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Application of Hybrid Plasmids Carrying Glycolysis Genes to ATP Production by *Escherichia coli*

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The closely linked structural genes of phosphofructokinase (*pfkA*) and triosephosphate isomerase (*tpi*) of *Escherichia coli* were separately cloned onto plasmid pBR322. By gene dosage effects, transformed cells of *E. coli* C600 with these pBR322 hybrid plasmids showed 7- and 16-fold increases in the specific activities of phosphofructokinase and triosephosphate isomerase, respectively, over the specific activities in C600. Dried preparations of *E. coli* cells dosed with these genes showed appreciably high ATP-regenerating activity.

We have been studying the construction of a bioreactor system for the production of useful compounds (9–12). This reactor system consists of two reaction processes. One is an ATP-regenerating process catalyzed by sequential enzyme reactions in glycolysis. In this process, ATP is continuously regenerated from AMP or ADP by consuming glucose and converting it to ethanol. The other is a substrate-converting process. In this system, various substrates are converted into useful compounds by utilizing ATP regenerated by glycolysis. By coupling of these two processes, chemically, medically, and nutritionally useful compounds have been produced efficiently.

The glycolytic pathways of yeast cells have been preferred as an ATP-regenerating system since yeast cells possess high glycolytic activity. However, it is believed that the efficiency of substrate conversion varies depending on the ATP-regenerating activity. So to construct a more efficient ATP-regenerating system, the improvement of the activity of glycolytic enzymes of microbial cells was investigated by applying the effects of plasmid-mediated gene dosage.

For this purpose, *Escherichia coli* cells were used as a model system to construct the strains carrying a hybrid plasmid for glycolysis genes. *E. coli* C600 carrying hybrid ColE1 plasmid pLC16-4 for the structural genes *pfkA* and *tpi*, the genes for phosphofructokinase (PFK) and triosephosphate isomerase (TPI), respectively, was constructed as shown previously (17). The cells dosed with such genes showed high ATP-regenerating activity. To elucidate this effect, genes *pfkA* and *tpi* were separately cloned onto pBR322, and the individual effect of each gene on ATP-regenerating activity was examined.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used were *E. coli* K-12 derivatives, C600 (F^- *thr leu thi lacY supE tonA*) and ET2036 [F^- Δ(*gal-bio-uvrB*) φ80h Δ(*rha-pfkA-tpi*)] (16). Strain JA200(pLC16-4) was obtained from the Clarke and Carbon clone bank (3). The plasmid used was pBR322 (2).

Preparation of plasmid DNA. Crude plasmid DNA was prepared by the method of Meyers et al. (15). Covalently closed circular DNA was purified by ethidium bromide-cesium chloride equilibrium centrifugation.

Restriction endonuclease digestion, ligation, and transformation. All restriction endonucleases were purchased from Takara Shuzo Co., Ltd., Kyoto, Japan. Digestion was carried out under the conditions recommended by Takara. Ligation of DNA fragments was performed at 10°C for 15 h with T_4 DNA ligase (Miles Laboratories, Inc., Elkhart, Ind.) in 66 mM Tris-hydrochloride (pH 7.5) containing 1.0 mM EDTA, 10 mM dithiothreitol, 0.1 mM ATP, and 0.1 mg of bovine serum albumin per ml. Transformation was carried out by the method of Cohen et al. (4).

Gel electrophoresis. DNA fragments generated by restriction endonuclease digestion were analyzed in 1.0% agarose gels. The running buffer was the same as that described by Helling et al. (8). Polyacrylamide gel electrophoresis was carried out by the method of Davis (5).

Medium. EMB agar medium (1.0% peptone, 0.1% yeast extract, 0.5% NaCl, 0.2% K_2HPO_4, 0.006% methylene blue, 0.04% eosin Y, 1.0% carbohydrate, 1.5% agar) was used to determine the ability of cells to assimilate mannitol and glycerol.

Preparations of dried *E. coli* cells. *E. coli* cells were grown at 28°C on a reciprocal shaker in 500 ml of LB broth (1.0% peptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose; pH 7.2). Early-stationary-phase cells were harvested and washed three times with distilled water. The wet cells obtained were first fan dried at 25°C for 8 h (60% of moisture) and then completely dried over P_2O_5 for 24 h in a vacuum

desiccator. Dried cells were ground down with a mortar and pestle and stored at −20°C.

