Analysis of the *Escherichia coli* *proBA* locus by DNA and protein sequencing

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ABSTRACT

A 2.9 kb DNA fragment carrying the *Escherichia coli* *proBA* region, which encodes the first two enzymes of the proline biosynthetic pathway, was subcloned onto an expression plasmid carrying both the bacteriophage lambda P_L promoter (λP_L) and the lambda gene encoding a thermolabile cI repressor protein (cI857). Derepression of the λP_L promoter by thermal inactivation of the cI857 repressor protein resulted in the simultaneous overproduction of the *proB* (γ -glutamyl kinase) and *proA* (γ -glutamyl phosphate reductase) gene products. Nucleotide sequence analysis of the *proBA* locus allowed gene assignments consistent with the NH_2 and $COOH$ -terminal analyses and amino acid compositions of homogeneous preparations of the *proB* and *proA* proteins. The contiguous nature of the *proB* and *proA* genes suggests that the two genes constitute an operon in which *proB* precedes *proA*.

INTRODUCTION

In *Escherichia coli*, conversion of glutamate to proline is mediated by the sequential action of three enzymes; γ -glutamyl kinase (ATP: L-glutamate 5-phosphotransferase; EC 2.7.2.11), γ -glutamyl phosphate reductase [L-glutamate 5-semialdehyde: $NADP^+$ oxidoreductase (phosphorylating); EC 1.2.1.41], and Δ^1 -pyrroline-5-carboxylate reductase (L-proline: $NAD(P)^+$ 5-oxidoreductase; EC 1.5.1.2). These enzymes are the gene products of the *proB*, *proA* and *proC* loci, respectively (1,2). The first enzyme in the proline biosynthetic pathway, γ -glutamyl kinase (GK), catalyzes the ATP-dependent γ -phosphorylation of glutamate to form γ -glutamyl phosphate and ADP (3). The second enzyme of the proline pathway, γ -glutamyl phosphate reductase (GPR), catalyzes the NADPH-dependent reduction of γ -glutamyl phosphate to glutamate 5-semialdehyde and $NADP^+$ (4). L-glutamate 5-semialdehyde undergoes a spontaneous cyclization to form Δ^1 -pyrroline-5-carboxylate which is then converted to proline through the action of the third enzyme in the proline pathway, Δ^1 -pyrroline-5-carboxylate reductase (2,5).

The primary goal of our research efforts has been to genetically engineer the Gram-negative bacterium, *E. coli*, to overproduce free amino acids such as

proline. The overproduction of amino acids via fermentation is partially dependent upon overcoming the normal cellular regulatory circuits such as feedback inhibition and repression. Only GK, the first enzyme in the proline biosynthetic pathway, is subject to allosteric feedback inhibition by proline (3). Although it is unclear from the literature whether proB is subject to repression (1,6,10), neither proA nor proC appear to be regulated in this fashion. In a previous publication (7), we reported the nucleotide sequence of the proC gene, and the overproduction, purification to homogeneity and characterization of the corresponding gene product, Δ^1 -pyrroline-5-carboxylate reductase. Evidence presented suggested that transcriptional control of the proC gene might occur by either autogenous regulation or by positive activation.

As part of our continuing studies concerning the regulation of the proline biosynthetic pathway, we now report the overproduction, purification to homogeneity and partial characterization of the gene products of the proB and proA loci, as well as the nucleotide sequence of the proBA region. The proB gene we describe encodes a GK enzyme which is resistant to feedback inhibition by proline.

MATERIALS AND METHODS

Enzymes

Restriction endonucleases, T_4 -polynucleotide kinase, bacterial-alkaline phosphatase, T_4 -DNA ligase, terminal transferase, and E. coli DNA polymerase I (large fragment) were all supplied by Bethesda Research Laboratories, Inc., and were used as suggested by the manufacturer.

Bacterial Strains and Plasmids

E. coli K12 strain K802 (hsdR supE lacY galK metA) and the ColEI hybrid plasmid pLC7-19 were obtained from the E. coli Genetic Stock Center (Yale University). The expression plasmid pGW7 has already been described (7).

Construction and Screening of pGW7-proBA Recombinants

Plasmid pLC7-19 was obtained from the Clarke and Carbon collection of hybrid plasmids (8). We selected for a spontaneous mutation, mapping within the proB locus of pLC7-19, which conferred cellular resistance to 100 μ g/ml of DHP. GK activity, from strains harboring plasmids which conferred DHP resistance, was considerably more resistant to the inhibitory effects of proline than the GK activity from wild-type strains (9).

A 3.0 kb PstI DNA fragment, isolated from the modified pLC7-19 plasmid, was identified as encoding both the affected proB locus and the wild-type proA locus (10). The 3.0 kb PstI DNA fragment was subcloned onto the plasmid

pBR322, and the desired recombinant transformants were identified by the nutritional complementation of proline-auxotrophic *E. coli*. The proline prototrophic transformants were then transferred to lawns of proline-auxotrophic *E. coli* and tested for the ability to "cross-feed". Transformants which supported the nutritional requirement of the lawn, and which were therefore capable of overproducing proline, were plated on DHP-containing plates and shown to be resistant to the anti-metabolite. Restriction analysis of the isolated plasmid DNA confirmed the insertion of the 3.0 kb *Pst*I DNA fragment. This recombinant plasmid (pBR322-*proBA*) was the primary source of *proBA* DNA for sequence analysis and other *in vitro* manipulations.

The plasmid pBR322-*proBA* was digested sequentially with *Pst*I and *Bgl*II, and the resulting fragments were fractionated by electrophoresis on a 1.2% agarose gel. The 2.9 kb DNA fragment, containing the functionally intact *proBA* genes, was recovered by electrophoretic elution and purified by precipitation with spermine (11).

