTOTTAACTACC ATAAATC 310 .CCCTGTCATA .CGCTGTCATA .CGCTGTCATA 360 AAGCTTGCTACGC AAGCTTCCACGC CACCTTTCAC | ICCACAGAAA ICCATACAAAAA ICCATACAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA | 280 | 290 TCTG.AGTTA TCC.AGATTA CCCCGGGGTTA 340 A.TTTCATT A.ATTAGTA AGCTGTCGGA 390 GCACAAAGTT GCACAA.GTT GCACAAGTT | GTTTTCT GTTTTCT AATTTCT TTT 350 CATTTTC CATTTTT CATTTTT CATTTTT CATTTCA CGAGTTG GCAAC GCAAC GCAAC |
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putA

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.

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.

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 exudates 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-Tn5 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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