ATP-forming reaction. The reaction mixture (0.3 ml) containing 100 mM glucose 6-phosphate (G6P), 30 mM $MgSO_4 \cdot 7H_2O$, 300 mM potassium phosphate buffer (pH 8.0), 50 mM AMP, and 100 mg of dried cells per ml was incubated at 28°C with shaking. The reaction was terminated by immersing the test tube in boiling water for 3 min. Immediately after cooling, the cell debris was removed by centrifugation at 3,000 × *g* for 10 min, and the clear supernatants were used for the analysis of reactants.

Enzyme assay. All the strains were grown in LB broth to early stationary phase. The cells were harvested, washed twice with 20 mM Tris-hydrochloride buffer (pH 7.6), and then suspended in the same buffer. This cell suspension was treated on an ultrasonicator (Kubota model 200M). The disrupted cells were centrifuged for 30 min at 16,000 × *g*, and the resultant supernatant was dialyzed against 100 volumes of 20 mM Tris-hydrochloride buffer (pH 7.6) at 4°C overnight. The dialysates were used for the assay of enzyme activities. PFK activity was measured by the enzymatic method of Foy and Bhattacharjee (6). TPI activity was also measured enzymatically by the method of Beisenherz (1). To determine the positions of protein bands for both PFK and TPI in polyacrylamide gel, the gel was cut into slices 2 mm thick after electrophoresis at 4°C. Then, each gel slice was ground down with a mortar and pestle at 4°C, and the resultant gel homogenates were used directly for enzyme assay.

Analyses. G6P, fructose 6-phosphate (F6P), and fructose 1,6-bisphosphate (FBP) were measured by the method of Latzco and Gibbs (13). AMP and ADP-ATP were determined by measuring their optical densities at 260 nm after extracting them with 0.01 M HCl (pH 2.0) from paper chromatograms. The paper chromatograph was developed on Toyo filter paper no. 53 with a solvent system consisting of 95% ethanol and 1 M ammonium acetate (2:1, vol/vol) (10). ATP was also measured on an ATP photometer (SAI Technology Co., San Diego, Calif.; model 3000). Protein was determined by the method of Lowry et al. (14).

RESULTS

Effect of plasmid pLC16-4 on ATP formation. Hybrid ColE1 plasmids for *E. coli* glycolysis genes were selected from the Clarke and Carbon clone bank by Thomson et al. (18). Among these plasmids, plasmid pLC16-4, which contains a structural gene (*pfkA*) for PFK, was examined, since PFK is thought to be a regulatory enzyme in the glycolytic pathway. In addition to *pfkA*, this plasmid contains a structural gene (*tpi*) for TPI.

At first, *E. coli* C600(pLC16-4) was constructed by transformation, and the specific activities of PFK and TPI in the crude extracts were determined. *E. coli* C600(pLC16-4) showed high activities of PFK and TPI: 6- and 11-fold more, respectively, than those in strain C600 (Table 1). The increase in activities was considered to be caused by a plasmid carrying the *pfkA* and *tpi* genes as shown by Thomson et al. (18, 19).

TABLE 1. PFK and TPI activities of transformants[a]

Strain	Enzyme activity (μmol/min per mg of protein)	
	PFK	TPI
C600	0.45	3.32
C600(pLC16-4)[b]	2.91	35.0
C600(pBR322)	0.47	4.33
C600(pGE7)	3.09	3.41
C600(pGE42)	2.39	2.33
C600(pGE51)	0.40	53.4

[a] Cells were grown in LB broth to early stationary phase. Preparation of crude cell extracts and assay conditions were the same as described in the text.
[b] Data from reference 17.

Dried cells prepared from strain C600(pLC16-4) showed the high ATP-forming activity. In the reaction with strain C600(pLC16-4), 95% of the initial AMP (20 mM) was phosphorylated to ADP (20%) plus ATP (75%) after 3 h of incubation, whereas with strain C600, only 50% of the initial AMP was phosphorylated to ADP (25%) plus ATP (25%) (Fig. 1). For the elucidation of the effect of each enzyme activity on ATP formation, subcloning of *pfkA* and *tpi* genes was investigated.

Subcloning of *pfkA* and *tpi* genes from pLC16-4. (i) Restriction cleavage map of pLC16-4. For construction of a restriction map of pLC16-4, the plasmid was digested with five restriction endonucleases, *Eco*RI, *Hin*dIII, *Pst*I, *Bam*HI, and *Sal*I, and the DNA fragments generated were analyzed by agarose gel electrophoresis (data not shown). Plasmid pLC16-4 had a molecular mass of 8.4 megadaltons (Md) and consisted of a ColE1 DNA region (4.2 Md) and an *E. coli* chromosomal DNA region (4.2 Md) inserted into the *Eco*RI site of ColE1 DNA by the polydeoxyadenylate-polydeoxythymidylate method (3). In the chromosomal DNA region, single susceptible sites for *Eco*RI and *Hin*dIII and three susceptible sites for *Pst*I were found (Fig. 2). Plasmid pLC16-4 had no susceptible sites for *Bam*HI and *Sal*I. Based on these data, the restriction map of pLC16-4 was constructed as shown in Fig. 2.