The staggered ends of *Bam*HI cleaved pGW7 and purified 2.9 kb *proBA* DNAs were made flush by the action of *E. coli* DNA polymerase I (large fragment). The DNAs were ligated together and used to transform strain K802. DHP resistant transformants were selected at 30°C on minimal agar plates containing DHP at 100 ug/ml. All transformants were ampicillin resistant and had the ability to excrete proline and thus "cross-feed" (some in a temperature dependent fashion) proline-requiring strains.

Total cellular protein prepared from the ampicillin and DHP resistant transformants was analyzed by SDS-polyacrylamide gel electrophoresis as described (7). The specific activities of GK and GPR as a function of temperature induction of the λP_L promoter were also measured. Finally, plasmid DNAs containing the 2.9 kb *proBA* DNA fragment were analyzed for insert-fragment copy number and orientation.

Cell-free Extract Preparation, Enzyme Assays and Enzyme Purification

Cells were suspended in 50mM Tris base-1mM dithiothreitol, pH 7.2, and disrupted by ultrasonic treatment as previously described (9).

The assay for GK activity in crude extracts was performed as described (1) except that the reaction mixture contained the following in a final volume of 0.25ml at pH 7.0: L-glutamate, 50mM; ATP, 10mM; $MgCl_2$, 20mM; L-methionine D,L-sulfoximine, 2.5mM; hydroxylamine HCl, 100 mM; Tris base, 50mM; and enzyme plus water. Inclusion of the glutamine synthetase inhibitor, methionine sulfoximine, greatly decreased background activity and did not interfere with GK activity (9). The assay for GK activity in purified preparations was

dependent upon the addition of GPR (9, see Discussion), and was performed as described (9). GPR activity was measured as described (1) except that the concentration of the substrate, D,L- Δ^1 -pyrroline-5-carboxylate, was increased from 1mM to 2.5mM. The synthesis of D,L- Δ^1 -pyrroline-5-carboxylate was accomplished as described (12).

Purification of GK and GPR

GK was purified to homogeneity by sequential chromatography on DEAE-cellulose, Procion Red-agarose, Cibacron Blue-agarose, and Hydroxylapatite (9). Homogenous GPR was prepared by DEAE-cellulose and Hydroxylapatite chromatography as described (9). Both purified enzymes were essentially free of contaminating protein as judged by SDS-polyacrylamide gel electrophoresis.

DNA Sequencing of the 3.0 kb proBA Region

The DNA sequence of the 3.0 kb PstI restriction fragment encoding the proBA genes was determined utilizing both the chemical modification procedure of Maxam and Gilbert (13), and the dideoxy chain-termination method of Sanger et al. (14). The five base-specific chemical modification reactions (G, G + A, A > C, C + T, and C) were performed essentially as described (13). For dideoxy sequencing, DNA restriction fragments generated by digestion of the 3.0 kb PstI fragment with AluI and TaqI were cloned into the SmaI and AccI sites of M13-mp8 (15), respectively. In addition, the entire 3.0 kb PstI fragment was cloned into the PstI site of M13-mp8.

Amino Acid Analysis

Amino acid compositions of the purified GK and GPR proteins were determined as previously described (7). NH_2 -terminal amino acid sequencing (GK and GPR) was performed on an updated Beckman 890C sequencer using polybrene (16). COOH-terminal (GPR) analysis was performed as described previously (17) utilizing carboxypeptidase Y.

RESULTS

Overproduction of GK and GPR

The proBA locus was cloned onto the expression plasmid pGW7, to facilitate the production and purification of the proBA gene products. Transformants were identified that overproduced both GK and GPR in response to temperature elevation. One transformant in particular exhibited enzyme activities that were significantly higher than other transformants when the temperature increased to 42°C. Plasmid pGW7-pro(BA)₂, isolated from this transformant, was analyzed by restriction enzyme cleavage and was found to contain a tandem repeat of the 2.9 kb proBA DNA fragment inserted at the BamHI site in pGW7 (Figure 1). Transformants which possessed temperature modulated

Figure 1. Diagram of the Plasmid pGW7-pro(BA)₂ Used to Overproduce the proB and proA Gene Products. The insertion of the direct repeat of the 2.9 kb DNA fragment, encoding the proB (GK) and proA (GPR) gene products, into the BamHI site of the expression plasmid pGW7 is described under Materials and Methods. Thick line, DNA derived from phage λ; thin line, DNA derived from pBR322. Arrows indicate the direction of transcription initiating from the λ_P and λ_P^R promoters as well as the direction of transcription of the proBA genes.

levels of enzyme activities lower than that obtained in transformants harboring pGW7-pro(BA)₂ were found to contain plasmid DNA with a single copy of the 2.9 kb proBA fragment oriented as shown for pGW7-pro(BA)₂ in Figure 1. Another class of transformants was found to contain a single copy of the 2.9 kb DNA fragment inserted in pGW7 in the opposite orientation as that found in pGW7-pro(BA)₂. Transformants harboring this plasmid construct, pGW7-proBA', were found to possess elevated levels of GK and GPR that were not enhanced following derepression of the λ P_T promoter.

