Rapid Paper

Application of Plasmid to ATP
Production by *E. coli*Makoto SHIMOSAKA, Yasuki FUKUDA
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A plasmid pLC16-4 which contained an *E. coli* phosphofructokinase (*pfk*) structural gene was introduced into *E. coli* C600. It was designated as C600 (pLC16-4). By gene dosage effect the C600 (pLC16-4) showed a 7-fold higher level of phosphofructokinase than the original strain *E. coli* C-600. By the use of this plasmid-carrying *E. coli* C600 (pLC16-4), a high level of ATP was produced in our ATP formation system: about 75% of 20 mM AMP was phosphorylated to ATP in a 3 hr reaction, while only 25% of AMP was converted to ATP by the original strain C600.

Escherichia coli which carries a hybrid plasmid containing a gene coded for some enzyme produces more of this enzyme than *E. coli* which does not. For example, cells carrying a hybrid ColE1 plasmid which contains a *trp* operon gene (ColE1-*trp*) produces a tryptophan synthetic enzyme at a high level.¹⁾ An almost identical effect of gene dosage was reported by Fraenkel *et al.*²⁾ about a plasmid which contained a *pfk* structural gene. The phosphofructokinase (EC 2.7.1.11) coded by the *pfk* structural gene is a main regulatory enzyme in the glycolytic pathway. The hybrid ColE1-*pfk* plasmid (pLC16-4)²⁾ was screened from the Clarke and Carbon clone bank.³⁾

We introduced this pLC16-4 plasmid into an *E. coli* cell and examined the effect of the *pfk* gene dosage on the flux of the glycolytic pathway and an ATP formation in our system.^{4~9)}

MATERIALS AND METHODS

Strains. *E. coli* JA200 carrying a pLC16-4^{2,3)} was obtained from the Clarke and Carbon clone bank. *E. coli* C600 (F⁻, thr, leu, thi) was obtained from Dr. Oka and used through these experiments.

Preparation of a Plasmid pLC16-4. A cleared lysate was prepared from *E. coli* JA200 carrying a pLC16-4 by the method of Clewell *et al.*,¹⁰⁾ successively the plasmid DNA was purified by a ethidium bromide-caesium chloride equilibrium gradient centrifugation.

Transformation. *E. coli* C600 was transformed with a pLC16-4 by the method of Cohen *et al.*¹¹⁾ and colicin immune (col^{imm}) cells were selected.

Preparation of dried cells of E. coli. The original *E. coli* C600 and *E. coli* C600 carrying a pLC16-4 were grown in the medium containing 0.3% peptone, 0.1% yeast extract, 0.2% KH₂PO₄, 0.3% (NH₄)₂HPO₄, 0.1% MgSO₄·7H₂O, 5.0% glucose at 28°C for 12 hr on a reciprocal shaker. Five hundred ml of the above medium was put in a 2-liter Sakaguchi flask. The cells were harvested by centrifugation at 9,000 × *g* for 10 min, then washed three times with distilled water to remove the broth. These wet cells were first fan-dried for 8 hr at 25°C (60% of moisture), then completely dried over P₂O₅ for 24 hr in a vacuum desiccator.⁴⁾

Assay for ATP formation.^{4~9)} The reaction mixture (0.5 ml) containing 200 mM glucose or glucose-6-phosphate, 30 mM MgSO₄·7H₂O, 300 mM potassium phosphate buffer (pH 8.0), 20 mM AMP, 100 mg/ml of dried cells was shaken at 28°C. The reaction was terminated by immersing the test tube in boiling water for 3 min. After cell debris was removed by centrifugation at 3,000 × *g* for 10 min, the supernatant was analysed.

Analyses. Glucose was measured at 500 nm with "glucostat" reagent (Fujisawa Co., Ltd., Japan) and glucose-6-phosphate was measured by the method of Latzko *et al.*¹²⁾ AMP, ADP and ATP were determined by measuring their optical densities at 260 nm after extracting their spots with 0.01 N HCl from paper chromatograms.⁴⁾ The paper chromatography was developed on Toyo filter paper No. 53 with a solvent system consisting of 95% ethanol and 1 M ammonium acetate (2:1, by volume). ATP was measured also by an ATP photometer (SAI Technology Co. Model 3000). Phosphofructokinase activity was measured by the method of Foy and Bhattacharjee.¹³⁾ Protein was determined by the method of Lowry *et al.*¹⁴⁾

RESULTS AND DISCUSSION

The plasmid pLC16-4 efficiently transform-

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ed strain C600 to colicin immune cell (col^{imm} , 3×10^5 transformants/ μg DNA). After analyzing the cleared lysate by an agarose gel electrophoresis, we confirmed that this transformant carries a plasmid which is identical to pLC16-4 in size (data are not shown here). We used this transformant (denoted C600 (pLC16-4)) as a *pfk* gene dosed cell and the original C600 as a control cell.

A crude extract from C-600 (pLC16-4) attained about a 7-fold higher level of phosphofructokinase activity than that from C600 as shown in Table I. This increase of enzyme level is probably due to a plasmid-carried *pfk* gene, since Fraenkel *et al.*²⁾ also reported the same gene dosage effect.

TABLE I. PHOSPHOFRUCTOKINASE ACTIVITY OF C600 AND C600 (pLC16-4) CELLS

The enzyme activity was determined with the dialyzed cell extracts as described in MATERIALS AND METHODS.

Strain	Activity ($\mu\text{mol}/\text{min}/\text{mg}$ -protein)
C600	0.45
C600 (pLC16-4)	2.91

Using this transformant, we tried to produce ATP in our system which had been done with yeast.⁴⁻⁹⁾ The system worked as well with *E. coli* as with yeast. In this reaction with C600 (pLC16-4), 95% of the initial AMP (20 mM) was phosphorylated to ADP (20%) plus ATP (75%) after a 3 hr incubation, while in the reaction with the original C600, only 50% of the initial AMP was phosphorylated to ADP (25%) plus ATP (25%) and no more phosphorylation was observed by further incubation (Fig. 1). The optimal conditions for ATP production are now under investigation. This effective production of ATP probably resulted from the fact that the flux of the glycolytic pathway was activated by the increase of phosphofructokinase activity. Although the plasmid pLC16-4 also contains a structural gene (*tpi*) coded for triosephosphate isomerase (EC. 5.3.1.1), the dosage effect of this gene has not been estimated yet. As can be seen in Fig. 1,

FIG. 1. ATP formation by Dried C600 (Left) and C600 (pLC16-4) (Right) Cells. ATP-forming reaction was carried out as described in MATERIALS AND METHODS using glucose (above) or glucose-6-phosphate (below) as energy sources. Symbols: (○), AMP; (◐), ADP; (●), ATP; (■), glucose; (□), glucose-6-phosphate.

glucose-6-phosphate was more effectively used than glucose by C600(pLC16-4). When glucose was used as energy source, a small amount of ATP was produced. Perhaps the enzyme activity which converts glucose to glucose-6-phosphate was inactivated in the drying process. Therefore we are now investigating the effects of *pfk* or *tpi* gene dosage and the conversion mechanism of carbohydrates added to the reaction mixture as energy sources.

Thus, we have succeeded in the application of plasmid to ATP production, and we plan to study the combination of this ATP production with phosphorylation of various substances. Some useful materials such as CDP-choline and/or various sugar nucleotides can be produced in this way.

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