

Cloning and Characterization of the Gene Encoding Inorganic Pyrophosphatase of *Escherichia coli* K-12

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***Escherichia coli* K-12 gene *ppa* encoding inorganic pyrophosphatase (PPase) was cloned and sequenced. The 5' end of the *ppa* mRNA was identified by primer extension mapping. A typical *E. coli* sigma 70 promoter was identified immediately upstream of the mRNA 5' end. The structural gene of *ppa* contains 528 base pairs, from which a 175-amino-acid translation product, M_r 19,572, was deduced. The deduced amino acid composition perfectly fitted with that of PPase as previously determined (P. Burton, D. C. Hall, and J. Josse, *J. Biol. Chem.* 245:4346-4351, 1970). Furthermore, the partial amino acid sequence (residues 1 to 108) of *E. coli* PPase determined by S. A. Cohen (Ph.D. thesis, University of Chicago, 1978) was the same as that deduced from the nucleotide sequence. This is the first report of the cloning of a PPase gene.**

Inorganic pyrophosphatase (EC 3.6.1.1; PPase) is ubiquitous in nature and plays an important role in energy metabolism, providing a thermodynamic pull for biosynthetic reactions such as protein, RNA, and DNA synthesis (22). According to Peller (33), nucleic acid syntheses would be energetically impossible in vivo if they were not coupled to the hydrolysis of pyrophosphate (PP_i), catalyzed by PPase. In addition, various findings suggest that PPase might have important roles not only in the regulation of macromolecular synthesis and growth (1, 9, 14, 21) but also in evolutionary events by affecting the accuracy by which DNA molecules are copied during chromosome duplication (2, 18, 28).

While the occurrence, reaction mechanism, and structural and kinetic properties of this important housekeeping enzyme have been studied extensively (5, 41), the regulation of PPase has received little attention. We have for some years studied the role of PP_i and regulation of PPase in bacteria (24-26). To obtain a better insight into these topics we cloned and characterized the *Escherichia coli ppa* gene encoding PPase.

(A preliminary report has already been presented at the Fourth European Congress on Biotechnology (16).)

MATERIALS AND METHODS

Chemicals. Restriction endonucleases, T4 DNA ligase, exonuclease III (from *E. coli* B), and alkaline phosphatase from calf intestine were bought from Boehringer Mannheim, Mannheim, Federal Republic of West Germany. Antibiotics, egg white lysozyme, and low-gelling-temperature (LGT) agarose (type VII) were obtained from Sigma Chemical Company, St. Louis, Mo. Mung bean nuclease was from Promega Biotec, Madison, Wis. DNA polymerase I (Klenow fragment) and [³⁵S]dATP (500 Ci/mmol) were purchased from Du Pont, Boston, Mass.

Bacterial strains, plasmids, and culture conditions. The following strains of *Escherichia coli* were used. K-12 is a wild-type strain from the collections of this laboratory. Strain JP5 was produced by mutagenizing *E. coli* K-12 with NTG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine). The isolation

and characterization of this mutant will be published later, but in this connection it is important that this strain contains only about 15% of the normal PPase activity. To obtain stable transformants, we constructed a *recA* derivative of JP5, designated strain RT4, by general transduction with phage P1 (31) grown on *E. coli* JC10240 (6), obtained from the *E. coli* Genetic Stock Center. Strain RT4 was used as a host in the cloning.

The plasmid pOU61 was donated by Sören Molin (Technical University of Denmark, Copenhagen, Denmark) in *E. coli* host CSH50. This plasmid has a copy number of 1 to 2 per genome equivalent at 30°C, but at 42°C several thousand copies of the plasmid are produced in one cell (27). The plasmid encodes β-lactamase.

The strains were grown in LB broth or on LA plates (29). Ampicillin (25 μg/ml) or tetracycline (15 μg/ml) was added when required. Growth was followed by measuring the light scattering of the cultures at 550 nm.

