Divergent Structure and Regulatory Mechanism of Proline Catabolic Systems: Characterization of the *putAP* Proline Catabolic Operon of *Pseudomonas aeruginosa* PAO1 and Its Regulation by PruR, an AraC/XylS Family Protein

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Pseudomonas aeruginosa PAO1 utilizes proline as the sole source of carbon and nitrogen via a bifunctional enzyme (the *putA* gene product) that has both proline dehydrogenase (EC 1.5.99.8) and pyrroline 5-carboxylate dehydrogenase (EC 1.5.1.12) activities. We characterized the *pruR-putAP* loci encoding the proline catabolic system of this strain. In contrast to the *putA* and *putP* (encoding proline permease) genes of other gram-negative bacteria, which are located at divergent or separate loci, Northern blotting demonstrated that the two genes form an operon in strain PAO1. While the phylogenetic lineage of the PutP protein of strain PAO1 was related to that of the origin (80% identity to the *P. putida* counterpart), PutA of PAO1 (PutA_{PAO}) was rather distantly related (47% identity) to the *P. putida* counterpart. Moreover, unlike the PutA proteins of *P. putida* and enteric bacteria, PutA_{PAO} appeared to lack a regulatory function. Upstream of the *putAP* operon, the divergent PA0781 gene specified a hypothetical outer membrane protein with a molecular weight of 74,202. This gene appeared to be dispensable for proline utilization as indicated by the normal growth of a knockout mutant of PA0781 on medium containing proline. The *pruR* (proline utilization regulator) gene immediately upstream of PA0781 encoded a transcriptional activator of the AraC/XylS protein family and mediated the proline-responsive expression of *putAP*. Primer extension studies identified a PruR-dependent promoter responsive to proline in the 5'-flanking region of *putA*. Thus, the proline utilization system of *P. aeruginosa* differs from that of *P. putida* with respect to *putA* structure, the organization of the *putAP* genes, and the regulatory mechanism of *putA* expression.

A proline catabolic enzyme of *Sinorhizobium meliloti* contributes to root colonization as well as to the establishment of a symbiotic interaction with roots (15, 16, 42). Proline catabolic enzymes also participate in the utilization of octopines and nopalines by *Agrobacterium tumefaciens* (5) and in the development of nodules induced by *S. meliloti* (15, 16, 42). Since proline is extruded along with other organic compounds from plant roots into soil at concentrations sufficient to induce a proline catabolic enzyme (1, 3, 44), root-colonizing bacteria may use proline for growth and sustenance in the rhizosphere.

Root-colonizing fluorescent pseudomonads such as *Pseudomonas aeruginosa*, *P. fluorescens*, and *P. putida* efficiently utilize proline as the sole source of carbon and nitrogen (25, 37, 44). In *P. aeruginosa* PAO1 and *P. putida*, proline is catabolically converted, as in other gram-negative bacteria, into glutamate by a bifunctional catabolic enzyme (encoded by the *putA* gene) that has proline dehydrogenase (ProDH; EC 1.5.99.8) and Δ^1 -pyrroline-5-carboxylate dehydrogenase (P5CDH; EC 1.5.1.12) activities. The resulting glutamate is subsequently channeled into the tricarboxylic acid cycle via 2-ketoglutarate (23, 24, 30, 44). In addition to the catalytic function, PutA proteins of *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, and

P. putida control their expression and that of the divergent *putP* gene (encoding a proline permease) by acting as a repressor in the absence of proline (2, 23, 27, 40, 44). The PutA of *S. meliloti* also autoregulates its synthesis (36); the *putP* gene of this strain has not been identified but appears to be located separately from *putA* (4). Proline abolishes the repressive effect of PutA on *putAP* expression by preventing its binding to the control sites of the promoters (2, 27, 40). A leucine zipper-like sequence of the C-terminal region has been proposed as a DNA-binding motif interacting with the control sites, and ProDH activity and PutA phosphorylation are associated with the DNA-binding activity (2, 20, 27). In contrast, *A. tumefaciens*, *Rhodobacter capsulatus*, and probably *Bradyrhizobium japonicum* use another mechanism to regulate *putA* expression. In these bacteria, the PutR protein of an Lrp-type transcriptional activator essentially controls *putA* expression with proline as a coinducer (4, 17, 39). Thus, gene organization and the regulatory mechanism of *putA* differ within the family *Rhizobiaceae* (36).

P. aeruginosa PAO6087 (*pru::Tn5-751*) has been isolated from *P. aeruginosa* PAO6049, a derivative of strain PAO1, as a mutant that is unable to utilize proline as a source of carbon and of nitrogen (33). Sequence analysis in this study revealed that the *pru::Tn5-751* gene encodes an AraC/XylS-family regulatory protein (9, 22, 41) and that it is located close to the *putAP* genes. In this system, the *putA* and *putP* genes constitute an operon and PruR mediates the proline-responsive expression of this operon. The PutA protein of strain PAO1 appears