TABLE 1. Specific activities of γ -glutamyl kinase and γ -glutamyl phosphate reductase as a function of increasing temperature^a

Plasmid ^b	Temperature	Specific Activity ^c	
		γ -glutamyl kinase	γ -glutamyl phosphate reductase
None	30°C	0.592 $\pm$ 0.16	2.09 $\pm$ 0.50
	37°C	0.498 $\pm$ 0.16	1.77 $\pm$ 0.65
	42°C	0.525 $\pm$ 0.18	1.35 $\pm$ 0.60
pGW7- <u>proBA</u> '	30°C	9.59 $\pm$ 2.2	59.73 $\pm$ 10
	37°C	7.30 $\pm$ 1.8	47.34 $\pm$ 12
	42°C	5.15 $\pm$ 1.1	25.96 $\pm$ 5
pGW7- <u>pro</u> (BA) ₂	30°C	22.54 $\pm$ 2.2	125.0 $\pm$ 14
	37°C	25.45 $\pm$ 2.5	175.7 $\pm$ 20
	42°C	50.41 $\pm$ 12.4	613.0 $\pm$ 20

^aStrain K802 with and without plasmid was grown at 30°C in complex broth with and without ampicillin, respectively. At an optical density of 0.65–0.80, at 660 nm, 230ml portions of each culture were incubated at 30°C, 37°C, and 42°C for a period of two hours. Enzyme activity was assayed as described in Materials and Methods.

^bpGW7-proBA' and pGW7-pro(BA)₂ contain the proBA region cloned in opposite orientations. pGW7-proBA' contains a single copy of the 2.9 kb proBA region in the wrong orientation relative to P_L. pGW7-pro(BA)₂ contains two copies of the 2.9 kb fragment in a direct tandem repeat oriented as to be placed under the control of the λ P_L promoter (see Figure 1).

^cAll values represent the average of three independent experiments $\pm$ the standard deviation. γ -Glutamyl kinase activity is expressed as nanomoles product formed per minute per milligram of protein. γ -Glutamyl phosphate reductase activity is expressed as nanomoles NADP reduced per minute per milligram of protein.

binding of ribosomes to either the proB or the proA ribosomal binding site (see Discussion).

As the temperature of the pGW7-proBA' cell culture was increased from 30°C to 42°C both GK and GPR exhibited a decrease in their specific activities of 46% and 57%, respectively. The observed decreases in specific activity is consistent with the proBA DNA insert being situated in the opposite orientation as required for transcription from λ P_L. The transcriptional activity of RNA polymerase molecules initiating at the proB promoter would be opposed by RNA polymerase molecules initiating at the λ P_L promoter and transcribing in the opposite direction.

Enzymatic analysis of cells harboring the plasmid pGW7-pro(BA)₂ and grown at 30°C revealed a 2.2-fold increase in the specific activities of GK and GPR

Figure 2. SDS-polyacrylamide Gel Electrophoretic Analysis of Proteins Accumulated During Induction of the λP_L Promoter. *E. coli* strain K802 with and without plasmid was grown as described in Table 1. Following the two hour incubation at either 30°C, 37°C or 42°C, 0.1 ml aliquots of each of the nine cultures were subjected to centrifugation in an Eppendorf microfuge. The cell pellets were resuspended in electrophoresis sample buffer, boiled for five minutes and electrophoresed on a 7.5%-20% polyacrylamide gradient slab gel containing 0.1% SDS (34). After electrophoresis the protein bands were visualized by staining with Coomassie Brilliant Blue R-250. Arrows on left indicate the position of protein molecular weight markers. Arrows on right indicate the location of γ -glutamyl kinase (GK) and γ -glutamyl phosphate reductase (GPR).

as compared to cells containing pGW7-proBA'. This increase is consistent with the gene dosage effect expected for a plasmid containing a tandem repeat of the proBA DNA fragment. Shifting the temperature of the culture from 30°C to 42°C resulted in an additional 2-fold and 5-fold increase, respectively, in the specific activities of GK and GPR. Overall this corresponded to a respective 100-fold and 350-fold increase in comparison to wild-type cells, and is indicative of control by the λP_L promoter.

Total soluble protein obtained from cells grown at either 30°C, 37°C, or 42°C was fractionated by SDS-polyacrylamide gel electrophoresis and the abundant proteins were detected by staining with Coomassie Brilliant Blue (Figure 2). Cells containing no plasmid and cells containing the plasmid pGW7-proBA' showed similar protein profiles. Analysis of cells containing the plasmid pGW7-pro(BA)₂ revealed the plasmid-specified accumulation of two major protein species of molecular weight 37k and 41.5k. These protein bands were prominent even when protein was examined from cells grown at 30°C, and the intensity of staining increased further as the temperature of the culture was increased to 42°C. The appearance and accumulation of the two proteins were concomitant with the observed increases in enzymatic activity (Table 1), as well as with the appearance (as detected by SDS-polyacrylamide gel electrophoresis) of two ³⁵S-methionine labeled proteins of molecular weights of 37k and 41.5k. These proteins were synthesized preferentially during temperature induction of ³⁵S-methionine pulse-labeled cells harboring pGW7-pro(AB)₂ (data not shown).

A comparison of the electrophoretic mobilities of the purified GK and GPR enzymes with the total soluble protein extracted from temperature induced cells harboring pGW7-pro(BA)₂ was made by SDS-polyacrylamide gel electrophoresis. The induced protein of molecular weight 41.5k comigrated with purified GPR, and the induced protein of molecular weight of 37.0k comigrated with purified GK. The molecular weight of GPR is somewhat lower than that previously reported (18). Densitometric analysis of the Coomassie stained SDS-polyacrylamide gels indicated that GK and GPR together represented approximately 20-25% of the total soluble protein in the cell, with GPR accumulating in a 2 to 3-fold molar excess over GK (See Discussion).

Nucleotide Sequence of the 3.0 kb DNA Fragment Encoding GK and GPR

The strategy employed in obtaining the DNA sequence of the *E. coli* proBA region is shown in Figure 3. Analysis of the DNA sequence (Figure 4) revealed two tandem open reading frames encoding amino acid sequences that correlate well with those determined for the proB and proA gene products (Table 2, Figure 4). Each of the two open reading frames show a pattern of codon usage (data not shown) similar to that observed for the combined codon distribution of 52 *E. coli* genes (19).