DNA isolation and cloning methods. *E. coli* CSH50 was cultured at 30°C to an A_{550} of 0.5. Then the cultivation was continued at 42°C for 2 h to amplify the plasmid pOU61. The cells were collected by centrifugation and lysed by lysozyme and alkali, and the plasmid pOU61 was isolated and purified by centrifugation to equilibrium in cesium chloride-ethidium bromide density gradients according to Maniatis et al. (29). Chromosomal DNA was isolated from the wild-type *E. coli* K-12 cells as described by Silhavy et al. (39).

Restriction endonuclease digestions of the plasmid and chromosomal DNA were performed as suggested by the suppliers. Other DNA manipulations, including isolation of restriction fragments from 0.7% LGT agarose gel, dephosphorylation of the linearized vector with calf intestinal phosphatase, and ligations, were carried out as described previously (29). *E. coli* RT4 cells were made competent by the calcium chloride procedure and transformed as described by Maniatis et al. (29). The transformants were grown on LA plates which contained ampicillin (25 μg/ml) by incubating at 30°C for 30 h. The colonies were screened by the magnesium pyrophosphate overlay test (23).

RNA methods. RNA was isolated as described by Igo and Losick (19). The 5' end of the *ppa* transcript was mapped by using the primer extension technique (30).

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TABLE 1. Specific activities of PPase in *E. coli* strains used in this work^a

Strain	Sp act (mkat/kg) of PPase in cells grown at:	
	30°C	42°C
K-12	10	10
RT4	1.5	1.5
RT4(pEV1)	53	400
RT4(pTP1)	60	580

^a K-12 is the wild-type strain used as a donor and RT4 (*ppa recA*) was used as a recipient strain in the cloning experiment. RT4(pEV1) is the primary transformant from the cloning experiment containing the plasmid pEV1, which is a derivative of pOU61 with a 4-kb DNA fragment inserted into the *Bam*HI site. RT4(pTP1) contains the plasmid pTP1, which is a deletant of pEV1 with a 1.3-kb DNA fragment inserted into the *Bam*HI site of pOU61. Owing to the amplification of the pOU61 derivatives at 42°C (27), the specific activities were measured from the cells grown at 30°C and for 2 h at 42°C, respectively.

Enzyme assay. PPase activity of the cell samples was determined by the method of Heinonen and Lahti (15).

DNA sequencing. Sequence analysis was done by the dideoxy chain termination method (36). For the sequence determination, an overlapping series of deletions were prepared for both orientations of the insert DNA in M13mp18 by unidirectional digestion with exonuclease III (17).

Computer analysis. Searches for open reading frames and terminator regions of *ppa* transcription, derivation of the amino acid sequence from the nucleotide sequence, and studies on codon usage were carried out by the Sequence Analysis Software Package supplied by the University of Wisconsin Genetics Computer Group (8).

RESULTS

Cloning strategy. Our strategy for the cloning of gene *ppa* encoding *E. coli* PPase was as follows. The chromosomal DNA isolated from *E. coli* K-12 was partially digested by *Sau*3A1. The digest was fractionated by gel electrophoresis in LGT agarose, and DNA fragments of 2 to 9 kb were extracted from the melted gel as described by Maniatis et al. (29). This fragment pool was ligated into the *Bam*HI site of the vector pOU61. The plasmids were transformed into strain RT4 (*ppa recA*), and ampicillin-resistant transformants were screened for high PPase activity by the magnesium pyrophosphate overlay test (23).

Out of the 4,500 transformants obtained, 1 had 35-fold-higher PPase activity than the recipient strain, RT4. This

clone, designated RT4(pEV1), had fivefold-increased PPase activity compared with the wild-type *E. coli* K-12 (Table 1). PPase activity of this clone was further increased sevenfold when the strain was grown for 2 h at 42°C. When the plasmid pEV1 isolated from this clone was retransformed into *E. coli* RT4, colonies with high PPase activity were obtained. Hence, it was evident that the plasmid pEV1 contained the *ppa* gene.