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant phenotypes or genotypes ^a	Reference or source
Strains		
<i>P. aeruginosa</i>		
PAO1	Wild type	38
PAO4484	<i>ntrC::FRT</i>	30
PAO4502	<i>putA::FRT</i> -Gm	This work
PAO4503	<i>putP::ΩSp</i> /Sm	This work
PAO4517	PA0781::ΩSp/Sm	This work
PAO4528	<i>putA::FRT</i>	This work
PAO4534	<i>purR::FRT</i> -Gm <i>putA::FRT</i>	This work
PAO4547	<i>putA::ΩSp</i> /Sm	This work
PAO6087	<i>pruR::Tn5-751</i> (= <i>pru::Tn5-751</i>) <i>met-9011</i> <i>amiE200</i> <i>strA</i>	33
<i>E. coli</i>		
DH5α	F [−] /endA1 <i>hsdR17</i> (r _k [−] m _k ⁺) <i>supE44</i> <i>thi-1</i> <i>recA1</i> <i>gyrA</i> <i>relA1</i> Δ(<i>lacI</i> ZYA-argF)U169 <i>deoR</i> [φ80dlacΔ(<i>lacZ</i>)M15]	Bethesda Research Laboratories
HB101	<i>supE44</i> <i>hsdS20</i> (r _B [−] m _B [−]) <i>recA13</i> <i>ara-14</i> <i>proA2</i> <i>lacY</i> <i>galK2</i> <i>rpsL20</i> <i>xyl-5</i> <i>mtl-1</i>	35
XL1-Blue	<i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>hsdR17</i> <i>supE44</i> <i>recA1</i> <i>lac</i> [F', <i>proAB</i> <i>lacI</i> ^a ZΔM15 Tn10 (Tc ^r)]	Stratagene
Plasmids		
pBluescript II KS(+)	Ap ^r , ColE1 replicon	Stratagene
pEX18Ap	Ap ^r , ColE1 replicon, <i>oriT</i> <i>sacB</i>	12
pFLP2	Ap ^r , ColE1 replicon, <i>Flp</i> <i>oriT</i> <i>sacB</i>	12
pHP45ΩSp/Sm	Ap ^r , ColE1 replicon, ΩSp/Sm	8
pME6015	Tc ^r , pACYC177 and pVS1 replicons, ' <i>lacZ</i>	S. Heeb
pNIC6011	Ap ^r Cb ^r , pACYC177 and pVS1 replicons, <i>mob</i>	30
pNIT6012	Tc ^r , pACYC177 and pVS1 replicons, <i>mob</i>	30
pPS858	Ap ^r Gm ^r , ColE1 replicon carrying a <i>FRT</i> -Gm ^r cassette	12
pRK2013	Km ^r , ColE1 replicon, <i>tra</i> (RK2)	6
pUC118	Ap ^r , ColE1 replicon	43
pYI358	Ap ^r Cb ^r , pNIC6011 derivative carrying a 5.9-kb 'PA0779- <i>pruR</i> -PA0781- <i>putA</i> ' region	This work
pYI366	Ap ^r Km ^r , a pBluescript II KS(+) derivative carrying a 8.0-kb ' <i>pruR</i> -PA0781- <i>putA</i> - <i>putP</i> ' region	This work
pYI367	Ap ^r , pEX18Ap derivative carrying the 4.3-kb <i>EcoRI</i> - <i>KpnI</i> <i>putA</i> fragment from pYI366	This work
pYI368	Ap ^r Gm ^r , pYI367 derivative having the <i>FRT</i> -Gm ^r cassette from pPS858	This work
pYI369	Tc ^r , pNIT6012 derivative having the 3.8-kb <i>KpnI</i> - <i>XhoI</i> fragment of the PA0781- <i>putA</i> region	This work
pYI370	To ^r , pNIT6012 derivative carrying the <i>putA</i> -His ₆ fusion as a <i>KpnI</i> - <i>XhoI</i> fragment	This work
pYI378	Tc ^r , pME6015 derivative carrying the <i>putA</i> '-' <i>lacZ</i> fusion	This work
pYI388	Ap ^r , pEX18Ap derivative carrying the 3.5-kb <i>XhoI</i> - <i>PstI</i> fragment containing <i>putP</i>	This work
pYI389	Ap ^r , pYI388 derivative carrying the <i>FRT</i> -Gm ^r cassette on <i>putP</i>	This work
pYI415	Ap ^r , pEX18Ap derivative carrying the 2.6-kb <i>EcoRI</i> fragment containing PA0781	This work
pYI416	Ap ^r Sp ^r Sm ^r , pYI415 with an insertion of ΩSp/Sm at the <i>BbsI</i> site on PA0781	This work
pYI425	Ap ^r , pEX18Ap derivative carrying 2.2-kb <i>XhoI</i> - <i>SalI</i> fragment containing <i>pruR</i>	This work
pYI426	Ap ^r Gm ^r , pYI425 derivative carrying the <i>FRT</i> -Gm ^r cassette on <i>pruR</i>	This work

^a Ap, ampicillin; Cb, carbenicillin; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline; Tp, trimethoprim; Ω, omega interposon (8); *FRT*, Flp recombinase target (12).

to lack a repressor function and is distantly related to the *P. putida* counterpart in terms of its primary structure.

MATERIALS AND METHODS

Strains, plasmids, and media. The strains and plasmids used in this study are listed in Table 1. *E. coli* and *P. aeruginosa* PAO strains were grown in Luria-Bertani medium (35) and nutrient yeast-extract medium or minimal medium P (MMP) supplemented with 20 mM (each) carbon and nitrogen sources (10). Antibiotics were added as described when necessary (12).

