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Regulation of Proline Utilization in *Salmonella typhimurium*: a Membrane-Associated Dehydrogenase Binds DNA In Vitro

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The PutA protein is a membrane-associated enzyme that catalyzes the degradation of proline to glutamate. Genetic evidence suggests that in the absence of proline, the PutA protein also represses transcription of the *putA* and *putP* genes. To directly determine whether PutA protein binds to the *put* control region, we analyzed gel retardation of *put* control region DNA by purified PutA protein in vitro. The *put* control region is 420 bp. Purified PutA protein bound specifically to several nonoverlapping fragments of control region DNA, indicating the presence of multiple binding sites in the control region. Electrophoretic abnormalities and behavior of circularly permuted fragments of control region DNA indicate that it contains a region of intrinsically curved DNA. To determine whether the multiple binding sites or the DNA curvature are important in vivo, two types of deletions were constructed: (i) deletions that removed sequences predicted to contribute to DNA curvature as well as potential operator sites and (ii) deletions that removed only potential operator sites. Both types of deletions increased expression of the *put* genes but were still induced by proline, indicating that multiple *cis* elements are involved in repression. These data suggest a model for *put* repression that invokes the formation of a complex between PutA protein molecules bound at different sites in the control region, brought into proximity by a loop of curved DNA.

Proline can be used as the sole source of carbon and nitrogen by *Salmonella typhimurium*. Proline utilization requires the two divergently transcribed genes that form the *put* operon: the *putP* gene, encoding the major proline permease, and the *putA* gene, encoding a multifunctional protein that catalyzes the degradation of proline to glutamate (12). The PutA protein has two distinct enzymatic activities: proline oxidase and pyrroline-5-carboxylate dehydrogenase (17, 21). The proline oxidase reaction couples proline oxidation to the reduction of a tightly associated flavin adenine dinucleotide cofactor, and reoxidation of the reduced flavin adenine dinucleotide requires association of the enzyme with the electron transport chain in the membrane. Thus, PutA protein must become membrane associated at this stage in proline catabolism (17, 24).

Expression of the *put* operon is induced by proline. The phenotypes of several types of *putA* mutants indicate that the PutA protein is also involved in the regulation of the *put* operon (16). (i) Mutations that disrupt the *putA* gene cause loss of enzymatic activity and constitutive expression of *putP*. Such mutants are designated *putA*(A^-C^-), where A^- indicates the absence of enzymatic activity and C^- indicates the absence of regulatory activity. (ii) There are three classes of *putA* missense mutants: *putA*(A^-C^-), which show the same phenotype as the null mutants; *putA*(A^-C^+), which retain only the regulatory activity; and *putA*(A^+C^-), which retain only the enzymatic activity. These three types of mutations map throughout the *putA* gene, indicating that both regulatory and enzymatic properties are located in a single polypeptide. (iii) Haploid *lac* operon fusions to the *putA* gene show constitutive expression of β-galactosidase, but proline-dependent regulation is restored when the *putA* gene is supplied in *trans*, demonstrating that repression occurs at the transcriptional level (14). Taken together, these results strongly suggest that the PutA protein negatively regulates transcription of the *put* operon.

The *put* control region was identified by isolation of *cis*-acting regulatory mutants (8). Promoter up mutations for *putA* and *putP* map in a region between the two structural genes. Operator constitutive mutants which overexpress both the *putA* and *putP* genes also map in the same deletion intervals. DNA sequence analysis indicates that the region between the *putA* and *putP* structural genes is 420 bp long (7). Immediately upstream of the *putA* gene is a potential promoter for *putA* and a potential promoter for *putP*. Between these two promoters is a 27-bp sequence of dyad symmetry that could serve as an operator site (Fig. 1).

On the basis of these results, a model was proposed to explain how the PutA protein might regulate gene expression. In the presence of proline, the PutA protein becomes membrane associated and the *put* genes are fully expressed; however, in the absence of proline the PutA protein may remain in the cytoplasm, where it is able to bind the operator site between the *putA* and the *putP* promoters, precluding expression of both genes. This model accounts for most of the observations except that it does not explain the function of the approximately 380-bp untranslated leader sequence for *putP*. The regulatory region is highly conserved in *S. typhimurium*, *Escherichia coli*, and *Klebsiella aerogenes* (1, 8, 19). Such long untranslated leader sequences without a function are not common in procaryotic systems.

Thus, we expected that additional features of the control region may be involved in regulation of the *put* operon. In this study, we demonstrate that PutA protein binds to multiple sites in the *put* control region in vitro and that the DNA in the *put* control region is curved.