Subcloning. The plasmid pEV1 contains a 4-kb DNA fragment, inserted into the *Bam*HI site of pOU61. Because *E. coli* PPase was known to consist of six identical subunits with a molecular weight of approximately 20,000 (43), the length of the structural gene was estimated to be about 500 to 600 bp. Hence, a DNA fragment of 1 to 2 kb was thought to be long enough to contain the whole *ppa* gene with its possible regulatory regions. For this region, the 4-kb fragment encoding PPase was digested partially by *Sau*3A1 restriction enzyme. Fragments 1 to 2 kb long were isolated from LGT agarose gel, ligated into the *Bam*HI site of pOU61, and transformed into the RT4 strain. Screening of the transformants indicated several colonies with high PPase activity. For further studies we selected one clone, RT4(pTP1) (Table 1).

Plasmid pTP1 contains a 1.3 kb DNA fragment inserted into the *Bam*HI site of pOU61. When this fragment was religated into the *Bam*HI site of pOU61 and retransformed into RT4, colonies with high PPase activities were again obtained, whereas RT4 transformed with pOU61 produced only colonies with a low level of PPase activity. Hence, it is evident that the plasmid pTP1 contains the intact *E. coli* gene encoding inorganic pyrophosphatase. To our knowledge this is the first report of cloning of any PPase gene.

Nucleotide sequence of the gene encoding *E. coli* inorganic pyrophosphatase. For the sequence determination, the *Bam*HI fragment of 1.3 kb encoding *E. coli* PPase was transferred from the plasmid pTP1 into the *Bam*HI site of the replicative form of the M13mp18 vector. Exonuclease III was used to create a series of controlled unidirectional deletions of the 1.3-kb DNA fragment for both orientations in M13mp18. A partial restriction map and the strategy of nucleotide sequence analysis are depicted in Fig. 1. The entire sequence was determined for both strands from the overlapping exonuclease III deletants of the 1.3-kb fragment. The data in Fig. 2 show 291 nucleotides (nt) of the upstream flanking sequence, a 528-nt coding region, and 375 nt of the 3'-flanking sequence. The nucleotide sequence is numbered by designating the transcription start point as nt +1.

The 5' end of *ppa* mRNA was determined by the primer extension method (30). A 19-mer oligonucleotide primer

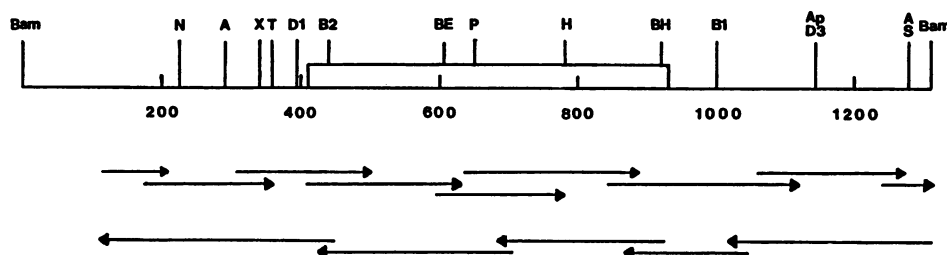


FIG. 1. Sequencing strategy and partial restriction map of *E. coli ppa*. The partial restriction map is that of the 1.3-kb DNA fragment cloned into the *Bam*HI site of the vector, pOU61. Bam, *Bam*HI; N, *Nru*I; A, *Acc*I; X, *Xmn*I; T, *Tth*32; D1, *Dra*I; B2, *Bgl*II; BE, *Bst*EII; P, *Pst*I; H, *Hpa*I; BH, *Bss*HII; B1, *Bgl*I; Ap, *Apa*LI; D3, *Dra*III; S, *Sal*I. The open reading frame encoding *E. coli* PPase is shown by an open bar. The arrows indicate the extent and direction of the sequencing of the fragments obtained after exonuclease III treatment; those pointing to the right indicate the noncoding strand; those pointing to the left indicate the coding strand.