The DNA sequence upstream from the 5'-terminus of the proB gene, from nucleotide position 150-214, is extremely AT-rich (86%) as compared to the *E. coli* genome (49% AT-rich). This region and flanking sequences contain multiple dyad symmetries (Figure 4).

Figure 3. Sequencing Strategy for the proBA Region. The arrows denote sequencing direction and length of sequences obtained in individual experiments. Dashed arrows indicate sequence data obtained from strand-separated DNA fragments; bold arrows indicate sequence data obtained by the dideoxy chain-termination method; thin arrows indicate sequence data obtained by the Maxam and Gilbert method. Also shown are the restriction enzymes used to generate the various sequence start sites (•), as well as the codons for the initiation and termination of translation of the proB and proA gene products.

The nature of the DNA sequence upstream from the proB coding region precludes firm assignments of promoter regions. A 50 bp region encompassing the 5'-terminus of the proB gene was compared with published (20) E. coli promoter DNA sequences (Figure 5). The putative -10 region of the proB gene is homologous with the -10 region of the str and uvrB (P1) genes, and differs from the -10 region of several other genes by only a single nucleotide. The sequence ATTTTC, which precedes the putative -10 region of proB, is homologous with the sequence present at the analogous region in the T5-₂₅ gene. The significance of this homology is uncertain since this region has not been implicated in any regulatory control of transcription. Finally, both λ PRE and proB contain the sequence CGTT(Pyr)GTTTG, centered at position -35.

A putative Shine-Dalgarno sequence, GAG, precedes the proB initiator methionine codon and an open reading frame is terminated by a single TAA nonsense codon at position 1455 (Figure 4).

Two putative proA Shine-Dalgarno sites have been identified within the 14 bp intergenic region between proB and proA (Figure 4). The sequence TAAGGAG is complementary to the 3'-region of the 16S rRNA considered to be involved in the initiation of translation. This intergenic sequence presumably functions in the termination of translation of the proB gene product in addition to the initiation of translation of the proA gene product (see Discussion). An additional ribosomal binding site within the intergenic region might occur at AGG.

The proA gene encodes 407 amino acids and terminates at nucleotide

CTGCAGCCTGTACAGATGCAGATGCCACAATGCCCATCACCCTAATGCCAGAGTGCTCTTTTTCATTT 69
 TCAATTCCTGATTTTAATTAACGCGCGAATATTACGCGGGAGAGTCCCGTTGAAAACAGGAAAGTTTTAAC 140
 CTGAGATTGTTAAAGATATATTACAGATTAATAATTCTTAAATGTGGTAATTTATTAATCTGTAATA 211
 AAAGCGTAAACAACCTGCCGCTAGGCTGCTGATCCCGCGCAACAAAACGCCATGCTTTGCTCGCAGATGGTT 282
 GGCAACCGACGACAGTCTGCTAAAACGTTCTTTGATATCATTTCCTAAAATTGAATGGCAGAGAATC 353
 proB→
 MET SER ASP SER GLN THR LEU VAL VAL LYS LEU GLY THR SER VAL LEU THR GLY 407
 ATG AGT GAC AGC CAG ACG CTG GTG GTA AAA CTC GGC ACC AGT GTG CTA ACA GGC
 Chi site
 GLY SER ARG ARG LEU ASN ARG ALA HIS ILE VAL GLU LEU VAL ARG GLN CYS ALA 461
 GGA TCG CGC CGT CTG AAC CGT GCC CAT ATC GTT GAA CTT GTT CGC CAG TGC GCG
 GLN LEU HIS ALA ALA GLY HIS ARG ILE VAL ILE VAL THR SER GLY ALA ILE ALA 515
 CAG TTA CAT GCC GCC GGG CAT CGG ATT GTT ATT GTG ACG TCG GGC GCG ATC GCC
 ALA GLY ARG GLU HIS LEU GLY TYR PRO GLU LEU PRO ALA THR ILE ALA SER LYS 569
 GCC GGA CGT GAG CAC CTG GGT TAC CCG GAA CTG CCA GCG ACC ATC GCC TCG AAA
 GLN LEU LEU ALA ALA VAL GLY GLN SER ARG LEU ILE GLN LEU TRP GLU GLN LEU 623
 CAA CTG CTG GCG GCG GTA GGG CAG AGT CGA CTG ATT CAA CTG TGG GAA CAG CTG
 PHE SER ILE TYR GLY ILE HIS VAL GLY GLN MET LEU LEU THR ARG ALA ASP MET 677
 TTT TCG ATT TAT GGC ATT CAC GTC GGG CAA ATG CTG CTG ACC CGT GCT GAT ATG
 GLU ASP ARG GLU ARG PHE LEU ASN ALA ARG ASP THR LEU ARG ALA LEU LEU ASP 731
 GAA GAC CGT GAA CGC TTC CTG AAC GCC CGC GAC ACC CTG CGA GCG TTG CTC GAT
 ASN ASN ILE VAL PRO VAL ILE ASN GLU ASN ASP ALA VAL ALA THR ALA ALA ILE 785
 AAC AAT ATC GTT CCG GTA ATC AAT GAG AAC GAT GCT GTC GCT ACG GCA GCG ATT
 LYS VAL GLY ASP ASN ASP ASN LEU SER ALA LEU ALA ALA ILE LEU ALA GLY ALA 839
 AAG GTC GGC GAT AAC GAT AAC CTT TCT GCG CTG GCG GCG ATT CTT GCG GGT GCC
 ASP LYS LEU LEU LEU LEU THR ASP GLN LYS GLY LEU TYR THR ALA ASP PRO ARG 893
 GAT AAA CTG TTG CTG CTG ACC GAT CAA AAA GGT TTG TAT ACC GCT GAC CCG CGC
 SER ASN PRO GLN ALA GLU LEU ILE LYS ASP VAL TYR GLY ILE ASP ASP ALA LEU 947
 AGC AAT CCG CAG GCA GAA CTG ATT AAA GAT GTT TAC GGC ATT GAT GAC GCA CTG
 ARG ALA ILE ALA GLY ASP SER VAL SER GLY LEU GLY THR GLY GLY MET SER THR 1001
 CGC GCG ATT GCC GGT GAC AGC GTT TCA GGC CTC GGA ACT GGC GGC ATG AGT ACC