Cloning of the *pruR*-*putAP* region. Strain PAO6087 (*pru::Tn5-751*) was isolated as a mutant unable to utilize proline (Pru[−] phenotype) as a source of carbon and nitrogen from a mutant library of *P. aeruginosa* PAO6049 (*met-9011* *amiE200* *strA*). This library was constructed using the transposon Tn5-751 (34). To clone an allele of *pru::Tn5-751*, we constructed a library of the partially digested (5- to 10-kb) *Sau3AI* chromosomal DNA of *P. aeruginosa* PAO1 by using the *E. coli*-*Pseudomonas* shuttle vector pNIC6011 in *E. coli* XL1-Blue as described previously (11, 28). We then conjugated library plasmids into strain PAO6087 (*pru::Tn5-751*) from *E. coli* XL1-Blue (28, 30). Among several Pru⁺ transconjugants selected on MMP agar supplemented with 20 mM proline, we selected one transconjugant harboring plasmid pYI358 with an insert of a 5.9-kb chromosomal DNA segment (Fig. 1) for further analysis. We cloned the PAO871-*putA* region downstream of *pruR* as a 8.0-kb *aph* (Km^r)-*putP* fragment into the

plasmid pBluescript II KS(+) (Stratagene) from the chromosomal DNA of strain PAO6087 by using restriction endonucleases *Bam*HI and *Ban*III, which cut the transposon around the center and cut *putP* at nucleotide position (nt) 795 (Fig. 1), respectively. The *putP* region was amplified by PCR using KOD Dash DNA polymerase (Toyobo Biochemicals), the chromosomal DNA of strain PAO1 as a template, and oligonucleotide primers (5'-GCATCGACGAGCTGAAGAAAG AGAATTC-3', nt −688 to −660 of *putP*; and 5'-TGAAGGCATCTCTCGAA TGGCTGAGGCTG-3', complementary to the region 193 to 221 bp downstream of *putP*). After digestion with both *Xho*I and *Pst*I, the DNA fragment was cloned into plasmid pEX18Ap (12) between the *Sal*I (compatible with the *Xho*I site) and *Pst*I sites, to produce plasmid pYI388 (Fig. 1). The nucleotide sequence of the cloned fragment was verified by DNA sequencing.

Construction of strains and plasmids. Knockout mutants of PA0781, *putA*, and *putP* were constructed by the method of Hoang et al. (12). DNA fragments containing a gene of interest were cloned into plasmid pEX18Ap (*mob*⁺ *sacB*) (12). An ΩSp/Sm interposon (8) or a DNA cassette (*FRT*-Gm) containing *FRT* and the gentamicin resistance (Gm^r) gene (12) was then inserted into a target gene on the plasmids at an appropriate site (Fig. 1). Target genes on the PAO1 chromosome were replaced with the corresponding knockout genes on plasmids by recombination as described previously (12, 28, 30). We eliminated the Gm^r sequence of the *FRT*-Gm cassette integrated into the chromosome by introducing plasmid pFLP2 carrying Flp recombinase (12) into mutant cells. The plasmids used for construction of knockout mutants are listed in Table 1.

FIG. 1. Structure of *prurR-putP* loci of PAO strains and plasmids. Gene names (PA) correspond to annotation of the Pseudomonas Genome Project (www.pseudomonas.com). DNA cassettes, Tn5-751, and histidine-codon tag sequences are not drawn to scale. Thin solid and broken lines indicate chromosomal and plasmid sequences, respectively. Strains PAO4502, PAO4528, and PAO4547 carry *FRT*-Gm, *FRT* and Ω Sp/Sm cassettes on *putA*, respectively. Strain PAO4534 has *FRT* and *FRT*-Gm inserts on *putA* and *prurR*, respectively. Only relevant restriction sites are indicated: E, *EcoRI*; Ba, *BamHI*; Bb, *BbsI*; Bg, *BglI*; Bn, *BanIII*; K, *KpnI*; Pm, *PmaCI*; Sa, *SaI*; Sc, *SacI*; Su, *Sau3AI*; Sm, *SmaI*; Xh, *XhoI*.

To facilitate purification of the PutA protein so that the amino terminal could be sequenced, we constructed a *putA* gene tagged with six histidine codons (*putA*-His₆). The 3.8-kb *KpnI*-*XhoI* fragment of plasmid pYI366 containing the 5'-flanking region and the ProDH domain of *putA* (Fig. 1) was first cloned into plasmid pNIT6012 (30). The carboxyl-terminal half of the PSCDH domain was then amplified by PCR using plasmid pYI366 (*putA*) (Table 1) as a template, and primers 5'-CCGGCAAGGCCCTCGACGTGTTGCA-3' (nt 1817 to 1841, upstream of the *XhoI* site, of *putA*) and 5'-GCTGCTGTCGCTGGCCGACGCC GAGCACCACCACCACCTGACTCGAG-3' (complementary to the terminal *putA* coding sequence followed by six histidine codons [bold type], the TGA termination codon, and the *XhoI* site [underlined]). We verified the nucleotides by sequencing and then rejoined the DNA fragment to the 3' end of *putA* to produce functional *putA* in plasmid pYI370 (Fig. 1). For fusion assays of the *putA* promoter, the 5'-flanking region of *putA* was amplified by PCR using pYI370 as the template and primers designed to add *Bam*HI and *Pst*I sites (underlined) at the ends: 5'-CCGGATCCGGCTCCATGGTCACTGGGCTCGGA CTT-3' (corresponding to nt -459 to -435 of *putA*) and 5' CCCTGCAGTTTG AACATCACGCCCTCTCTTG-3' (complementary to nt -15 to +9 of *putA*). The amplified fragment was cloned into plasmid pME6015 (*'lacZ'*) (Table 1) between the *Bam*HI and *Pst*I sites to produce plasmid pYI378 (*putA*::*'lacZ'*).