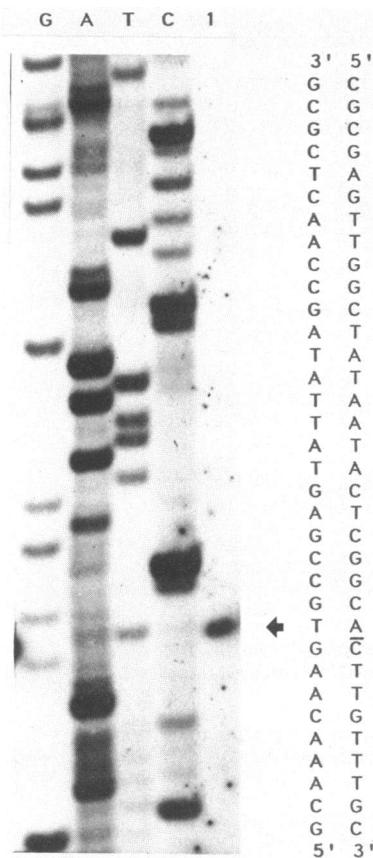


FIG. 3. Primer extension mapping of the *ppa* mRNA 5' termini. Reverse transcriptase and 5'-³²P-labeled 19-mer oligonucleotide complementary to nt 44 to 62 of *ppa* mRNA (Fig. 2) were added to total *E. coli* RNA isolated from RT4(pTP1). The cDNA product was electrophoresed through a polyacrylamide-urea sequencing gel (lane 1). Lanes G-C contain the products of Sanger sequencing reactions, using the M13 clone of *ppa* as the template and the same primer. The sequence from the sequencing ladder is shown, together with the complementary strand on which is marked the likely transcription initiation site.

characteristic of *E. coli* sigma 70-dependent promoters (13). Furthermore, 6 bp upstream from the initiation codon of the *ppa* gene there was an AGGAAA (Fig. 2) that closely resembled the *E. coli* Shine-Dalgarno sequence (38, 40).

The rho-independent termination sequence of the *ppa* gene (at nt 580 to 630) predicted by the TERMINATOR program supplied by the University of Wisconsin Genetics Computer group (8) is shown in Fig. 2. It includes a CGGGC region, a dyad symmetry, and a stretch of thymine residues (Fig. 2) that are shared by many *E. coli* terminator sequences (3). However, crucial signals for transcription termination reside primarily in the RNA structure (34). Transcript of this terminator could form the stem-and-loop structure shown in Fig. 4. This structure contains a stem of 11 bp, and it is followed by a stretch of U residues as is typical in rho-independent terminators in *E. coli* (3, 34). The stability of stem-and-loop structures can be expressed as ΔG^0 of the formation of the structure. On the basis of the values given by Freier et al. (10), we calculated the ΔG^0 for the putative terminator of the *ppa* gene as -10.1 kcal/mol. This is in the range (-8.0 to -17.9 kcal/mol) of the ΔG^0 values calculated by us for several terminators of *E. coli* and its phages on the

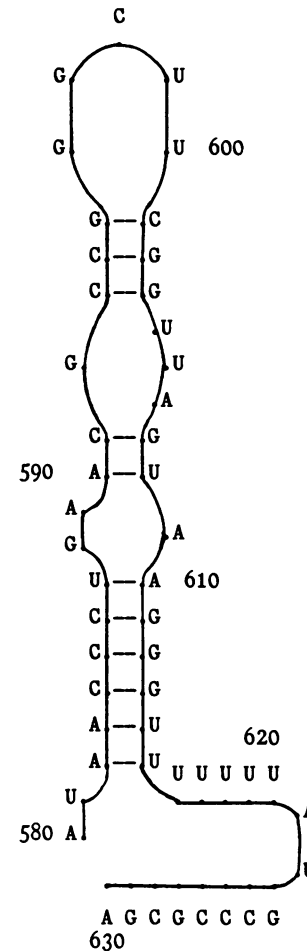


FIG. 4. Proposed secondary structure of the *E. coli ppa* mRNA 3' trailer sequence corresponding to the predicted terminator (nt 580 to 630).

basis of the sequences compiled by Rosenberg and Court (35).

Codon usage. Choice among synonymous codons is distinctly nonrandom in the *ppa* gene. It predominantly uses the codons ("optimal codons") generally preferred by highly expressed *E. coli* genes (Table 2). This is compatible with the fact that *E. coli* PPase is an abundant, housekeeping enzyme, the cellular content of which is approximately 1,000 molecules per genome (20; for comparison, see reference 11).