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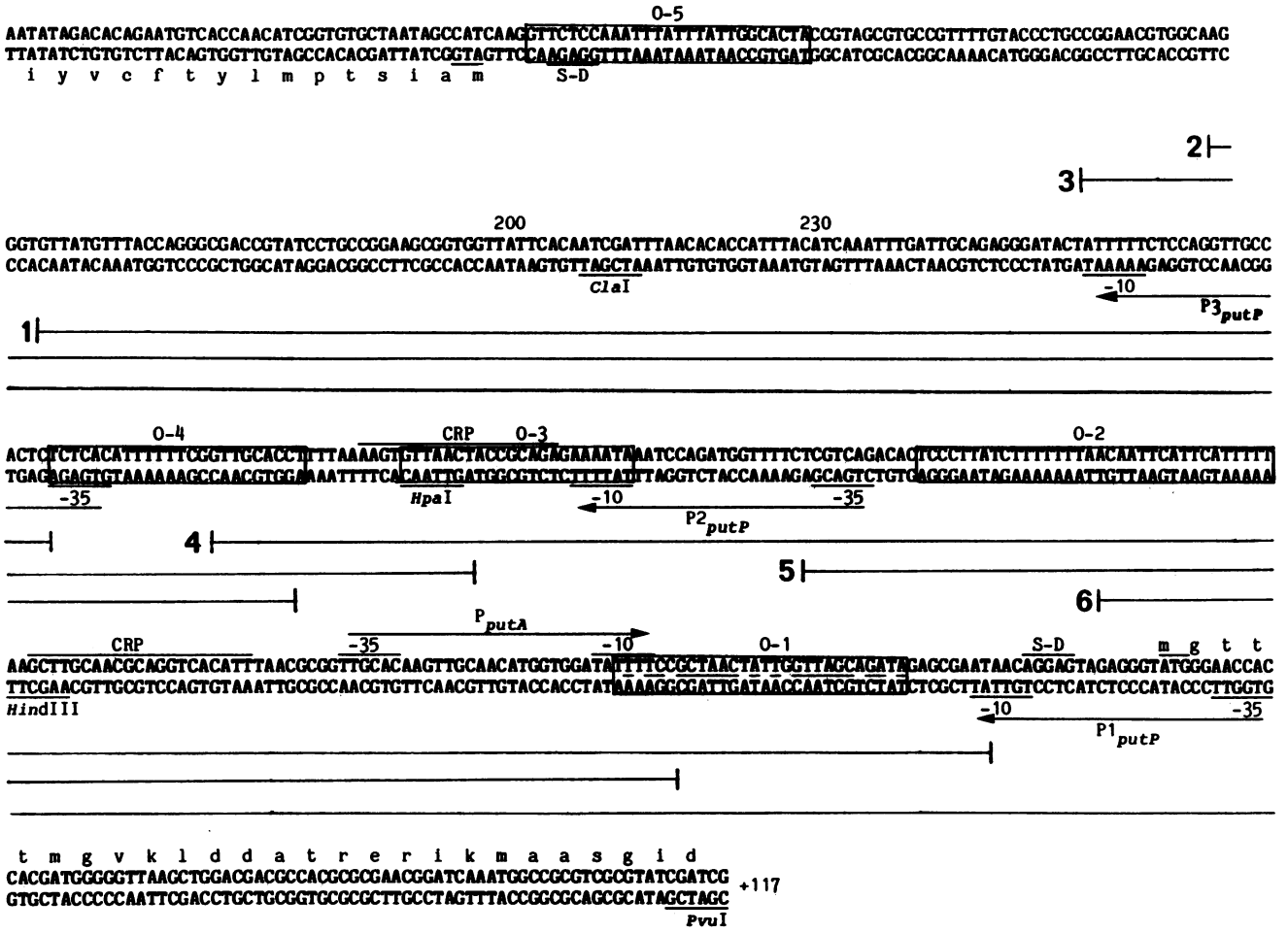

FIG. 1. DNA sequence of the control region from the *put* operon. Boxed sequences represent potential operators O-1 to O-5; arrows represent promoter sequences; positions 200 and 230 indicate sites predicted to curve. Lines under the sequence represent DNA removed in the indicated deletion: 1, ΔC1-3; 2, ΔC1-5; ΔC3-13; 4, ΔH3-4; 5 ΔH3-6; 6, ΔH3-7.

MATERIALS AND METHODS

Strains and media. All strains used were derivatives of *S. typhimurium* LT2. Strain MS62 contains a large deletion of the *put* operon (Δ*put-557*) that extends from a site within the *putP* gene to a site within the *putA* gene, resulting in a PutA$^-$ PutP$^-$ phenotype (12). Strain MS1210 has a Mu dJ insertion in the *putA* gene, resulting in a PutA$^-$ phenotype and a *putA*::*lacZ* operon fusion.

Nutrient broth (Difco Laboratories, Detroit, Mich.) with 0.5% NaCl (NB) was used as a rich medium. NCE (21) was used for the minimal medium. Minimal medium was supplemented with 0.6% sodium succinate (uninduced) or 0.6% sodium succinate and 0.2% proline (induced).

***Bal*31 deletions.** Plasmid pPC6, a pBR322 derivative that carries the entire *put* operon (7), was digested to completion with either *Cla*I or *Hin*dIII (Bethesda Research Laboratories, Inc. [BRL], Gaithersburg, Md.) restriction endonuclease. The linear DNAs were extracted with phenol-chloroform, ethanol precipitated (13), and then digested with *Bal*31 exonuclease as recommended by the supplier (New England BioLabs, Beverly, Mass.). The reactions were stopped after 1, 2, or 3 min by adding ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) to a final concentration of 20 μM. The resulting deletions were directly religated by using T4 DNA ligase.

Electroporation. Each of the *Bal*31 deletion plasmids was moved into *S. typhimurium* MS62 by electroporation. Cells were grown to mid-log phase, centrifuged at 4,000 × *g* for 10 min at 4°C, washed with 1 volume of 1 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), washed with 1/2 volume of the same buffer, then washed with 1/50 volume of 10% glycerol. The washed cells were resuspended in 1/500 of the original volume of 10% glycerol, and 40 μl of these prepared cells was used for each electroporation. Electroporation was done by using a Bio-Rad Gene Pulser (Bio-Rad, Richmond, Calif.). Electroporation conditions were as follows: capacitance, 25 μF; resistance, 200 Ω; and voltage, 2.5 kV. Typical time constants were approximately 4 ms. After electroporation, the cells were grown in NB for about 30 min, and then transformants containing the plasmid were selected on NB-ampicillin (50 μg/ml) plates.

DNA sequencing. The *Bal*31 deletion plasmids isolated from the transformants were purified, and the DNA sequence of the double-stranded plasmids was determined by using Sequenase (U.S. Biochemical Corp., Cleveland, Ohio).