LYS	LEU	GLN	ALA	ALA	ASP	VAL	ALA	CYS	ARG	ALA	GLY	ILE	ASP	THR	ILE	ILE	ALA	
AAA	TTG	CAG	GCC	GCT	GAC	GTG	GCT	TGC	CGT	GCG	GGT	ATC	GAC	ACC	ATT	ATT	GCC	1055
ALA	GLY	SER	LYS	PRO	GLY	VAL	ILE	GLY	ASP	VAL	MET	GLU	GLY	ILE	SER	VAL	GLY	
GCG	GGC	AGC	AAG	CCG	GGC	GTT	ATT	GGT	GAT	GTG	ATG	GAA	GGC	ATT	TCC	GTC	GGT	1109
THR	LEU	PHE	HIS	ALA	GLN	ALA	THR	PRO	LEU	GLU	ASN	ARG	LYS	ARG	TRP	ILE	PHE	
ACG	CTG	TTC	CAT	GCC	CAG	GCG	ACT	CCG	CTT	GAA	AAC	CGT	AAA	CGC	TGG	ATT	TTC	1163
GLY	ALA	PRO	PRO	ALA	GLY	GLU	ILE	THR	VAL	ASP	GLU	GLY	ALA	THR	ALA	ALA	ILE	
GGT	GCG	CCG	CCG	GCG	GGT	GAA	ATC	ACG	GTA	GAT	GAA	GGG	GCA	ACT	GCC	GCC	ATT	1217
LEU	GLU	ARG	GLY	SER	SER	LEU	LEU	PRO	LYS	GLY	ILE	LYS	SER	VAL	THR	GLY	ASN	
CTG	GAA	CGC	GGC	AGC	TCC	CTG	TTG	CCG	AAA	GGC	ATT	AAA	AGC	GTG	ACT	GGC	AAT	1271
PHE	SER	ARG	GLY	GLU	VAL	ILE	ARG	ILE	CYS	ASN	LEU	GLU	GLY	ARG	ASP	ILE	ALA	
TTC	TCG	CGT	GGT	GAA	GTC	ATC	CGC	ATT	TGC	AAC	CTC	GAA	GGC	CGC	GAT	ATC	GCC	1325
HIS	GLY	VAL	SER	ARG	TYR	ASN	SER	ASP	ALA	LEU	ARG	ARG	ILE	ALA	GLY	HIS	HIS	
CAC	GGC	GTC	AGT	CGT	TAC	AAC	AGC	GAT	GCA	TTA	CGC	CGT	ATT	GCC	GGA	CAC	CAC	1379
SER	GLN	GLU	ILE	ASP	ALA	ILE	LEU	GLY	TYR	GLU	TYR	GLY	PRO	VAL	ALA	VAL	HIS	
TCG	CAA	GAA	ATT	GAT	GCA	ATA	CTG	GGA	TAT	GAA	TAC	GGC	CCG	GTT	GCC	GTT	CAC	1433
ARG	ASP	ASP	MET	ILE	THR	ARG	***											
CGT	GAT	GAC	ATG	ATT	ACC	CGT	TAA	GGAGCAGGCTG	ATG	CTG	GAA	CAA	ATG	GGC	ATT			1489
ALA	ALA	LYS	GLN	ALA	SER	TYR	LYS	LEU	ALA	GLN	LEU	SER	SER	ARG	GLU	LYS	ASN	
GCC	GCG	AAG	CAA	GCC	TCG	TAT	AAA	TTA	GCG	CAA	CTC	TCC	AGC	CGC	GAA	AAA	AAT	1543
ARG	VAL	LEU	GLU	LYS	ILE	ALA	ASP	GLU	LEU	GLU	ALA	GLN	SER	GLU	ILE	ILE	LEU	
CGC	GTG	CTG	GAA	AAA	ATC	GCC	GAT	GAA	CTG	GAA	GCA	CAA	AGC	GAA	ATC	ATC	CTC	1597
ASN	ALA	ASN	ALA	GLN	ASP	VAL	ALA	ASP	ALA	ARG	ALA	ASN	GLY	LEU	SER	GLU	ALA	
AAC	GCT	AAC	GCC	CAG	GAT	GTT	GCT	GAC	GCG	CGA	GCC	AAT	GGC	CTT	AGC	GAA	GCG	1651
MET	LEU	ASP	ARG	LEU	ALA	LEU	THR	PRO	ALA	ARG	LEU	LYS	GLY	ILE	ALA	ASP	ASP	
ATG	CTT	GAC	CGT	CTG	GCA	CTG	ACG	CCC	GCA	CGG	CTG	AAA	GGC	ATT	GCC	GAC	GAT	1705
VAL	ARG	GLN	VAL	CYS	ASN	LEU	ALA	ASP	PRO	VAL	GLY	GLN	VAL	ILE	ASP	GLY	GLY	
GTA	CGT	CAG	GTG	TGC	AAC	CTC	GCC	GAT	CCG	GTG	GGG	CAG	GTA	ATC	GAT	GGC	GCG	1759