Derived amino acid sequence. The sequence shown in Fig. 2 contains an open reading frame of 528 nt, corresponding to the inorganic pyrophosphatase primary translation product. Serine is the amino-terminal amino acid residue in the mature *E. coli* PPase (4; S. A. Cohen, Ph.D. thesis, University of Chicago, 1978). Hence, Met shown in Fig. 2 is removed by an amidopeptidase, creating the new terminus of the seryl residue that was originally the second in the chain. The mature translation product contains 175 amino acid residues (Fig. 2), and it has a calculated molecular weight of 19,572. This is in good agreement with the subunit size of 20,000 determined for the purified *E. coli* PPase by Wong et al. (43). Amino acid composition of *E. coli* PPase in our

TABLE 2. Codon usage in *ppa*^a

Amino acid	Codon	No.	Amino acid	Codon	No.
Gly	GGG	0	Trp	TGG	2
Gly	GGA	0	End	TGA	0
Gly	<u>GGT</u>	6	Cys	TGT	0
Gly	<u>GGC</u>	3	Cys	TGC	2
Glu	GAG	4	End	TAG	0
Glu	<u>GAA</u>	11	End	TAA	0
Asp	GAT	5	Tyr	TAT	1
Asp	GAC	9	Tyr	<u>TAC</u>	7
Val	GTG	2	Leu	TTG	0
Val	<u>GTA</u>	1	Leu	TTA	1
Val	<u>GTT</u>	11	Phe	TTT	0
Val	GTC	2	Phe	<u>TTC</u>	6
Ala	GCG	5	Ser	TCG	0
Ala	<u>GCA</u>	4	Ser	TCA	0
Ala	<u>GCT</u>	4	Ser	<u>TCT</u>	2
Ala	GCC	3	Ser	<u>TCC</u>	2
Arg	AGG	0	Arg	CGG	0
Arg	AGA	0	Arg	CGA	0
Ser	AGT	0	Arg	CGT	2
Ser	<u>AGC</u>	4	Arg	<u>CGC</u>	2
Lys	AAG	3	Gln	<u>CAG</u>	1
Lys	<u>AAA</u>	13	Gln	CAA	1
Asn	AAT	1	His	CAT	0
Asn	<u>AAC</u>	6	His	<u>CAC</u>	5
Met	ATG	3	Leu	<u>CTG</u>	12
Ile	ATA	0	Leu	CTA	0
Ile	ATT	2	Leu	CTT	0
Ile	<u>ATC</u>	8	Leu	CTC	2
Thr	ACG	0	Pro	<u>CCG</u>	9
Thr	ACA	0	Pro	CCA	2
Thr	<u>ACT</u>	1	Pro	CCT	2
Thr	<u>ACC</u>	3	Pro	CCC	0

^a Preferred codons described in reference 7 are underlined.

derived amino acid sequence agrees very well with the data presented by Burton et al. (4) (Table 3).

Burton et al. (4) determined the amino- and carboxy-terminal amino acids of *E. coli* PPase with the result NH₂-Ser-Leu(Ile)-Leu(Ile) . . .Ala-Lys-COOH (the paper chromatographic systems employed in that study did not discriminate clearly between the phenylthiohydantoin of leucine and isoleucine). This is compatible with our sequence with the exception that the next-to-last amino acid in the carboxy terminus is asparagine instead of alanine (Fig. 2). Cohen (thesis) partially determined the amino acid sequence of *E. coli* PPase (residues 1 to 86, and a peptide containing 23 residues). In our work these two separate fragments were shown to form a continuous peptide covering the first 108 amino acids of the enzyme. The partial sequence presented by Cohen is the same as the amino-terminal sequence shown in Fig. 2 with the following exceptions: Glx-35 presented by Cohen is Glu-35; Glx-80 is Gln-80; Lys-81 is Pro-81; Gln-83 is Ser-83; and His-88 is Arg-88 (Fig. 2). The good agreement between the determined amino acid sequence of PPase and that predicted by our DNA sequence determination proves that our clone contains the structural gene of *E. coli* PPase.