Amplification of labeled DNA fragments. DNA from the *put* control region was amplified by the polymerase chain reaction (PCR), using ^{32}P-end-labeled primers (10). The PCR reaction mix contained 20 pmol of a labeled primer that would anneal to the 5′ end of one strand and 20 pmol of an unlabeled primer that would anneal to the 5′ end of the opposite strand of the template. In addition, the reaction mix contained 200 μM each deoxynucleoside triphosphate, 0.075 μg of plasmid template, 20 μl of 5× PCR buffer (50 mM Tris hydrochloride [pH 8.0], 15 mM $MgCl_2$, 0.25% Tween 20, 0.25% Nonidet P-40), and 2.5 U of *Taq* DNA polymerase (Promega, Madison, Wis.) in a final volume of 100 μl. Thirty cycles of amplification were done, with denaturation of the template at 93°C for 1 min, annealing of the template to the primer at 45°C for 2 min, and DNA polymerization at 72°C for 2 min. The primers were labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase. To label 200 pmol of primer, the following reactants were used: 1.2 mCi [γ-^{32}P]ATP (5,000 Ci/mmol; DuPont-New England Nuclear, Wilmington, Del.), 10 μl of 50 mM $MgCl_2$, 5 μl of 1 M Tris hydrochloride (pH 7.6), 5 μl of 200 mM 2-mercaptoethanol, and 15 U of T4 polynucleotide kinase (BRL) in a total volume of 50 μl. The reaction mix was incubated at 37°C for 45 min. An additional 15 U of T4 polynucleotide kinase was then added, and the reaction mix was incubated at 37°C for another 45 min. The labeled primers were separated from unincorporated [γ-^{32}P]ATP by filtration through Sep Pak C18 cartridges (Waters Millipore, Milford, Mass.) and resuspended in 10 μl of distilled H_2O. The φX174, lambda, and pBR322 DNAs used as controls for nonspecific DNA binding were directly end labeled by polynucleotide kinase. The *Hin*dIII-digested lambda (lambda/*Hin*dIII) standard (BRL) was further digested with the *Cla*I restriction endonuclease, and the pBR322 DNA was digested with *Hae*III (BRL). Then the three samples of DNA (φX174/*Hae*III [BRL], lambda/*Hin*dIII/*Cla*I, and pBR322/*Hae*III) were treated with 10 U of bacterial alkaline phosphatase (Boehringer Mannheim, Indianapolis, Ind.) at 60°C for 1 h prior to end labeling with 300 μCi of [γ-^{32}P]ATP and 15 U of T4 polynucleotide kinase as described above. The unincorporated labeled phosphate was then removed by ethanol precipitation (13).

Gel retardation assays. PutA protein was purified as described previously (17). Purified PutA protein was stored in G buffer (30% glycerol, 70 mM Tris, pH 8.2) at −70°C to maintain its stability. Different molar ratios of PutA protein in G buffer were mixed with ^{32}P-labeled *put* control region DNA or nonspecific DNA and excess unlabeled plasmid DNA and were incubated at room temperature for 15 min. G buffer was added to each reaction to yield a final concentration of 1/10 G buffer (3% glycerol, 7 mM Tris, pH 8.2). The DNA fragments were then separated by electrophoresis on 10% polyacrylamide gels with a 30:0.8 acrylamide-to-bisacrylamide ratio. Electrophoresis was carried out in Tris-borate-EDTA buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA) at 15-V/cm constant voltage until the bromophenol blue dye in the φX174/*Hae*III standard reached the end of the gel. The gels were dried and autoradiographed.

Gel retardation of DNA fragments from the *put* control region was identical when the binding assay was done in a 1/10 dilution of G buffer, 1× medium salt restriction enzyme buffer (50 mM Tris, 50 mM NaCl, 10 mM $MgCl_2$), or 1× in vitro transcription buffer (50 mM Tris hydrochloride [pH 8.0], 5 mM $MgCl_2$, 5 mM dithiothreitol, 50 mM KCl, 0.05 mg of gelatin per ml). However, when the binding buffer contained NaCl concentrations of ≥80 mM, gel retardation was completely prevented. In addition, as observed for some other DNA-binding proteins (20), the indicator dyes bromophenol blue and xylene cyanol also inhibit gel retardation by PutA protein. A surprising observation was that use of a 38:2 acrylamide-to-bisacrylamide ratio prevented gel retardation by PutA protein, possibly because a smaller pore size inhibits migration of the large PutA protein.

A similar electrophoresis procedure was used to examine altered electrophoretic mobility due to intrinsic curvature of different subfragments of the *put* regulatory region. However, in this case the DNA produced by the PCR was not radioactively labeled, no protein was added, and electrophoresis was carried out at 4°C in 8 and 10% polyacrylamide gels.

Circular permutation assays. Fragments containing the regions of DNA predicted to curve were amplified from the *put* control region by using PCR as described above. The fragments from oligonucleotide 6 to oligonucleotide 8 and from oligonucleotide 5 to oligonucleotide 7 were cloned into the *Sal*I site of plasmid pBEND2 (26). The PCR-amplified fragments and the *Sal*I-digested vector were treated with Klenow fragment (BRL) and 50 μM each dATP, dCTP, dGTP, and dTTP for 1 h at 37°C, phenol extracted, and ethanol precipitated. The blunt-ended fragments were then ligated at 25°C for 18 h with T4 DNA ligase (BRL). The plasmid DNA was isolated and digested with the following enzymes: *Bgl*II, *Xho*I, *Eco*RV, *Pvu*II, *Sma*I, *Nru*I, and *Bam*HI (BRL). Electrophoresis of the digests was carried out in 10% polyacrylamide gels at 4°C.

Enzyme assays. Cells were grown to mid-log phase in NCE-succinate medium (uninduced conditions) and in NCE-succinate-proline medium (induced conditions). Proline oxidase was assayed as described by Dendinger and Brill (2). Proline oxidase activity is expressed as nanomoles of proline oxidized per minute per unit of optical density at 650 nm (OD_{650}) of the cell suspension. Proline transport was assayed as described by Ekena et al. (4). Proline transport activity is expressed as nanomoles of proline transported per minute per milligram of protein. β-Galactosidase was assayed by using the chloroform-sodium dodecyl sulfate procedure described by Miller (18). β-Galactosidase activity is expressed as nanomoles of *o*-nitrophenyl-β-D-galactoside (ONPG) hydrolyzed per minute per OD_{650} of the cell suspension.

Computer analysis. Computer analysis of *put* DNA sequences was done with the DNAstar (DNAstar, Madison, Wis.) software package. The predicted DNA bends were found with the BEND program; this program is based on the wedge model for curved DNA, in which a small bend at ApA dinucleotides distributed with helical periodicity in a DNA sequence is predicted to cause intrinsic curvature (22). For computing the helix trajectory, the tilt angle at the ApA wedge is taken as 2.4°, opening toward the AA dinucleotide, and the roll angle is taken as 8.4°, opening toward the major groove. The potential secondary operator sites were located by using the ALIGN program, based on the Wilbur-Lipman algorithm (23), to find sequences similar to the sequence originally proposed to be the unique *put* operator.

