INCREASED SYNTHESIS OF PHOSPHOENOLPYRUVATE CARBOXYLASE IN A STRAIN OF *ESCHERICHIA COLI* BEARING A ColE1-*ppc*$^+$ HYBRID PLASMID

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1. Introduction

Phosphoenolpyruvate carboxylase (PEPC) (EC 4.1.1.31) of *Escherichia coli* is an allosteric enzyme whose activity is regulated by multiple effectors [1]. The enzyme has M_r 361 000 with 4 presumably identical subunits [2]. Elucidation of the regulatory mechanism of the enzyme on molecular basis [1] has been hampered by the low level of the enzyme in the cells. To overcome this difficulty, we have attempted to elevate the enzyme level using ColE1 hybrid plasmids containing the gene for PEPC (*ppc*$^+$). We undertook the gene-cloning study also to determine the primary structure of PEPC from the base sequence of the cloned DNA.

We report that JA200/pLC20-10 is a fit strain for our purpose among the gene bank of [4]. The enzyme of this strain showed a higher level (~10-fold) than that of the wild strain. Several lines of evidence are presented which suggest the molecular identity of the enzymes from both strains.

Abbreviations: PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; M_r, relative molecular mass

2. Materials and methods

Mitomycin C was obtained from Kyowa Hakko Kogyo (Tokyo). Colicin E1 was partially purified by ammonium sulfate fractionation according to [5].

The strains used in this study, all of which are derivatives of *E. coli* K-12, are listed in table 1. The organisms were grown aerobically at 37°C. The media used are: (i) L broth [8]; (ii) the basal salts medium [9] supplemented with 1 ml Anraku's metal solution [10] per liter; and (iii) the 'enriched' medium [9]. For the cultivation of plasmid-bearing strains colicin E1 was also included in the media to provide continuous selective pressure to maintain these strains.

The cell-free extract was obtained by centrifugation of a sonicated cell suspension at 104 000 × *g* for 1 h, and its PEPC activity was assayed as in [3]. The rate of NADH oxidation depending on exogenously added PEP was taken as the enzyme activity. One unit of the enzyme was defined as the activity oxidizing 1 μmol NADH/min at 30°C.

PAGE and SDS–PAGE were performed in gels of 7.5% (w/v) acrylamide according to [11] and [12], respectively.

The other determinations were carried out as in [3,9].

Table 1
Bacterial strains

Strain	Relevant genotype	Source
W3110	F$^-$	[1]
BA197	HfrH/ColE1	T. Ikemura
AB1622	F$^-$, *ppc*, *thi*, *str*^{R}	[6]
PA342	F$^-$, *ppc*, *thi*, *arg*, *leu*, *his*, *thr*	[7]
JA200/pLC20-10	F$^+$, *recA*, *leu*, *thr*, Δ*trpE5*/ColE1-*ppc*$^+$	[4]
AB1622/pLC20-10	*ppc*, *thi*, *str*^{R}/ColE1-*ppc*$^+$	This work

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3. Results

3.1. *Identification of plasmid carrying the* ppc$^+$ *gene*

As a starting organism we used the series of 2100 cloned *E. coli* strains prepared in [4]. Each clone contains a ColE1 plasmid into which a fragment of *E. coli* chromosomal DNA had been inserted. About 20 copies of the independently replicating plasmid are usually present per cell, and this gene dosage effect is expected to result in equally elevated levels of the corresponding gene products [13].