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VAL GTA	LEU CTG	ASP GAC	SER AGC	GLY GGC	LEU CTG	ARG CGT	LEU CTT	GLU GAG	ARG CGT	ARG CGT	ARG CGC	VAL GTA	PRO CCG	LEU CTG	GLY GGG	VAL GTT	ILE ATT	1813
GLY GGC	VAL GTG	ILE ATT	TYR TAT	GLU GAA	ALA GCG	ARG CGC	PRO CCG	ASN AAC	VAL GTG	THR ACG	VAL GTT	ASP GAT	VAL GTC	ALA GCT	SER TCG	LEU CTG	CYS TGC	1867
LEU CTG	LYS AAA	THR ACC	GLY GGT	ASN AAT	ALA GCG	VAL GTG	ILE ATC	LEU CTG	ARG CGC	GLY GGT	GLY GGC	LYS AAA	GLU GAA	THR ACG	CYS TGT	ARG CGC	THR ACT	1921
ASN AAC	ALA GCT	ALA GCA	THR ACG	VAL GTG	ALA GCG	VAL GTG	ILE ATT	GLN CAG	ASP GAC	ALA GCC	LEU CTG	LYS AAA	SER TCC	CYS TGC	GLY GGC	LEU TTA	PRO CCG	1975
ALA GCG	GLY GGT	ALA GCC	VAL GTG	GLN CAG	ALA GCG	ILE ATT	ASP GAT	ASN AAT	PRO CCT	ASP GAC	ARG CGT	ALA GCG	LEU CTG	VAL GTC	SER AGT	GLU GAA	MET ATG	2029
LEU CTG	ARG CGT	MET ATG	ASP GAT	LYS AAA	TYR TAC	ILE ATC	ASP GAC	MET ATG	LEU CTG	ILE ATC	PRO CCG	ARG CGT	GLY GGT	GLY GGC	ALA GCT	GLY GGT	LEU TTG	2083
HIS CAT	LYS AAA	LEU CTG	CYS TGC	ARG CGT	GLU GAA	GLN CAG	SER TCG	THR ACA	ILE ATC	PRO CCG	VAL GTG	ILE ATC	THR ACA	GLY GGT	GLY GGT	ILE ATA	GLY GGC	2137
VAL GTA	CYS TGC	HIS CAT	ILE ATT	TYR TAC	VAL GTT	ASP GAT	GLU GAA	SER AGT	VAL GTA	GLU GAG	ILE ATC	ALA GCT	GLU GAA	ALA GCA	LEU TTA	LYS AAA	VAL GTG	2191
ILE ATC	VAL GTC	ASN AAC	ALA GCG	LYS AAA	THR ACT	GLN CAG	ARG CGT	PRO CCG	SER AGC	THR ACA	CYS TGT	ASN AAT	THR ACG	VAL GTT	GLU GAA	THR ACG	LEU TTG	2245
LEU CTG	VAL GTG	ASN AAT	LYS AAA	ASN AAC	ILE ATC	ALA GCC	ASP GAT	SER AGC	PHE TTC	LEU CTG	PRO CCC	ALA GCA	LEU TTA	SER AGC	LYS AAA	GLN CAA	MET ATG	2299
ALA GCG	GLU GAA	SER AGC	GLY GCG	VAL GTG	THR ACA	LEU TTA	HIS CAC	ALA GCA	ASP GAT	ALA GCA	ALA GCT	ALA GCA	LEU CTG	ALA GCG	GLN CAG	LEU TTG	GLN CAG	2353
ALA GCA	GLY GGC	PRO CCT	ALA GCG	LYS AAG	VAL GTG	VAL GTT	ALA GCT	VAL GTT	LYS AAA	ALA GCC	GLU GAA	GLU GAG	TYR TAT	ASP GAC	ASP GAT	GLU GAG	PHE TTT	2407
LEU CTG	SER TCA	LEU TTA	ASP GAT	LEU TTG	ASN AAC	VAL GTC	LYS AAA	ILE ATC	VAL GTC	SER AGC	ASP GAT	LEU CTT	ASP GAC	ASP GAT	ALA GCC	ILE ATC	ALA GCC	2461
HIS CAT	ILE ATT	ARG CGT	GLU GAA	HIS CAC	GLY GGC	THR ACA	GLN CAA	HIS CAC	SER TCC	ASP GAT	ALA GCG	ILE ATC	LEU CTG	THR ACC	ARG CGC	ASP GAT	MET ATG	2515
ARG CGC	ASN AAC	ALA GCC	GLN CAG	ARG CGT	PHE TTT	VAL GTT	ASN AAC	ALA GCC	SER TCT	THR ACG	ARG CGT	PHE TTT	THR ACC	ASP GAC	GLY GGC	GLY GGC	GLN CAG	2569

PHE GLY LEU GLY ALA GLU VAL ALA VAL SER THR GLN LYS LEU HIS ALA ARG GLY TTT GGT CTG GGT GCG GAA GTG GCG GTA AGC ACA CAA AAA CTC CAC GCG CGT GGC	2623
PRO MET GLY LEU GLU ALA LEU THR THR TYR LYS TRP ILE GLY ILE GLY ASP TYR CCA ATG GGG CTG GAA GCA CTG ACC ACT TAC AAG TGG ATC GGC ATT GGT GAT TAC	2677
THR ILE <u>ARG ALA</u> *** ACC ATT CGT GCG TAA ATAAAACCGGGTGATGCAAAAGTAGCCATTGATTACAAAGGCCATTGACG	2743
<u>CATCGCCCGGTTAGTTTAA</u> ACCTTGTCACCGTGATTACGTTTCGTGAACATGTCCTTTCAGGGCCGATAT	2814
AGCTCAGTTGGTAGAGCAGCGCATTCGTAATGCGAAGGTCGTAGGTTGCACTCTATTATCGGCACCATTA	2885
AAATCAAATTGTTACGTAAGATCTTATCATTCTCCACCAAAAAATTATCTTAATGTACAGCTGGTGTA	2956
GTAATTCTATCAACGAAGATCAATCTTACTACTGACCAAAAAGGCCTGATAGGCCTTCGCTCACTATAC	3027
ATCCTTGGCTGCAG	3041