RESULTS

PutA protein is a DNA-binding protein in vitro. Although genetic studies suggested that the *putA* gene encodes a *trans*-acting repressor for the *put* operon (14), there was no direct evidence that the PutA protein could bind to DNA. To determine whether purified PutA protein binds to the *put* control region DNA in vitro, we assayed DNA binding by using gel retardation assays (5, 6). The electrophoretic

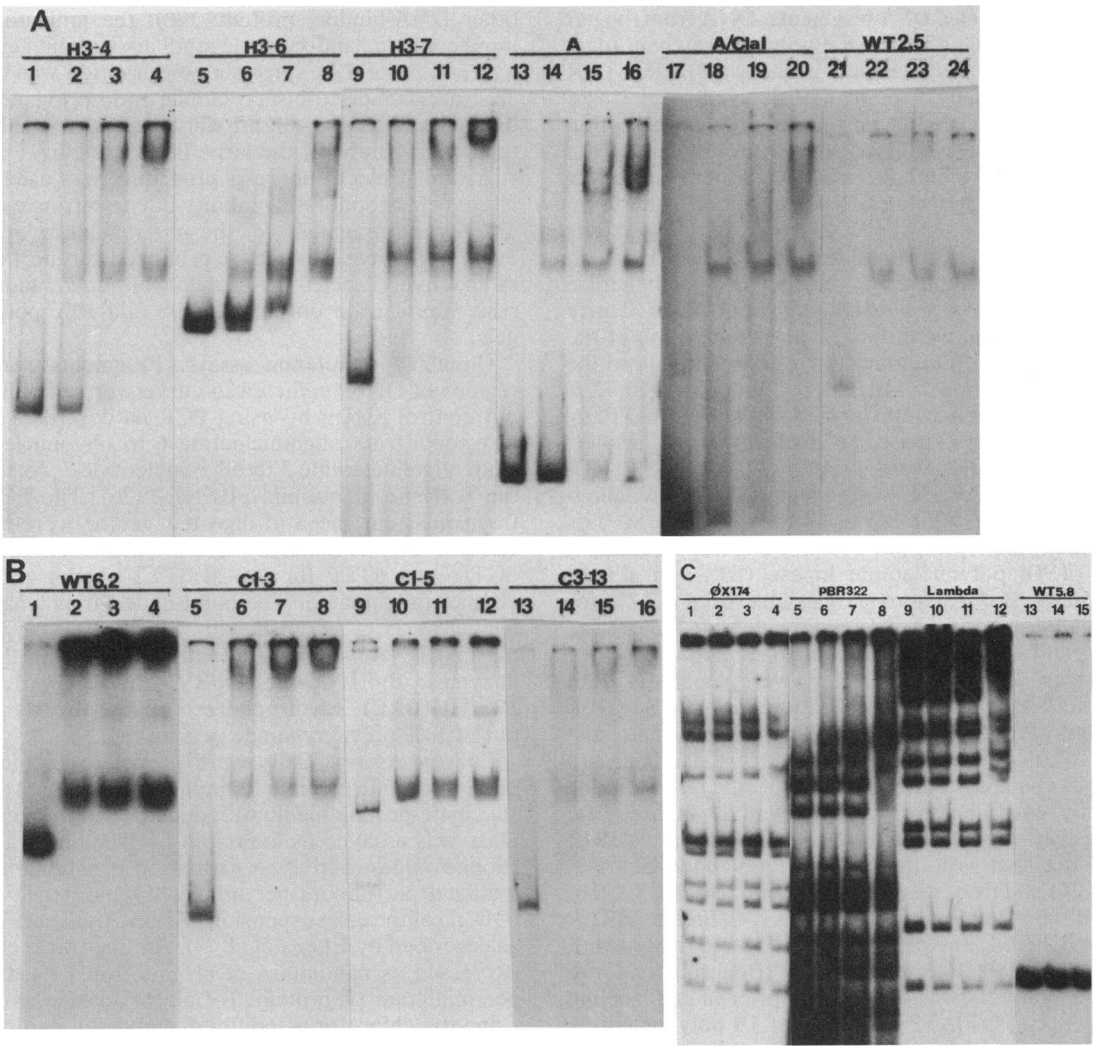

FIG. 2. Gel retardation by PutA protein. (A) Binding to *put* control region DNA subfragments. Lanes: 1 through 4, 0.36 nM DNA from deletion H3-4, amplified with oligonucleotides 6 and 2; 5 through 8, 0.27 nM DNA from deletion H3-6, amplified with oligonucleotides 6 and 4; 9 through 12, 0.30 nM DNA from deletion H3-7, amplified with oligonucleotides 6 and 2; 13 through 16, 0.59 nM DNA from the wild-type *put* control region, amplified with oligonucleotides 6 and 8 (fragment A); 17 through 20, the same fragment digested with *Cla*I; 21 through 24, 0.31 nM DNA from the wild-type *put* control region, amplified with oligonucleotides 2 and 5. Lanes 1, 5, 9, 13, 20, and 24 did not contain protein. For each DNA fragment, the subsequent lanes contained increasing concentrations of PutA protein: lanes 2, 6, 10, and 15 contained 28 nM PutA protein; lanes 3, 7, 11, and 14 contained 56 nM PutA protein; and lanes 4, 8, 12, and 13 contained 112 nM PutA protein. (B) Binding to DNA fragments obtained by amplification with oligonucleotides 6 and 2. Lanes: 1 through 4, 0.20 nM DNA amplified from the wild-type *put* control region; 5 through 8, 0.28 nM DNA amplified from the C1-3 deletion plasmid; 9 through 12, 0.32 nM DNA amplified from the C1-5 deletion plasmid; 13 through 16, 0.33 nM DNA amplified from the C3-13 deletion plasmid. Lanes 1, 5, 9, and 16 contained DNA without added protein. For each DNA fragment, the subsequent lanes contained increasing concentrations of PutA protein as described for panel A. (C) Nonspecific DNA binding. Lanes: 1 through 4, 0.66 nM ϕX174/*Hae*III DNA; 5 through 8, 0.61 nM pBR322/*Hae*III DNA; 9 through 12, 0.70 nM lambda/*Hin*dIII/*Cla*I DNA; 13 through 15, 0.91 nM DNA fragment from the wild-type *put* control region amplified with oligonucleotides 5 and 8. Lanes 1, 5, 9, and 13 did not contain any PutA protein. Subsequent lanes contained increasing concentrations of PutA protein: lanes 2, 6, and 10 contained 56 nM PutA protein; lanes 3, 7, 11, and 14 contained 112 nM PutA protein; and lanes 4, 8, 12, and 15 contained 224 nM PutA protein.