Determination of the Tn5-751 insertion site. To determine the insertion site of Tn5-751 on the PAO6087 (*pru*::Tn5-751) chromosome by nucleotide sequencing, the junction regions between the chromosome and the transposon were cloned as follows. The chromosomal DNA of strain PAO6087 was digested with *EcoRI*, which cleaves the Tn5-751 sequence into *aph* (Km^r) and *dfrII* (Tp^r) segments (34) (Fig. 1). The resulting *EcoRI* fragments containing the junctions were then ligated to the corresponding site of plasmid pUC118 (43), and the ligated DNA was transformed into *E. coli* DH5 α (14). Plasmid DNA was isolated from Km^r and Tp^r transformants, and the junction sequences in the inserts were determined using an IS50 (terminal inverted sequences of Tn5)-specific primer (5'-C GTTACCATGTTAGGAGG-3', complementary to nt 71 to 88 of IS50).

Enzyme purification and assays. Enzymes were prepared from cells grown in MMP supplemented with the indicated 20 mM carbon and nitrogen sources by passage through a French pressure cell (SLM Instruments) at 20,000 lb/in². ProDH was measured as described previously (24, 25). One unit was defined as the amount of enzyme required to generate 1 μ mol of product per min. To purify PutA_{PAO}-His₆ strain PAO4502 (*putA*::*FRT*-Gm) harboring plasmid pYI370

(*putA*-His₆) was grown to an optical density at 600 nm (OD₆₀₀) of 1.0 in 1 liter of MMP containing 20 mM proline and tetracycline at 50 μ g/ml. After centrifugation, sedimented cells were washed with 20 mM sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl, suspended in 50 ml of the same buffer containing 10 mM imidazole, and disrupted by passage through the French pressure cell. The cell extract obtained by centrifugation at 100,000 $\times$ g at 4°C for 1 h was applied to a HiTrap chelating HP column (1 ml; Amersham Pharmacia Biotech). The column was washed with 10 ml of sodium phosphate buffer containing 0.5 M NaCl and 10 mM imidazole, and then PutA_{PAO}-His₆ was eluted using a stepwise gradient of imidazole (50, 100, 150, 200, and 500 mM, each at 10 ml) in the sodium phosphate buffer. The amino-terminal sequence was determined using PutA_{PAO}-His₆ eluted with 150 mM imidazole. The activity of β -galactosidase was measured using *o*-nitrophenyl- β -galactopyranoside by the method of Miller (26), and is expressed as Miller units. The protein concentration was determined using a protein assay kit (Bio-Rad Laboratories) with bovine serum albumin as the standard.

Nucleotide and amino acid sequencing. Nucleotides were sequenced using a Dye Terminator cycle-sequencing kit (Perkin-Elmer) and an ABI 311A DNA sequencer (Perkin-Elmer). Purified PutA_{PAO}-His₆ (10 pmol) was resolved by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (19) and blotted onto a polyvinylidene difluoride membrane (Millipore). The amino-terminal sequence was then determined using an HP 10000A protein sequencer (Hewlett-Packard).

Northern blotting and primer extension. We isolated RNA from cells exponentially growing (OD₆₀₀ = 0.3) in MMP containing the indicated carbon and nitrogen sources as described previously (28). Samples of RNA (50 μ g) in 10% (wt/vol) glyoxal were resolved along with RNA markers (Toyobo Biochemicals) on 1.0% agarose HS (Nippon Gene) and blotted onto Hybond N⁺ nylon membranes (Amersham Pharmacia Biotech) using a GenVac blotter (Pharmacia LKB). Membrane transcripts of *putA* and *putP* were then detected using 1.3-kb *XhoI*-*SmaI* (a *SmaI* fragment excised from plasmid pYI388) and 0.5-kb *SuI* (isolated from pYI388) fragments (Fig. 1) labeled with fluorescein-dUTP, respectively, and the ECL detection system (version II; Amersham Pharmacia Biotech). Samples of RNA (20 μ g) were annealed with oligonucleotides (5'-TA GTTGGCGCTGATGACAGGGAAGAATTC -3', complementary to nt 55 to 83 of *putA*, or 5'-AGCGACATCACCAGTTGGTGATGCTCTGTG-3', complementary to nt 52 to 80 of *prurR*) and then labeled with ³²P at the 5' end using

[γ - 32 P]ATP (220 Bq/nmol) (Amersham Pharmacia Biotech) and polynucleotide kinase (Takara Shuzo) for primer extension studies. A complementary strand was synthesized using avian reverse transcriptase (RAV-2; Takara Shuzo) in the presence of deoxyribonucleotides as described previously (28) and resolved on 6% denatured polyacrylamide gels along with sequence ladders generated using the BcaBEST sequencing kit (Takara Shuzo), plasmid pYI358 DNA as the template, and the 32 P-end-labeled oligonucleotide primers. Radioactive DNA fragments on the gels were visualized on X-ray film.

RESULTS

Cloning and gene organization of the *pru::Tn5-751* region.