Figure 4. Nucleotide Sequence of the 3.04 kb *Pst*I DNA Fragment Containing the *proB*A Region. The 5'-end of the *phoE* gene (sense strand) is shown including the position of a putative Shine and Dalgarno sequence (underlined). Regions of dyad symmetry [axes indicated by (▼)] thought to be involved in the transcriptional control of either the *phoE* gene or the *proB* gene are indicated. The putative -10 region (dashed line) of the *proB* gene is indicated along with the putative Shine and Dalgarno sequences (underlined) and translation termination codons (asterisks) for the *proB* and *proA* genes. A region of dyad symmetry thought to be involved in the termination of transcription of the *proA* gene is indicated as well as the proposed stop-site of transcription (dashed line). Confirmation of a portion of the amino acid sequence deduced from the DNA sequence was obtained by NH_2 -terminal and COOH-terminal sequence analyses of purified γ -glutamyl kinase (*proB*) and γ -glutamyl phosphate reductase (*proA*) protein and is indicated by overlines.

position 2690 with TAA. A second nonsense codon, TGA, occurs further downstream, in phase with the first nonsense codon. This second codon is located within an inverted repeat sequence centered at position 2726. A possible RNA transcription termination structure with features characteristic of rho-independent terminators (19) is shown in Figure 6.

The secondary attachment site for bacteriophage lambda had already been sequenced (22), and mapped by complementation (23) to the *proB*A region. The sequence data presented here shows that the secondary attachment site (GTATAAA) resides in the N-terminal portion of the *proA* gene (Figure 4).

The calculated molecular weight for the GK (*proB*) and GPR (*proA*) monomers based upon the DNA sequence was 38,952 and 43,503, respectively. These values are consistent with those obtained from SDS-polyacrylamide gel electrophoretic analysis of total cellular protein (Figure 2). Based upon a molecular weight (9) of 236,000 and 268,000 for the holoenzymes of GK and GPR, respectively, GK

Figure 5. Homologies in the Nucleotide Sequences of the 5'-Regulatory Region of proB. The 5' sequences of the proB gene are compared with the -10 sequences of Str and λ PRE and the -35 sequence of T5-25. Underlined and boxed sequences are explained in text.

Amino Acid	GK Analysis		GPR Analysis	
	Protein ^a	DNA ^b	Protein ^a	DNA ^b
non-polar				
Ala	46	46	58	53
Gly	35	37	34	31
Ile	29	31	22	27
Leu	45	39	48	43
Met	4	6	8	9
Phe	7	5	2	5
Pro	10	11	20	12
Val	30	25	35	35
		200		215
polar				
Asn	n.d.	13	n.d.	16
Cys	1	3	n.d.	7
Gln	n.d.	13	n.d.	18
Ser	21	22	16	20
Thr	18	19	16	21
Trp	n.d.	2	n.d.	1
Tyr	8	7	5	7
		81		90
acidic				
Asp (Asx)	(40)	24 (37)	(46)	28 (44)
Glu (Glx)	(35)	17 (30)	(46)	23 (41)
		39		51
basic				
Arg	26	26	21	25
His	8	10	6	7
Lys	8	11	15	19
		47		51

^aData are amino acid residues, as determined by amino acid analysis, to the nearest whole number.

^b Composition determined from DNA sequence.

n.d., not determined.

Figure 5. Homologies in the Nucleotide Sequences of the 5'-Regulatory Region of proB. The 5' sequences of the proB gene are compared with the -10 sequences of Str and λ PRE and the -35 sequence of T5-25. Underlined and boxed sequences are explained in text.

Figure 6. A Possible RNA Transcription Termination Structure at the 3'-End of the *proA* Gene. The 3'-end of the *proA* gene has been represented as a sequence of ribonucleotides in a stem and loop secondary structure. The COOH-terminal alanine codon (GCG) is followed by both a UAA nonsense codon (***) and, further downstream and in phase, a UGA nonsense codon (***). A GC-rich sequence present in the stem, followed by a U-rich region are boxed. The termination structure has been drawn to maximize base pairing stability according to the rules of Tinoco et. al. (35). The stem has a ΔG value of -33.4 kcal while the entire stem and loop structure has a ΔG value of -31.4 kcal.

and GPR are each comprised of 6 identical subunits.

Amino Acid Sequence of GK and GPR

The complete amino acid sequence of GK and GPR is shown in Figure 4. NH_2 and COOH-terminal amino acid analysis of both purified enzymes corresponded with that predicted by the DNA sequence (Figure 4). Further confirmation of the putative protein sequences was provided by the amino acid composition obtained for both purified proteins which correlated well with that predicted from the DNA sequence (Table 2).

DISCUSSION

In this study the genes encoding GK (*proB*) and GPR (*proA*), two of the three enzymes in the proline biosynthetic pathway, were subcloned, identified by protein and DNA sequence analysis, and placed under the control of the highly active λP_L promoter. Though the *proB* and *proA* loci had been placed at about 6 min. on the *E. coli* linkage map (24), it was unclear whether we would find the *proB* and *proA* genes arranged contiguously (25) or separated by intervening genes (26, 27, 28). Complementation and enzymology studies employing plasmids obtained from the Clarke and Carbon collection of hybrid plasmids indicated that the *proB* and *proA* genes were within at least 20 kb of each other (1). Recently, several laboratories (10,29,30,31) have identified

a 3 kb E. coli DNA fragment which encodes GK and GPR activity. With this present report we now document that the proB and proA genes are indeed contiguous and very likely comprise a polycistronic operon. However, we cannot rule out the possibility (as some of the above studies might suggest) of genomic fluidity in the proBA region. During our analysis of the DNA sequence data obtained for the proBA region, we discovered the presence of a sequence within proB, GCTGGTGG (Figure 4), homologous to a chi site - a recombinational hotspot around which the rate of RecBC-promoted recombination is elevated (21). If the proBA region is subject to genomic fluidity then the presence of a sequence homologous to a hotspot of generalized recombination might explain the occurrence of strain specific gene rearrangements in the proBA region.