When PEPC-negative mutants such as AB1622 and PA342 are grown on sugars or glycerol, they require L-glutamate or C_4-dicarboxylate members of the tricarboxylic acid cycle as a supplement [6]. Plasmids carrying the *ppc*$^+$ gene can be identified as those which complement this auxotrophic phenotype when transferred to these mutants. For the F-mediated transfer of hybrid ColE1 plasmid to the recipient cells, strain AB1622 and each strain of JA200/pLC bearing hybrid ColE1 plasmids were mixed-cultivated in L broth medium in the wells of Micro Test II® tissue culture plate (Falcon) at 37°C for 14 h. Then, the agar plate for selection was inoculated with each culture. The medium used was the basal salts medium supplemented with 0.4% glucose, thiamine–HCl (3 μg/ml) and streptomycin sulfate (200 μg/ml). After incubation at 37°C for 3–4 days, several clones were obtained which were able to grow in the medium not containing L-glutamate. Among them, one of the clones (AB1622/pLC20-10) which was thought to have received a hybrid plasmid from JA200/pLC20-10 as judged by its resistance to colicin E1 (prepared from BA197), contained PEPC at a level ~10-times higher than that in the wild-type strain (W3110) (table 2). The enzyme level in the donor strain JA200/pLC20-10 was also found to be higher to the same extent.

Table 2
Specific activities of PEPC in hybrid plasmid-bearing strains

Strain	Specific activity of PEPC (units/mg protein)
AB1622	0.00
W3110	0.38 ± 0.04 (4)
AB1622/pLC20-10	3.9 ± 0.7 (4)
JA200/pLC20-10	4.0 ± 0.5 (4)

Each strain was grown in the basal salts medium containing 0.4% glucose, thiamine HCl (3 μg/ml) and the required amino acid(s). The supplemented amino acid for AB1622 was sodium L-glutamate (340 μg/ml), and those for JA200/pLC20-10 were L-leucine (67 μg/ml), L-threonine (67 μg/ml) and L-tryptophan (33 μg/ml). Data are given ±SD. The figures in parentheses represent the number of cultures tested

3.2. *Levels of the enzyme in the plasmid-bearing strains grown on various carbon sources*

Our previous studies on the control of enzyme synthesis with strain W showed that the enzyme was present to a high level when cells were grown under glycolytic conditions and to a low level when grown under gluconeogenic conditions; this represented nutritional induction by glucose, not repression by the C_4-dicarboxylate metabolites [9]. To examine whether a similar control mechanism is operative in the plasmid-bearing strains, the effects of carbon sources on the enzyme level were investigated. As seen from table 3, the enzyme levels in these strains were affected by carbon sources in an essentially similar manner to those in the reference strain not bearing the plasmid (W3110). These results indicate the intactness of the *ppc* gene of the hybrid plasmid in the control of its expression. Therefore, it can be inferred that the elevated levels of the enzyme are due to the presence of multiple copies of the intact *ppc* gene, namely, due to the gene dosage effect.

3.3. *Characterization of the enzyme in the plasmid-bearing strains*

The sensitivities of the enzymes in the plasmid-bearing strains to their allosteric effectors were determined. The enzyme from strain AB1622/pLC20-10 was activated by acetyl-CoA, fructose 1,6-bisphosphate, GTP, laurate and dioxane, and was inhibited by L-aspartate, showing that the enzyme encoded by the *ppc* gene in the plasmid has the normal sensitivity to all the effectors (not shown).

PAGE of the cell-free extracts from strain AB1622/pLC20-10 gave only one activity band whose mobility coincided exactly with those of the enzymes from strain W3110 and strain W (not shown). The position where the enzyme activity was found is indicated by an arrow in fig.1. The protein staining revealed a clear band at the position of the enzyme for the extracts from the plasmid-bearing strains but not on those from the strains not bearing the plasmid (fig.1). Upon SDS–PAGE a thick band with a mobility equal to PEPC from strain W was observed only for the extracts from the plasmid-bearing strains (fig.2). These results

Table 3
Levels of PEPC in hybrid plasmid-bearing strains grown on various carbon sources