mobility of a DNA fragment carrying the wild-type *put* control region was specifically retarded by purified PutA protein (Fig. 2B, WT6,2). At concentrations of PutA protein that bind to the *put* control region, PutA protein did not bind to DNA lacking the *put* control region, including ϕX174 DNA, phage lambda DNA, and pBR322 DNA (Fig. 2C). PutA protein bounds to the wild-type *put* control region at a molar ratio of approximately 100:1 (Fig. 2B). At molar ratios of PutA protein to DNA of approximately 800:1, some nonspecific DNA binding was observed (Fig. 2C), and at molar ratios of PutA protein to DNA of 1,600:1, considerable nonspecific binding was observed (data not shown). In addition, PutA protein did not bind to a fragment from within the *put* control region which lacks putative operator sites even at molar ratios of PutA protein to DNA of 800:1 (Fig. 2C, WT5,8). These results indicate that PutA protein is a DNA-binding protein specific for the *put* control region.

DNA sequence analysis of the *put* control region. The

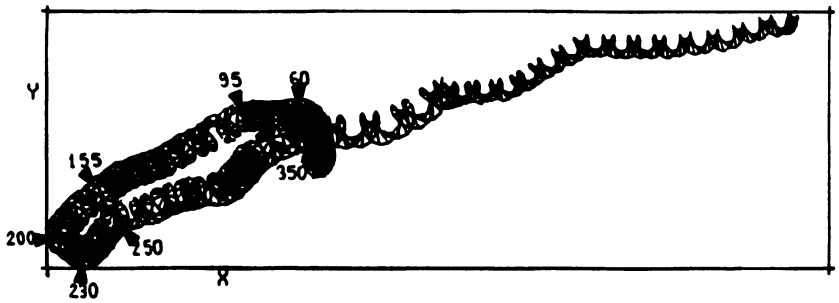

FIG. 3. Computer-predicted curvature of the *put* control region. The sites numbered 200 and 230 correspond to the same sites in Fig. 1, located at each side of the *Cla*I site.

prominent features of the *put* regulatory region are shown in Fig. 1. The 27-bp sequence of dyad symmetry labeled O-1 was originally proposed to be the unique operator site (6). It is possible that the long untranslated leader sequence contains secondary operator sites. To investigate this possibility, we searched the DNA sequence for similar sequences. The sequences O-2 through O-5 in Fig. 1 represent additional operatorlike sequences. Another role of this region may be to promote DNA curvature. Two major intrinsic bends are predicted by computer analysis to occur at each side of the *Cla*I site (Fig. 3), around the positions labeled 200 and 230 in Fig. 1. To determine whether these features actually have a role in *put* regulation, we made small deletions in the regulatory region (Fig. 4) and studied their effect on binding of purified PutA protein in vitro and on *put* expression in vivo.

PutA protein binds to multiple sites in the *put* control region. To determine whether PutA protein binds to multiple sites in the *put* control region, we assayed gel retardation of DNA fragments from each of the deletions by PutA protein. PutA protein bound to control region DNA from each of the deletion mutants (Fig. 2). Since none of the deletions removed the potential operator site O-5 (Fig. 4), we also tested PutA binding to DNA fragments produced by PCR amplification that contained only that potential operator site. PutA protein bound to the fragment made from primers 6 to 8 as well as the fragment made from primers 5 to 2, containing the rest of the *put* control region (Fig. 2A). Furthermore, PutA protein also bound to these fragments after digestion with *Cla*I, eliminating overlapping sequences shared by the fragments (Fig. 2A). These results indicate that there are multiple binding sites for the PutA protein in the *put* control region.

DNA from the *put* control region is curved. A second feature that may be involved in regulation is the potential bending of the *put* control region (Fig. 3). The presence of curved DNA in the *put* control region was tested by observing the electrophoretic mobility of different subfragments of the *put* control region. As DNA snakes through a gel during electrophoresis, it must constantly reorient to find the gel pores and pass through them. This rate-limiting step is slower for curved DNA than for typical B-form DNA when the size of the gel pores decreases (15). Different fragments of *put* control region DNA were obtained by PCR amplification, using primers that annealed to different sites in the control region (Fig. 4). The fragments amplified from oligonucleotide 6 to oligonucleotide 2 or to oligonucleotide 4 showed a large decrease in mobility as the polyacrylamide concentration was increased, as expected for curved DNA (Table 1). To confirm these results, we carried out circular permutations of *put* control region subfragments (25). To make the circular permutations, the two PCR-amplified subfragments from oligonucleotide 6 to oligonucleotide 8 and from oligonucleotide 5 to oligonucleotide 7 were cloned into

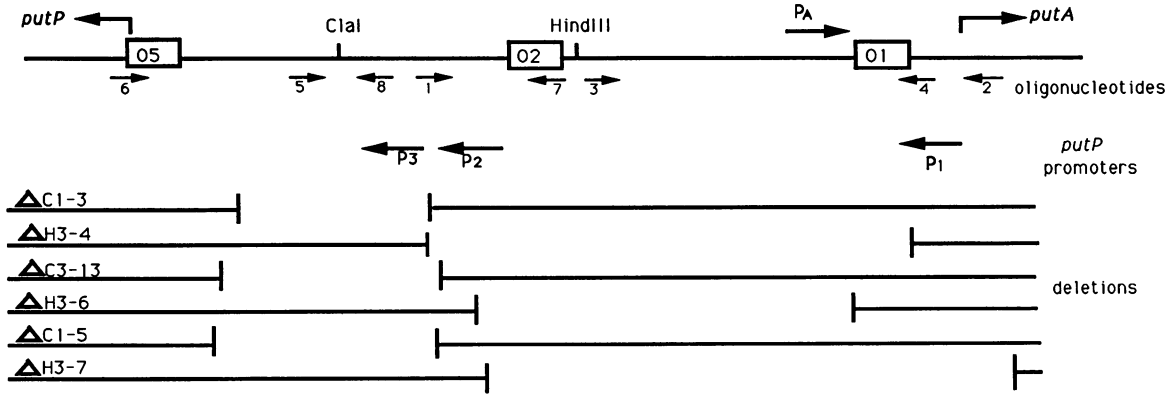

FIG. 4. Deletion mutations in the *put* control region. The top line with the boxed potential operator sites represents the *put* control region flanked by the beginning of the *putA* and *putP* structural genes. P_A indicates the *putA* promoter, and P_1 through P_3 indicate the *putP* potential promoters. The arrows numbered 1 to 8 represent the oligonucleotides used in the PCR amplifications. The positions of *Bal*31 deletions (ΔC1-3, ΔC1-5, ΔC3-13, ΔH3-4, ΔH3-6, and ΔH3-7) relative to the potential operator sites and promoters are shown as spaces between brackets that represents the DNA deleted in each case.