In the present study we found no evidence of a DNA sequence upstream from the 5'-end of the proB gene that corresponded to a transcription attenuator. Previously published results (6) had indicated that the synthesis of GK was repressed by proline. However, we as well as others (10,1) have found that the specific activity of GK is not affected by the addition of proline to the growth medium. In addition, the regulation of enzyme synthesis in proBA-lacZ fusion strains was found not to be influenced by the type of carbon source (i.e., proline or glucose) in the growth medium (10).

Several inverted repeats were identified upstream from the 5'-end of the proB gene (see Figure 4). During the course of this research we had identified, via a promoter assay vector, a promoter in close proximity to the proB promoter that stimulated transcription from the opposite strand (Don VanLeeuwen, unpublished results). A recent report (32), detailing the nucleotide sequence of the E. coli phoE gene, positioned the phoE gene on the opposite strand and adjacent to the proA gene. While our DNA sequence of this region agrees with that reported, it is clear from our research that the phoE gene actually abutts the proB gene (Figure 4). The close proximity of the phoE and proB 5'- transcription regulatory regions makes structural/functional assignments for the inverted repeats difficult. Apparently both the phoE and proB genes lack the canonical -35 sequence.

The concept of the proBA locus functioning as an operon (30,31) is supported by our observations that 1) analysis of the DNA sequence preceding the proA coding region failed to reveal attractive promoter sequences, 2) only 14 bp separate the COOH-terminal of proB from the NH₂-terminal of proA, with the proB termination codon most likely overlapping with the proA ribosomal binding site, and 3) the absence at the 3'-end of the proB gene of visible homology to a rho-independent terminator.

A rationale for the polycistronic nature of the proBA locus can also be constructed based upon an enzymological argument. The product of the reaction catalyzed by GK is a labile intermediate, γ -glutamyl phosphate, and it has been suggested (3) that GK and GPR form a molecular complex which ensures the direct transfer of γ -glutamyl phosphate from GK to GPR. The biosynthesis of both enzymes in close spacial proximity to one another would result in relatively high localized concentrations of both enzymes and this would facilitate the formation of a physical complex. In support of the existence of this presumptive complex, we have found that purified preparations of GK are enzymatically inactive in enzyme assays employing hydroxymate, but are enzymatically active following the addition of a homogenous preparation of GPR (9).

The existence of this enzymatic complex would lead to the requirement that cellular levels of GK and GPR either be tightly regulated in a stoichiometric fashion, or with GPR being in molar excess over GK. Several lines of evidence indicate that GPR is synthesized in molar excess: (1) Hayzer reported (1) an almost 6-fold differential increase in the specific activity of GPR in comparison to GK in cells transformed with a ColEI hybrid plasmid containing the proBA region. (2) In this present communication we report a 2-fold differential increase in specific activity in favor of GPR when the proBA genes were cloned into the expression plasmid pGW7, and transformed cells were grown at 30°C or 37°C (Table I). At 42°C, with the λP_L promoter fully induced, we observed a 3-fold differential increase in specific activity in favor of GPR. (3) SDS-polyacrylamide gel electrophoresis also revealed a corresponding differential increase in the amounts of the two enzymes (Figure 2). Thus, it would appear that the synthesis of GPR is regulated in a fashion that ensures a molar excess of GPR over GK.

Transcriptional and translational regulatory mechanisms that could conceivably function to ensure either stoichiometric or molar excess amounts of GPR become apparent upon examination of the proBA DNA sequence. First, the polycistronic nature of the proBA genes would facilitate the synthesis of stoichiometric amounts of mRNA sequences encoding both proteins. Second, the overlap of the proB translation termination codon with the proA ribosomal binding site could allow translational coupling (33) i.e., the maintenance of ribosomal association with the mRNA throughout the translation of both protein encoding mRNA sequences. Translational reinitiation without ribosome release would help to ensure stoichiometric production of both polypeptides. Third, the potential availability of several ribosomal binding sites (one in proB and

two in proA) directing ribosomes to the GPR mRNA sequence would also ensure a molar excess of GPR. In addition, the extensive homology between the proA ribosomal binding site, TAAGGAG (versus GAG for proB), and the 3'-end of the 16S rRNA potentially portends an advantage in the competition for ribosomes.

The three genes coding for the enzymes of the proline biosynthetic pathway have been completely sequenced and characterized (7, present report). With the knowledge obtained from our studies we have constructed several vectors enabling the overproduction of proline in industrially significant quantities by strains of E. coli (10). In addition, proline is a primary osmoregulatory agent (36) whose overproduction in agriculturally significant organisms via genetic engineering technology might increase their osmotic tolerance and therefore their environmental tolerance and productivity. The ability to manipulate the expression of the enzymes of the proline biosynthetic pathway should result in a better understanding of osmoregulation in bacteria and animals as well as in plants. Finally, comparison of our sequence data of the mutant proB gene, responsible for conferring the DHP analog resistant phenotype, with the DNA sequence of the wild-type proB gene (manuscript in preparation) should facilitate the elucidation, and optimization by site-specific mutagenesis, of the structural and molecular interactions determining proline feedback resistance.

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