Carbon source[a] (%, w/v)	Specific activity of PEPC (units/mg protein)		
	W3110	AB1622/pLC20-10	JA200/pLC20-10
Glucose (0.40)	0.42 (1.0)[b]	4.5 (1.0)[b]	4.4 (1.0)[b]
Glycerol (0.41)	0.17 (0.40)	n.d.[c]	5.0 (1.14)
D,L-Lactate (0.40)	0.08 (0.19)	1.2 (0.27)	n.d.
D,L-Malate (0.90)	0.06 (0.14)	0.33 (0.07)	n.d.
L-Aspartate (0.44)	0.04 (0.10)	0.40 (0.09)	n.d.
'Enriched'	0.05 (0.12)	0.26 (0.06)	0.45 (0.10)
Glucose (0.40) + L-aspartate (0.44)	0.34 (0.81)	2.3 (0.51)	n.d.
Glucose (0.40) + 'enriched'	0.21 (0.50)	0.26 (0.06)	0.77 (0.18)

[a] Synthetic growth medium consisted of indicated carbon source, the basal salts medium, and the required supplements as in table 2
[b] The figures are parentheses represent the relative values taking the value of the glucose-grown cells as a standard for each strain
[c] Not determined

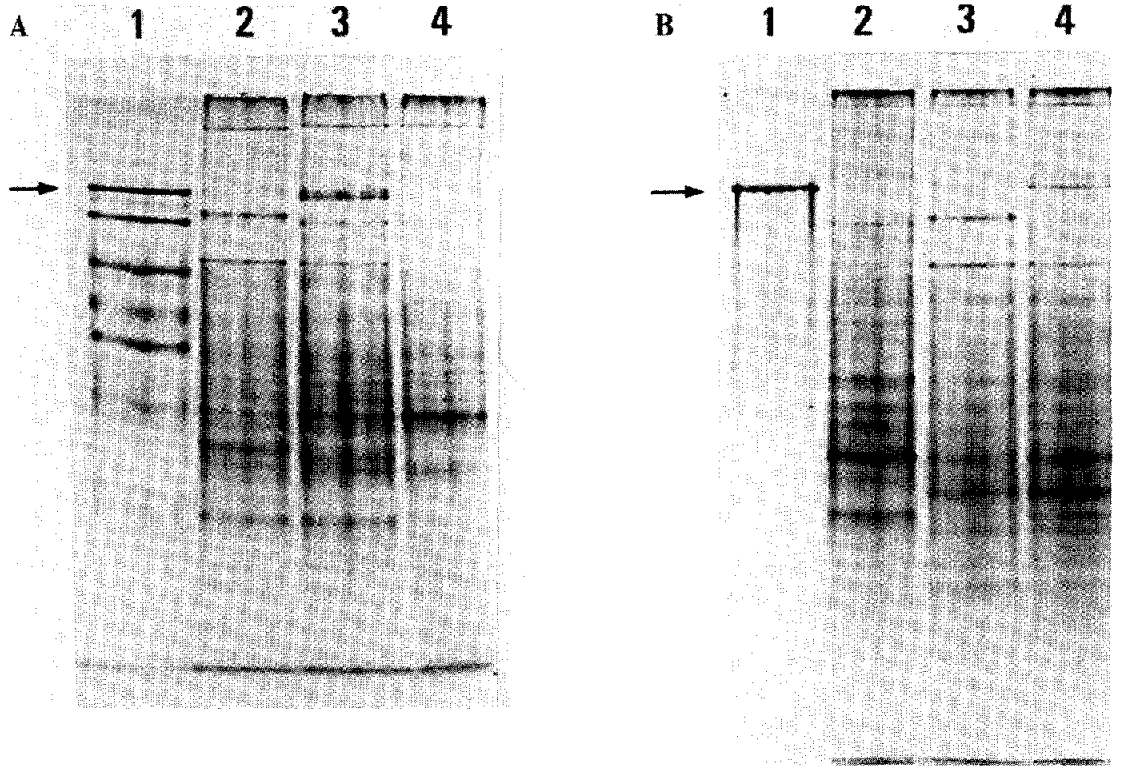

Fig.1. PAGE of cell-free extracts from hybrid plasmid-bearing strains. The cell-free extracts (100 μg protein each) which had been prepared for the experiment in table 2 were subjected to a PAGE (pH 8.9). (A) Slot (1), partially purified PEPC (100 μg protein) from strain W; (2) W3110; (3) AB1622/pLC20-10; (4) AB1622. (B) Slot (1), purified PEPC (10 μg protein) from strain W [2] (prepared by N. Fujita in this laboratory); (2) AB1622; (3) W3110; (4) JA200/pLC20-10. Proteins were stained with Coomassie brilliant blue. Arrows denote the location where the enzyme activity was detected as in [14].