TABLE 1. Electrophoretic mobility of DNA fragments from the *put* control region

PCR between fragments from oligonucleotides[a]:	Fragment length[b] (bp)	Apparent length[c]		% Change[d]
		8% gel	10% gel	
6 to 8	189	194	194	
6 to 7	307	365	370	1.3
6 to 4	432	520	540	3.8
6 to 2	474	580	620	6.9
3 to 2	130	155	155	
1 to 2	222	270	270	
5 to 2	327	400	400	

[a] The oligonucleotides used are shown in Fig. 4.
[b] Determined from the DNA sequence.
[c] Determined by comparison with φX174/*Hae*III and pBR322/*Hae*III size standards run on 8 and 10% nondenaturing polyacrylamide gels at 4°C.
[d] Percent change in observed length when the fragments were run in 8 and 10% polyacrylamide gels.

the *Sal*I site of the vector pBEND2 (Fig. 5A) (26) in the same orientation as the sequences shown in Fig. 1 and 4. Circular permutations of the 6-8 fragment exhibited equal mobilities (data not shown). However, circular permutations of the 5-7 fragment showed increased retardation as they were cut from the *Bgl*II site to the *Bam*HI site, located at the far left and far right, respectively, of the duplicated restriction sites (Fig. 5B). These results indicate that the DNA to the right of the *Cla*I site in the *put* control region is curved. Plotting and extrapolation of the electrophoretic mobilities of these fragments versus the distance of the cloned 5-7 fragment from the left and right ends of the circularly permuted fragments indicated that the curved DNA is located near the region of the computer-predicted curvature labeled 230 in Fig. 1 (Fig. 5C).

Sites throughout the *put* control region are involved in regulation of the *put* operon. To determine whether the removal of potential operator sites or DNA bending sites affected *put* regulation, we assayed expression of the *put* genes in each of the deletion mutants. Expression of *putA* was assayed by measuring proline oxidase (Table 2), and expression of *putP* was assayed by measuring proline transport (Table 3). Both the deletions from the *Cla*I site and those from the *Hin*dIII site affected *putA* and *putP* expression, but the effect on expression of the two genes differed as described below.

Each of the *Cla*I deletions showed a higher basal level of proline oxidase activity, but they were still induced by proline. The *Hin*dIII deletions removed the *putA* promoter predicted from sequence analysis (7) and eliminated proline oxidase activity, indicating that this is the *putA* promoter used in vivo. Oligonucleotide extension analysis further confirmed that this is the functional *putA* promoter (2). Thus, the *Cla*I deletions remove sites in the *put* control region, upstream of the *putA* promoter, that are involved in repression of the *putA* gene.

Except for deletion H3-7, expression of the *putP* gene in all of the deletion mutants was still induced by proline. Compared with the wild type and other deletions, deletions C1-5 and H3-4 caused higher basal levels of *putP* expression, C1-3 and C3-13 caused lower levels of *putP* expression, and H3-6 caused lower levels of *putP* expression. These results indicate that multiple sites in the *put* control region are involved in regulation of *putP* gene expression in vivo. Deletion H3-7, which removed the potential *putP* promoter P_1, did not express *putP*. However, deletion C1-5, which removed the potential promoter P_3, and deletion H3-4, which removed P_2, still expressed *putP*. These data strongly suggest that P_1 is the major *putP* promoter used in vivo.

Deletions that remove the *putA* promoter but retain PutA repressor function. Both deletions H3-4 and H3-6 remove the *putA* promoter and therefore lack proline oxidase activity. No apparent new promoters for *putA* are created in these deletions. However, to our surprise, *putP* expression in both H3-4 and H3-6 was induced by proline. This finding raised the question of whether the observed regulation is mediated by the PutA protein. To answer this question, the *putA* structural gene was disrupted with *Bal*31 deletions in plasmids carrying deletion H3-4 (Fig. 6), and their ability to regulate *put* gene expression in *trans* was tested. The original H3-4 deletion plasmid, the H3-4 plasmids containing the additional deletions in the *putA* structural gene, and the wild-type pPC6 plasmid were then moved into strain MS1210. MS1210 contains a chromosomal *putA*::*lac* operon fusion, and therefore expression of β-galactosidase is regulated from the *putA* promoter.

Expression of β-galactosidase from each these strains was assayed (Table 4). In the absence of a complementing plasmid, β-galactosidase was expressed constitutively from the chromosomal *putA*::*lac* operon fusion. When pPC6 or H3-4 was introduced, expression of β-galactosidase from the *putA*::*lac* operon fusion was regulated by proline. Thus, H3-4 produces a factor that can act in *trans* to repress the *putA*::*lacZ* fusion. This repressor either is less efficient than the one made from the wild-type plasmid pPC6 or is produced in lower amounts. Deletions in the *putA* structural gene disrupt this *trans*-acting repressor function. These results indicate that the *trans*-acting factor produced by H3-4 that represses *put* expression is encoded by the *putA* gene. The PutA protein made by H3-4 must be able to bind to the operator site(s) and also bind proline since it functions in regulation, but it is defective for proline oxidase activity.

DISCUSSION

Genetic analysis of the *putA* gene suggests that it encodes an unusual multifunctional protein that acts as membrane-associated dehydrogenase and also regulates transcription of the *put* operon. Although the phenotypes of *putA* mutants imply that the *putA* gene encodes a *trans*-acting repressor for the *put* operon, there was no direct evidence that the PutA protein binds to DNA. Therefore, we determined whether purified PutA protein binds to *put* control region DNA in vitro by gel retardation assays. The electrophoretic mobility of a DNA fragment carrying the *put* control region was

TABLE 2. Expression of the *putA* gene product

Strain	Proline oxidase activity[a]		Induction ratio[b]
	−Proline	+Proline	
LT2	1.6	18.0	11.2
MS62	2.3	1.9	
MS62 (pPC6)	4.0	20.1	5.0
MS62 (C1-3)	5.7	10.7	1.9
MS62 (C1-5)	10.9	20.8	1.9
MS62 (C3-13)	17.2	20.9	1.2
MS62 (H3-4)	2.3	1.6	
MS62 (H3-6)	1.9	1.9	
MS62(H3-7)	1.8	1.9	

[a] Expressed as nanomoles of proline oxidized per minute per OD_{650}.
[b] Induced activity divided by uninduced activity.