P. aeruginosa PAO6087 (*pru::Tn5-751*) was isolated as a mutant unable to grow on proline (Pru^- phenotype) by insertional mutagenesis using the transposon Tn5-751 (33, 34). To determine whether the observed Pru^- phenotype of strain PAO6087 is due to a lack of ProDH/P5CDH, we assayed ProDH activity in this mutant and in the wild-type strain PAO1 grown in MMP containing 20 mM glutamate (noninducible conditions) or in 20 mM each glutamate and proline (inducible conditions). Noninduced PAO1 cells had only 0.8 U of ProDH/mg of protein, and proline induced ProDH synthesis to 41 U/mg of protein in wild-type strain PAO1, in accordance with previous results (25, 30). In contrast, little proline-inducible ProDH was synthesized in PAO6087 cells (0.7 U/mg of protein) even when cultured under inducible conditions, indicating that the insertion of Tn5-751 had impaired the structure or the regulatory gene for ProDH and P5CDH in this mutant.

To identify the *pru::Tn5-751* gene, we first cloned the corresponding chromosomal DNA region of strain PAO1 as a 5.9-kb DNA fragment in plasmid pYI358 (Fig. 1) by functional complementation (Pru^+) of the mutation (see Materials and Methods). Nucleotide sequencing revealed that the insert in this plasmid carries a PAO1 chromosome segment spanning from the 3' portion of PA0779 through the 5' portion of *putA* (38) (Fig. 1). The PA0779 gene encodes a putative protease with an M_r of 89,549, resembling the *E. coli* (41% identity, accession no. AAA16837) and human (42% identity, accession no. CAA53625) ATP-dependent protease, LA. The second gene (PA0780), which is transcribed in the direction opposite to PA0779, specifies a protein (250 residues) with an M_r of 27,587 (Fig. 1). A search of PROSITE files (www.expasy.ch/prosite) revealed significant similarity (value of 29.0) between the C-terminal region (152 to 250 residues) and the PS0121124 profile of the AraC/XylS family DNA-binding domains: this value was within the range (between 30.74 and 12.52) defined for the AraC/XylS members (41). The Helix-Turn-Helix program (7) detected a diagnostic helix-turn-helix DNA-binding motif of the AraC/XylS family (9, 22), between residues 166 and 186. The third PA0781 gene, located opposite PA0780, encodes a hypothetical outer membrane protein (687 residues; M_r , 74,202) with similarity (about 50%) to hemin receptor proteins of PAO1 (PhuR; www.pseudomonas.com) and *Xylella fastidiosa* (AAF83194). The last gene on plasmid pYI358 was the 5' part of the *putA* gene, which is annotated as the gene for ProDH/P5CDH (www.pseudomonas.com). The *putA* gene (*putA*_{PAO}) of strain PAO1 is referred to as *pruA* and *pruB*, which encode ProDH and P5CDH, respectively (13, 25). The absence of a functional *putA*_{PAO} gene and the presence of the complete PA0780 and PA0781 genes on the cloned DNA frag-

ment suggested that either PA0780 or PA0781 could be an allele of *pru::Tn5-751*.

Next, we sequenced the nucleotides to determine the precise insertion site of *pru::Tn5-751*. *EcoRI* fragments carrying the *aph* (Km^r) or *dhrrl* (Tp^r) genes were separately cloned along with the adjacent chromosomal segment (Fig. 1) into plasmid pUC118 as described in Materials and Methods. Sequencing the junctions between the transposon and the chromosome sequences revealed that the Tn5-751 sequence is preceded by the upstream sequence of nt 564 of PA0780 and followed by the downstream sequence of nt 556 of this gene. Thus, the 9-bp target sequence (nt 556 to 564) that had been duplicated during transposition reactions (21) flanked the transposon, establishing the insertion of Tn5-751 on PA0780 between nt 564 and 565 (Fig. 1). We designated this gene *pruR* (proline utilization regulator), because it encodes an AraC/XylS-type transcriptional regulator controlling *putA* expression as described above.

Identification of a proline-responsive promoter for *putA*_{PAO} using primer extension. Since the Ω interposon with transcription termination signals (8) on PA0781 of strain PAO4517 (Fig. 1) did not affect proline utilization, *putA* would be transcribed downstream of the insertion site. We accordingly determined the 5' end of *putA*_{PAO} by primer extension using a 32 P-end-labeled oligonucleotide complementary to nt 55 to 83 of *putA*_{PAO} (the amino terminal of PutA was determined by sequencing, as described below) as a primer and RNA samples from PAO1 cells grown in MMP and glutamate or MMP and proline as templates. A 253-bp cDNA was synthesized only with the RNA sample from cells grown on proline (Fig. 2A). A comparison with sequencing ladders determined the 5' end of the transcript 170 bp upstream of *putA*_{PAO} (Fig. 2), allowing identification of the plausible -10 and -35 sequences of the σ^{70} promoter at appropriate distances (Fig. 2B). Similar primer extension studies using an oligonucleotide primer complementary to 52 to 80 of *pruR* did not detect *pruR* cDNA (data not shown), indicating very low-level expression of this gene.