DISCUSSION

E. coli PPase is synthesized constitutively. Thus, the composition of the cell culture medium generally exerts no specific effects on the level of the enzyme (20). However, the production of *E. coli* PPase can be stimulated 1.5- to 3-fold

TABLE 3. Amino acid composition of *E. coli* PPase

Amino acid	No. of residues in subunit	
	Predicted ^a	Reported ^b
Ala	16	16.8
Arg	4	4.2
Asn	7	21.3 (Asx)
Asp	14	
Cys	2	2.0
Gln	2	17.4 (Glx)
Glu	15	
Gly	9	9.3
His	5	5.0
Ile	10	10.2
Leu	15	15.6
Lys	16	16.3
Met	3	2.9
Phe	6	6.1
Pro	13	13.4
Ser	8	7.9
Thr	4	4.2
Trp	2	2.0
Tyr	8	7.9
Val	16	16.7

^a Deduced from the nucleotide sequence.

^b Determined from highly purified PPase by Burton et al. (4).

by partial inhibition of DNA synthesis (14). Possibly, inhibition of DNA synthesis affects PPase synthesis indirectly, by disturbing the nucleotide metabolism, for example (24). The rate of synthesis of a constitutive protein is set genetically by the natural efficiency of the promoter of its gene, the rate at which ribosomes read the messenger, and the sensitivity of its messenger to degradation by nucleases (42). In some cases constitutive synthesis is a result of autogenous regulation (32, 37). By the in vitro transcription-translation of pTP1, we will see whether autogenous regulation also has some role in the production of *E. coli* PPase. As mentioned above, the sequences and spacing of the elements in the 5'-flanking region of *ppa* closely match the consensus sequences of *E. coli* genes. Furthermore, the codon usage is clearly biased in *ppa* (Table 2). Hence, *ppa* seems to be both transcribed and translated efficiently in *E. coli*. This is compatible with the fact that PPase constitutes about 0.2% of the total soluble protein in this bacterium (20).

Mutants having an altered structure or regulation of PPase would be most valuable in the elucidation of the role of PPase in metabolism. However, very little has been published concerning such mutants. Josse and Wong (20) checked at random 5,000 colonies after mutagenesis and they found three strains with diminished PPase activity. One of them contained only 2% of the normal PPase activity, but the mutant grew as fast as the wild type. We have also put a considerable amount of time and effort into experiments aimed at isolation of PPase mutants of *E. coli* K-12 after mutagenesis in vivo. We have not found any strain completely devoid of PPase activity, but some strains with slightly higher or lower levels of PPase activity compared with that of the wild type have been obtained (24). One of these mutants (RT4) with low PPase activity was used as a host in the cloning of *E. coli ppa* as described in this paper.

Now that we have the *ppa* gene cloned, we can try to produce a null mutant by replacing the wild-type gene present in the genome with a nonfunctional mutant constructed in vitro by the method described by Gutterman and Koshland (12), for example. However, we believe that a *ppa* null mutant is not viable, and hence it cannot be isolated. In

the case of essential enzymes, temperature-sensitive mutants are generally produced to circumvent this problem. We have made several attempts to isolate such PPase mutants with no success (24). One reason for our failure may be the unusually high thermostability of *E. coli* PPase (20).

As we planned the strategy for cloning the *E. coli ppa* gene, we thought that expression of high levels of PPase might be harmful or toxic to the cell. However, this is not the case. The strains listed in Table 2 all grew equally well in a rich medium, even at 42°C for a few hours, although the expressed levels of PPase ranged from about 6 times lower to 60 times higher than that of the wild type (K-12). After 2 h of growth at 42°C, the cells gradually started to die owing to the extensive amplification of the plasmid (27). Even then there was no difference in growth between the RT4 strain containing the pOU61 plasmid or its derivative pTP1 that contains the *ppa* gene.

It is logical to expect that the intracellular PP_i concentration would be dependent on the level of PPase. We have studied the relationship between PP_i and PPase in different growth conditions and also in conditions where the production of PPase is stimulated (24). No clear correlation has been found so far between the intracellular PP_i concentration and the level of PPase (24). However, this project is still in progress, and it will be interesting to see what the PP_i content is in the strains listed in Table 1, for example.

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