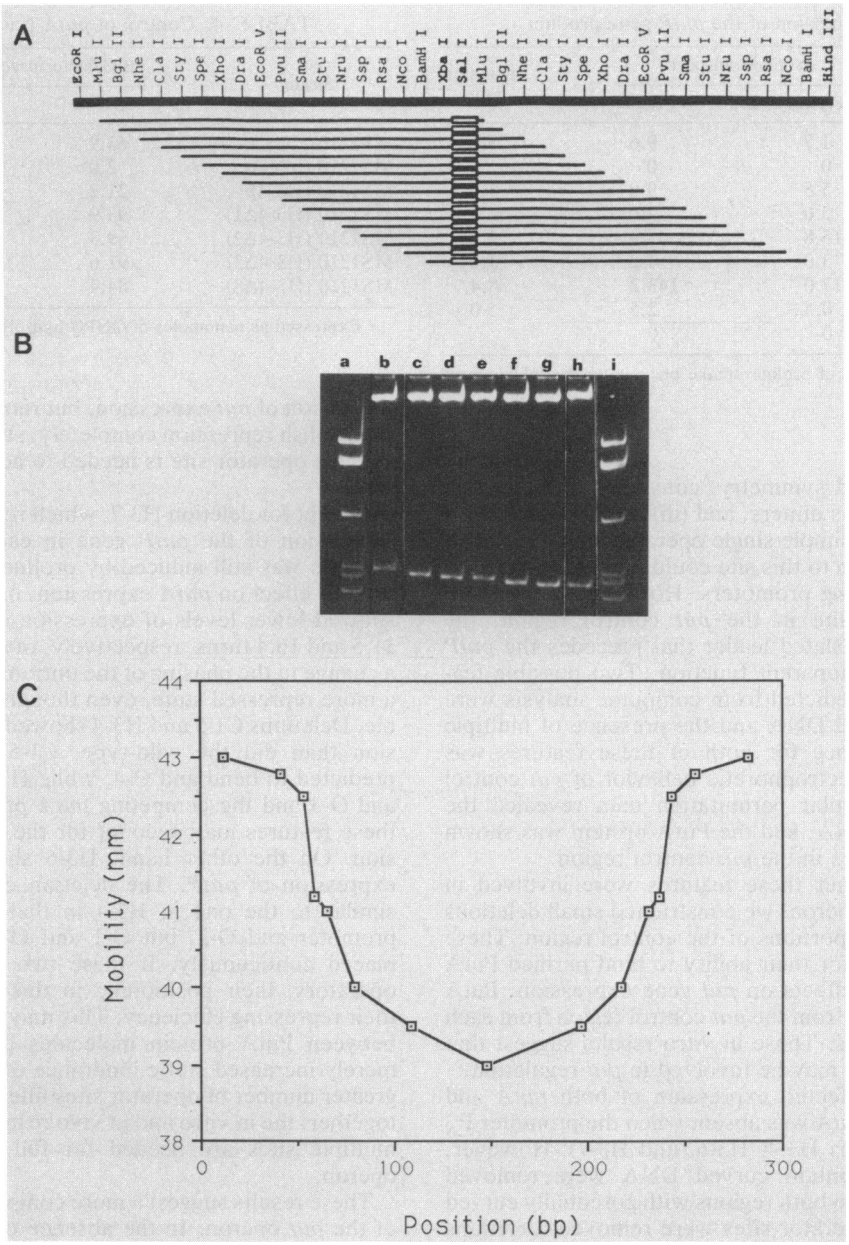

FIG. 5. Curvature of *put* control region DNA. (A) Order of the pBEND2 restriction sites used for circular permutations. The 236-bp fragment of the vector located between the *Eco*RI and *Hin*dIII sites of pBR322 is shown with the 17 duplicated restriction sites. (B) Circular permutations of the *put* control region fragment obtained by PCR amplification from oligonucleotide 5 to oligonucleotide 7 (see Fig. 4). Lanes a and i contain ϕX174/*Hae*III DNA size markers. Other lanes contain the following restriction digests: b, *Bgl*II; c, *Xho*I; d, *Eco*RV; e, *Pvu*II; f, *Sma*I; g, *Nru*I; and h, *Bam*HI. (C) Relative mobility of the 5-7 DNA fragment versus its distance from the ends of the circularly permuted 295-bp fragments (120 bp from the vector and 175 bp from the 5-7 fragment).

specifically retarded by purified PutA protein. Several lines of evidence indicate that the gel retardation is due to PutA protein: (i) in every purified PutA preparation, the DNA-binding activity was directly proportional to the PutA enzymatic activity (11); (ii) if the PutA protein was removed by immunoprecipitation, the gel retardation activity was lost (11); (iii) when protein was purified from *putA* deletion mutants in an identical fashion, the DNA-binding activity was absent (11); (iv) the concentration of PutA protein required for gel retardation in vitro was very similar to the concentration of PutA protein under repressed conditions in vivo (12); and (v) extensive genetic evidence indicates that the PutA protein functions as a repressor in vivo (14, 16). These results indicate that the *putA* gene encodes a DNA-binding protein specific for the *put* control region.

Regulation of the *put* operon was originally proposed to be the result of interactions between the PutA repressor protein and a single operator site. The best candidate for this operator site was the sequence labeled O-1 in Fig. 1. The reasons for this proposal were (i) this sequence is located between the two putative promoters, in agreement with the genetic mapping of operator constitutive mutants (7); (ii) the

TABLE 3. Expression of the *putP* gene product

Strain	Proline transport[a]		Induction ratio
	−Proline	+Proline	
LT2	1.7	9.6	5.6
MS62	0	0	
MS62 (pPC6)	5.5	9.0	1.6
MS62 (C1-3)	5.0	7.0	1.4
MS62 (C1-5)	15.8	45.9	2.9
MS62 (C3-13)	3.0	4.0	1.3
MS62 (H3-4)	17.0	143.2	8.4
MS62 (H3-6)	0.5	2.5	5.0
MS62 (H3-7)	0	0	

[a] Expressed as nanomoles of proline uptake per minute per milligram of protein.