The *putAP* operon. The relatively long intergenic space (312 bp) between *putA*_{PAO} and *putP*_{PAO} (Fig. 1) and the potential rho-independent transcription terminator with a stem (10 bp)-loop (2 bp) structure (-29.3 kcal/mol) located 10 bp downstream of *putA*_{PAO} suggested that the *putAP*_{PAO} genes are transcribed by either mechanism: transcription from the *putA*_{PAO} promoter as a single unit that is attenuated by the stem-loop to reduce *putP*_{PAO} expression or separately from the *putA*_{PAO} and *putP*_{PAO} promoters. To establish the transcription mechanism of *putA*_{PAO}*P*_{PAO} we analyzed the *putA*_{PAO} and *putP*_{PAO} transcripts by Northern blotting using the RNA samples prepared as described above. Probing with the *putA*_{PAO} sequence (nt 1894 to 3195 from the translation initiation codon) detected major and minor transcripts of 3.3 and 5.2 kb, respectively (Fig. 3), in RNA samples from PAO1 cells grown in MMP plus proline. The latter was also detected as a unique transcript when probed with the *putP*_{PAO} sequence (nt 569 to 1076), indicating that the *putA*_{PAO} promoter transcribes *putP*_{PAO}. This was confirmed by a genetic approach. Plasmid pYI366 (*putA*⁺) (Fig. 1) complemented the Pru^+ phenotype of strain PAO4502 (*putA::FRT*-Gm) (Fig. 1) but not that of strain PAO4547 (*putA:: Ω Sp/Sm*) (Fig. 1), in which transcription terminators of an Ω interposon in *putA* prevent downstream transcription. None of the transcripts was detected in the RNA

FIG. 2. Primer extension analysis of the *putA*_{PAO} transcript (A) and structure of the *putA*_{PAO} promoter region (B). (A) The complementary DNA strand was synthesized from RNA samples extracted from PAO1 cells grown in MMP supplemented with 20 mM glutamate (lane 1) or 20 mM proline (lane 2) and a ³²P-end-labeled oligonucleotide primer corresponding to the *putA* sequence from positions +55 to +83. Sequence ladders (lanes G, A, T, and C) were generated using the same primer and plasmid pYI358 as template. (B) The first six amino acid residues were determined by amino acid sequencing. The 5' end of the *putA*_{PAO} transcript (+1) is identified by an asterisk. Inferred -35 and -10 sequences resembling consensus -35 and -10 sequences of σ^{70} promoters are underlined. SD, Shine-Dalgarno sequence.

sample from PAO1 cells grown in MMP with glutamate (data not shown).

Structural features of PutA. The amino terminal inferred for PutA_{PAO} corresponds to around residue 70 of the PutA proteins of *P. putida* and *E. coli*, and the predicted PutA_{PAO} coding frame has another seven possible translation initiation sites (four ATG and three GTG codons) in the 5'-flanking region (463 bp). To purify PutA_{PAO} for amino-terminal sequencing, we constructed plasmid pYI370 carrying the *putA*_{PAO} gene tagged with six histidine codons (*putA*_{PAO}-His₆) (Fig. 1). When introduced into strain PAO4502 (*putA*::*FRT*-Gm), this fusion gene restored the Pru⁺ phenotype of the mutant, confirming that PutA_{PAO}-His₆ was formed as an active enzyme. We accordingly purified PutA_{PAO}-His₆ from this strain by using a Ni²⁺-chelate column and determined the amino-terminal sequence. The first seven amino acids determined (M-F-K-A-S-H-V) perfectly matched the amino-terminal sequence assigned to PutA_{PAO} (Fig. 2B). Although PutA_{PAO} has substantial similarity to known PutA proteins of other bacteria, in contrast to PutP (PutP_{PAO}) of this strain (see below), PutA_{PAO} was no more similar to PutA of *P. putida* (47% identity) and was distantly related to all known bacterial PutA proteins (44 to 47% identity). Moreover, PutA_{PAO} (1,060 residues) was also truncated by approximately 250 residues at the carboxyl-terminal region compared with its counterparts in enteric bacteria (1,321 residues) and *P. putida* (1,315 residues).

PutA_{PAO} has no autorepressor function. To examine whether PutA_{PAO} autoregulates its expression, we constructed the *putA* null mutant, PAO4502 (*putA*::*FRT*-Gm), by introducing a *FRT*-Gm^r cassette (12) into this gene at the *Sac*I site (Fig. 1) and introduced plasmid pYI378 carrying a *putA*_{PAO}::*lacZ*

fusion into this mutant. We did not detect β -galactosidase activity of the fusion in wild-type strain PAO1 cultured in MMP plus glutamate, but proline (i.e., growth in MMP plus proline or MMP plus glutamate and proline) induced fusion expression to high levels (Table 2). In agreement with the results of the ProDH and P5CDH assays (25), this proline-inducible expression was not affected by the presence of either succinate, a catabolite repressor for *P. aeruginosa*, or ammonia (Table 2). The fusion levels were comparable between the

FIG. 3. Northern blots of *putAP* transcripts. RNA was extracted from PAO1 cells grown in MMP with 20 mM proline, as described in the legend to Fig. 2, blotted onto Hybond N⁺ nylon membrane, and then probed with 1.3-kb *putA*_{PAO} (lane 1) or 0.5-kb *putP*_{PAO} (lane 2) regions isolated from plasmid pYI388 as *Sma*I or *Stu*I fragments (Fig. 1). The molecular sizes of transcripts were determined by comparison with RNA markers and are indicated by arrowheads.