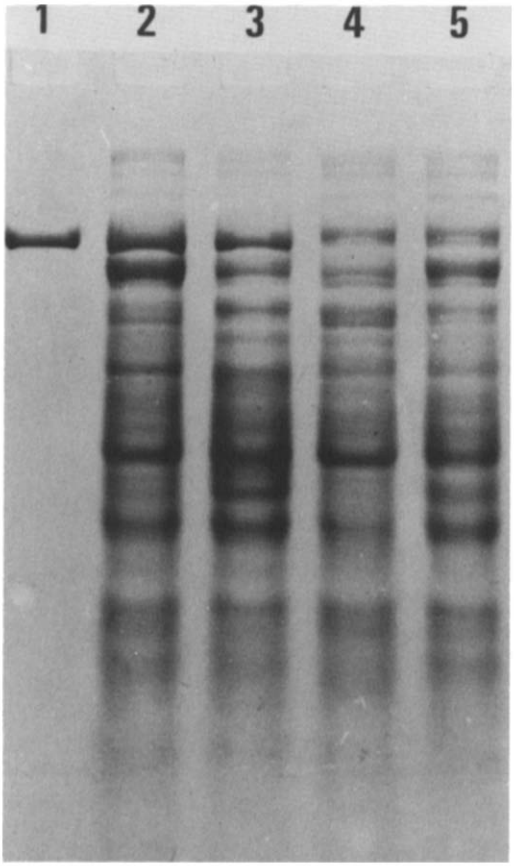

Fig.2. SDS–PAGE of cell-free extracts from hybrid plasmid-bearing strains: (1) purified PEPC from strain W; (2) JA200/pLC20-10; (3) AB1622/pLC20-10; (4) AB1622; (5) W3110.

strongly suggest that the enzyme encoded by the plasmid gene is identical with the wild-type enzyme encoded by the chromosomal gene in such molecular properties as electrophoretic mobility and molecular weight of its polypeptide chains. Furthermore, it can be inferred that the increased level of the enzyme in the plasmid-bearing strains are due to the increased number of the enzyme molecule. The content of the enzyme in the cell-free extracts from JA200/pLC20-10 was ~2% as protein according to the densitometer tracing of the electrophoretogram in fig.1B (slot 4) (measured by S. Ishijima in this laboratory).

4. Discussion

Hybrid plasmid pLC20-10 was identified as the one carrying the *ppc*$^+$ gene here. This was further supported by the fact that the purified plasmid DNA had an ability to transform PEPC-negative mutant (PA342) with a frequency of 2×10^4 transformants/μg DNA. The plasmid DNA was $\sim 1.2 \times 10^7$ M_r as measured by agarose gel electrophoresis (details will be published elsewhere).

This plasmid had been reported to carry the *argH*$^+$ gene [4]. The M_r measured in [4] using the electron microscope was in good accord with that estimated by us. Accordingly, the plasmid must carry the *ppc*$^+$ and *argH*$^+$ genes, both of which are known to be located at the same map position of 89 min on *E. coli* chromosome [15]. This work strongly suggests that the enzyme produced by the plasmid-bearing strains is identical with that produced by the wild-type strain in molecular properties. Thus the former strains can be utilized as good sources for large scale preparation of the enzyme. We have already shown that 150 mg of the enzyme is obtainable from 130 g wet cells of JA200/pLC20-10 (details will be published elsewhere).

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