TABLE 4. Control of *putA* transcription in *trans*

Strain	β-Galactosidase activity[a]		Induction ratio
	−Proline	+Proline	
MS1210	63.9	28.1	
MS1210 (pPC6)	2.0	10.1	5.0
MS1210 (H3-4)	21.2	41.5	2.0
MS1210 (H3-4Δ1)	93.9	43.5	
MS1210 (H3-4Δ2)	69.3	60.7	
MS1210 (H3-4Δ3)	91.6	74.5	
MS1210 (H3-4Δ8)	84.4	51.1	

[a] Expressed as nanomoles of ONPG hydrolyzed per minute per OD_{650}.

sequence showed dyad symmetry, consistent with the fact that PutA protein forms dimers; and (iii) the position of this sequence suggested a simple single operator model in which binding of PutA protein to this site could preclude transcription from both diverging promoters. However, if this were the only regulatory site in the *put* control region, the conserved long untranslated leader that precedes the *putP* gene would have no apparent function. Two possible features of this region predicted from computer analysis were the presence of curved DNA and the presence of multiple operator sites. Evidence for both of these features was obtained: abnormal electrophoretic behavior of *put* control region DNA and circular permutation data revealed the presence of curved DNA, and the PutA protein was shown to bind to multiple sites in the *put* control region.

To determine whether these features were involved in regulation of the *put* operon, we constructed small deletions that removed specific portions of the control region. These deletions were tested for their ability to bind purified PutA protein and for their effects on *put* gene expression. PutA protein bound to DNA from the *put* control region from each of the deletion mutants. These in vitro results suggest that multiple operator sites may be involved in *put* regulation.

All the deletions affected expression of both *putA* and *putP*. Expression of *putA* was absent when the promoter P_A was removed (deletions H3-4, H3-6, and H3-7). However, when regions that contain curved DNA were removed (deletion C1-3), or when both regions with potentially curved DNA and potential operator sites were removed (deletions C1-5 and C3-13), the basal level of *putA* expression was higher but still induced by proline. Thus, the sites containing potential operators or curved DNA are required for full repression of *put* expression, but removal of these sites does not abolish repression completely, suggesting that more than a single operator site is needed to achieve full repression in vivo.

Except for deletion H3-7, which removes the P_1 promoter, expression of the *putP* gene in each one of the deletion mutants was still induced by proline. However, in contrast to their effect on *putA* expression, deletions C1-3 and C3-13 showed lower levels of expression of *putP*. They removed 11.5 and 16.4 turns, respectively, of a helix; this could cause a change in the phasing of the untranslated leader that favors a more repressed state, even though induction is still possible. Deletions C1-5 and H3-4 showed higher levels of expression than did the wild-type. C1-5 is missing the region predicted to bend and O-4, while H3-4 is missing O-1, O-2, and O-3 and the competing *putA* promoter. Some or all of these features may account for the increased *putP* expression. On the other hand, H3-6 showed a lower level of expression of *putP*. The deletion created in H3-6 is very similar to the one in H3-4 in that it takes out the *putA* promoter and O-2, but O-1 and O-3 are still present and placed contiguously. If these two sites actually work as operators, their positioning in this deletion may increase their repressing efficiency. This may be due to cooperativity between PutA protein molecules bound to both sites or merely increased steric hindrance of RNA polymerase by a greater number of operator sites filled with repressor. Taken together, the in vitro and in vivo results strongly suggest that multiple sites are needed for full repression of the *put* operon.

These results suggest a more complex model for regulation of the *put* operon. In the absence of proline, PutA protein may accumulate in the cytoplasm and bind to multiple operator sites in the *put* control region. PutA protein bound to different sites may interact, promoted by the DNA flexi-

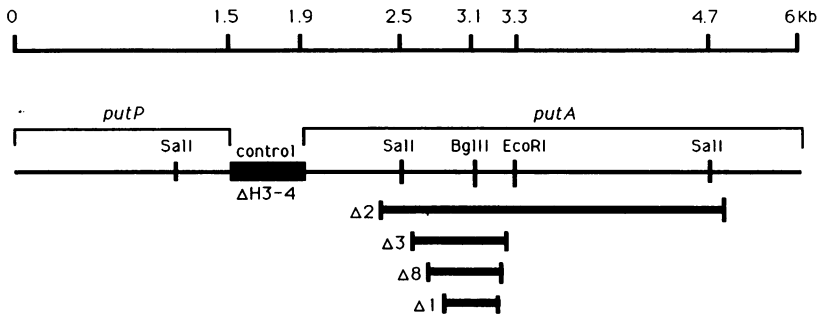

FIG. 6. Additional deletions in the *putA* structural gene made on the H3-4 deletion plasmid. The brackets at each side of the *put* control region represent the two structural genes, and the line at the top indicates the scale.

bility in the curved untranslated leader. Under inducing conditions, proline oxidation would reduce the tightly associated flavin adenine dinucleotide coenzyme, inducing the PutA protein to become membrane associated and abandon the operator sites, thus permitting expression of the *put* genes.

In some respects, regulation of the *put* operon may be similar to regulation of the *ara* operon. The *ara* control region contains multiple operator sites that regulate transcription of the divergent *araC* and *araBAD* genes. Proper contact between AraC proteins bound at different operator sites is facilitated by looping of the long stretch of DNA that separates them (9). Likewise, the *put* operon also has multiple operator sites separated by a long untranslated sequence containing curved DNA that could facilitate DNA looping. Although the mechanism of regulation of the *put* operon may share some features with regulation of the *ara* operon, the PutA repressor is unique. The molecular size of the PutA protein monomer is 132 kDa, much larger than other characterized repressor proteins. Furthermore, the PutA protein functions both as a repressor protein and as an enzyme that catalyzes two different catabolic steps in the oxidation of proline, one of which requires membrane association. Therefore, the PutA protein must bind proline, flavin adenine dinucleotide, NAD, the membrane, and DNA. Since PutA protein is quite different from other known repressors, we are now trying to determine precisely how it interacts with DNA.

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