TABLE 2. Regulation of *putA*'-'*lacZ* fusion by PutA and PruR^a

Enzyme source	β -Galactosidase activity (10^2 Miller units) ^b				
	Glu	Pro	Glu + Pro	Pro + Suc	Pro + NH ₄
PAO1 (wild type)	<0.1	62 $\pm$ 1	49 $\pm$ 2	66 $\pm$ 2	59 $\pm$ 2
PAO4502 (<i>putA</i> :: <i>FRT</i> -Gm)	<0.1	NG ^c	96 $\pm$ 6	NG	NG
PAO6087 (<i>pruR</i> ::Tn5-751)	<0.1	NG	<0.1	NG	NG
PAO4534 (<i>pruR</i> :: <i>FRT</i> -Gm)	<0.1	NG	<0.1	NG	NG
<i>putA</i> :: <i>FRT</i>)					

^a Cells harboring pYI378 (*putA*_{PAO}'::'*lacZ*) were grown in MMP medium supplemented with 20 mM carbon and nitrogen sources.

^b Activities of β -galactosidase were measured in cell extracts prepared as described previously (26, 28) and are represented as averages of three measurements with standard errors. Carbon and nitrogen sources added to MMP medium: Glu, glutamate; Pro, proline; Suc, succinate.

^c NG, no growth or very poor growth (see Fig. 4).

wild-type strain PAO1 and the *ntnC* mutant PAO4484 (*ntnC*::*FRT*) (data not shown). Thus, *putA*_{PAO} expression appeared to be independent of both carbon and nitrogen regulation.

The amount of β -galactosidase activity was negligible in strain PAO4502 (*putA*::*FRT*-Gm)/pYI378 (*putA*'::'*lacZ*) cells cultured in MMP with glutamate (Table 2), indicating that PutA_{PAO} does not repress its expression. When strain PAO4502 was cultured in MMP plus glutamate and proline, the fusion level in PAO4502 doubled compared with that in the wild-type strain PAO1 (Table 2). This increased expression was completely abolished by a *pruR* mutation, as in strain PAO4534 (*putA*::*FRT* *pruR*::*FRT*-Gm) (Table 2). When the fusion plasmid was harbored in the *pruR* mutant (PAO6087), the induction effect of proline on fusion expression was almost completely abolished (Table 2), indicating that PruR primarily controls the proline-responsive expression of *putA*_{PAO} at the level of transcription.

Functions of PA0781 and *putP* in proline utilization. The *putP*_{PAO} gene is located 312 bp downstream of *putA*_{PAO} (Fig. 1). PutP_{PAO} is highly homologous to the PutP proteins of *P. fluorescens* (84% identity), *P. putida* (80% identity), and *E. coli* (77% identity). To examine the possible role of PA0781, which encodes a putative outer membrane protein, and the contribution of *putP*_{PAO} to proline transport, we constructed knockout mutants PAO4517 (PA0781:: Ω Sp/Sm) and PAO4503 (*putP*:: Ω Sp/Sm) by inserting an Ω Sp/Sm cassette (8) into PA0781 (at the *BbsI* site) and *putP* (at the *BanIII* site), respectively (Fig. 1). Strain PAO4517 (PA0781:: Ω Sp/Sm) proliferated normally on proline (Fig. 4). In contrast, the growth of strain PAO4503 (*putP*:: Ω Sp/Sm) on proline was significantly handicapped (Fig. 4). Thus, PA0781 appears to be irrelevant to proline utilization and PutP_{PAO} is the major proline transporter for proline utilization in strain PAO1, as it is in enteric bacteria (23). Proline supporting the residual growth of the *putP* mutant (Fig. 4) might be taken up by basal levels of ubiquitous osmoregulated proline/glycine betaine transporters. In fact, strain PAO1 has the PA5370 and PA4343 genes, which are homologous to the osmoregulated proline transport *proP* and *proU* genes of *E. coli*, respectively, as well as PA3236, an orthologue of the osmoprotection proline/glycine-betaine periplasmic binding protein genes (www.pseudomonas.com).

FIG. 4. The growth of *putAP* and *pruR* mutants in proline medium is defective. Cultures were grown in MMP with 20 mM proline as carbon and nitrogen sources. Cell growth was measured as described previously (30) and is expressed as absorbance at 660 nm (A_{660}). Open circles, wild-type strain PAO1; solid circles, PAO4517 (PA0781); open triangles, PAO6087 (*pru*::Tn5-751); solid triangles, PAO4503 (*putP*:: Ω Sp/Sm); open squares, PAO4528 (*putA*::*FRT*).

DISCUSSION

The characteristic features of the *pruR*-*putA*_{PAO}*P*_{PAO} system of *P. aeruginosa* PAO1 disclosed in this study include the structure of ProDH/P5DH (the *putA*_{PAO} product), the organization of the *putA*_{PAO}*P*_{PAO} genes, and regulation of the *putA*_{PAO} promoter by a transcriptional activator of the AraC/XylS family. Compared with PutA proteins of enteric bacteria and of *P. putida*, PutA_{PAO} is truncated at both amino and carboxyl terminals. PutA of *B. japonicum*, which is supposed to be regulated by an Lrp-type transcriptional activator (4), also lacks these regions. Different roles of the terminal regions in repressor function have been suggested. Ling et al. (20) have postulated that a leucine zipper (bZIP)-like sequence (having four conserved leucine residues at intervals of 6 amino acids) in the carboxyl-terminal region of the *E. coli* and *S. enterica* serovar Typhimurium PutA proteins constitutes a possible DNA-binding motif, although this putative bZIP sequence does not have adjacent basic regions that interact with target DNA (18, 32). Similar sequences were also located at the corresponding regions of PutA proteins from *P. putida*, *R. capsulatus*, *A. tumefaciens*, and *S. meliloti*. However, the PutA proteins of these bacteria have distinct regulatory properties. PutA proteins of the enteric bacteria, *P. putida*, and *S. meliloti* are powerful repressors of *putA* expression (36, 44). In contrast, expression of *putA* is increased in *putA* mutants of *A. tumefaciens* and *R. capsulatus*, but only when exogenous proline and functional PutR are present (4, 17). Cho and Winans (4) proposed that the increased expression of *putA* in a *putA* mutant of *A. tumefaciens* could be a consequence of elevated cellular concentrations of proline due to the absence of the proline catabolic enzyme; elevated proline levels could in turn exert an induction effect on *putA* expression through the PutR

function. The situation may be analogous in a *putA*_{PAO} background, where *putA*_{PAO}::'*lacZ*' expression is increased only when both exogenous proline and functional *pruR* are present (Table 2). Since the PutA of *A. tumefaciens* lacks the amino-terminal sequence of 68 amino acids, the authors have postulated involvement of this deleted region in the regulation. However, although the PutA of *S. meliloti* has a shorter (by 2 amino acids) amino terminal than does the PutA of *A. tumefaciens*, it has apparent repressor properties (36). Thus, the present data seem to exclude the involvement of the amino- and carboxyl-terminal domains in the regulatory function of PutA, although a substitution(s) or small deletion(s) in the carboxyl-terminal regions of the *R. capsulatus* and *A. tumefaciens* PutA proteins might abolish the repressor function. Alternatively, PutA_{PAO} and PutA proteins without the repressor function might not undergo autophosphorylation, which is associated with the repressor activity (31). The location of phosphorylation sites and a kinase domain on PutA remain unknown.

Statistical analysis of the *putP* genes and the control regions among *S. enterica* serovar Typhimurium and *E. coli* strains has suggested horizontal transfer of a segment of *putP* and a cluster of control sites to a particular group of these bacteria from an unrelated, unknown source (29). Sequence diversity is also evident among PutA proteins of the family *Rhizobiaceae*, where PutA of *B. japonicum* are only distantly related to its *A. tumefaciens* and *S. meliloti* counterparts, which belong to the same family. While the phylogenetic relationships of PutP_{PAO} and other PutP proteins are relevant to that of the origin, PutA_{PAO} appears to be located at a phylogenetic position unrelated to that of the origin. The *putA* genes of these bacteria are regulated by different mechanisms. The *putA* gene of *S. meliloti* is autoregulated, as are the *putA* genes of enteric bacteria and *P. putida*, whereas the *putA* genes of *A. tumefaciens* and *B. japonicum* are controlled by the Lrp-type transcriptional activator encoded by the adjacent *putR* gene (4, 17, 36). Our identification of the *pruR* gene (encoding an AraC/XylS-type transcriptional activator) provides evidence that the regulatory mechanism of the proline catabolic genes is also totally different even within the same genus.

The *putAP* genes of *P. putida* and enteric bacteria are divergently located, and they share an intergenic promoter region controlled by PutA and proline (23, 44). On the other hand, the *putP* genes of *A. tumefaciens*, *B. japonicum*, *S. meliloti*, and *R. capsulatus* have not been identified but appear to be separated from *putA*. Thus, *putA*_{PAO}*P*_{PAO} is the first operon of the proline catabolic genes to be demonstrated by Northern blotting (Fig. 3). Transcripts from the *putA*_{PAO} promoter appear to be prevented by the stem-loop (−29.3 kcal/mol) at 10 bp downstream of *putA*, and only a small fraction of these transcripts can progress into *putP*_{PAO} over the attenuation signal (Fig. 3). These different amounts of transcripts allow the required high levels of expression of the catabolic enzyme PutA_{PAO} and the low levels of expression of the membrane protein PutP_{PAO}, which is cytotoxic at high levels.

The *putA* and *putP* promoters of *Klebsiella pneumoniae* and *K. aerogenes* are controlled by carbon and nitrogen sources through cAMP-Crp and Nac, respectively (23). In accordance with involvement of the PruR of the AraC/XylS family, which activates σ^{70} -dependent promoters (Fig. 2B), the *putA*_{PAO} pro-

motor is not controlled by nitrogen (Table 2) and does not require the NtrC function. The absence of nitrogen control has also been demonstrated with the *putA* promoter of *R. capsulatus*, which requires PutR, an Lrp-type transcriptional activator, for σ^{70} promoters (17). Expression of the *putA*_{PAO}*P*_{PAO} operon also appears not to be subject to catabolite repression by succinate (25) (Table 2). The independence of the *putA*_{PAO} promoter from carbon and nitrogen control implies the importance of proline as a nutrient for *P. aeruginosa* PAO1. The diversity of the structure, organization, and regulatory mechanism of the proline catabolic genes between *P. aeruginosa* and *P. putida* suggests that different genetic processes have been involved in the development of proline catabolism systems in these closely related species.

ACKNOWLEDGMENTS

We are grateful to D. Haas for advice regarding the manuscript and for providing strain PAO6087. We also thank M. Gut-Rella, S. Heeb, and H. Schweizer for the gifts of strain PAO6087, plasmid pME6015, and plasmids pEX18 and pPS858, respectively.

This study was supported in part by a grant-in-aid from the Ministry of Education, Culture, Sports and Sciences (grant 14360060) to Y.I. Y.N. is a domestic research fellow supported by the Japan Science and Technology Corporation.

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