(ii) Subcloning of *pfkA* and *tpi* genes. To construct a recombinant plasmid expressing only one gene (*pfkA* or *tpi*), various DNA fragments derived from the *E. coli* chromosomal DNA region of pLC16-4 by restriction endonuclease digestion were cloned onto pBR322. Gene expression was examined by using *E. coli* ET2036, which is deficient in both PFK and TPI activities (16). Deficiency in PFK and TPI activities results in a loss of the ability to grow on

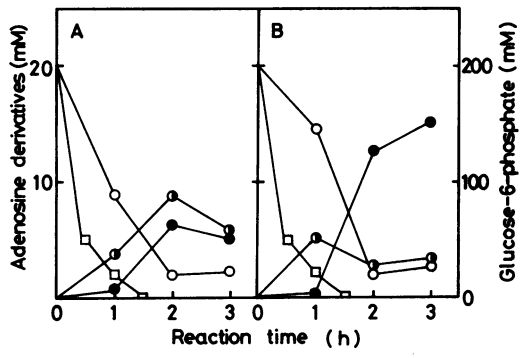

FIG. 1. ATP formation with dried cells of *E. coli* C600 (A) and C600(pLC16-4) (B). Data are taken from reference 17. Preparation of dried cells and the concentration of the reaction mixture were somewhat different from those described in the text. *E. coli* cells were grown in 0.3% peptone–0.1% yeast extract–0.2% KH_2PO_4–0.3% $(NH_4)_2HPO_4$–0.1% $MgSO_4 \cdot 7H_2O$–5.0% glucose in place of LB broth. The reaction mixture (0.5 ml) consisted of 200 mM G6P, 30 mM $MgSO_4 \cdot 7H_2O$, 300 mM potassium phosphate buffer (pH 8.0), 20 mM AMP, and 100 mg of dried cells per ml. At 1-h intervals, the reaction was terminated and the concentrations of AMP (○), ADP (◑), ATP (●), and G6P (□) were determined.

mannitol and glycerol, respectively (7). Therefore, if strain ET2036 was transformed with a plasmid containing both *pfkA* and *tpi* genes, it could grow on both mannitol and glycerol as carbon sources. The *E. coli* ET2036 transformed with pLC16-4 formed red colonies on both EMB-mannitol and EMB-glycerol agar media, although strain ET2036 formed white colonies on both media. By testing the colony color of strain ET2036 transformed with the constructed recombinant plasmid on both media, the presence of PFK and TPI activity expressed by the plasmid-carrying gene was investigated.

The hybrid pBR322 plasmid, designated pGE51, contained the 3.6-Md DNA fragment derived from the *E. coli* chromosomal DNA region by double digestion with *Eco*RI and *Hin*dIII (Fig. 2). Strain ET2036 carrying pGE51 expressed only TPI activity, indicating that the 3.6-Md DNA fragment contained only the *tpi* gene and that the *pfkA* gene was cleaved by either *Eco*RI or *Hin*dIII. Then the *Pst*I-digested DNA fragment (5.3 Md) containing the *Eco*RI site of pLC16-4 was investigated. Since this 5.3-Md fragment contained an *ori* region of ColE1, an autonomously replicating plasmid could be constructed by intramolecular ligation between the *Pst*I sites at the ends (Fig. 2). This plasmid (designated pGE42) enabled strain ET2036 to express only PFK activity. The *Pst*I-digested 5.3-Md DNA fragment was also cloned at the *Pst*I site of pBR322 (Fig. 2). This plasmid (designated pGE7) also expressed PFK activity in strain ET2036. Although pGE7 contained two *ori* regions (one cloned from the ColE1 DNA and the other from pBR322 DNA), it was stably maintained in Rec^+ cells such as strain C600.

Gene dosage effects of *pfkA* and *tpi* on each enzyme activity. As described above, hybrid plasmids expressing either *pfkA* (pGE7 and pGE42) or *tpi* (pGE51) were constructed. To estimate the gene dosage effects of these hybrid plasmids, each plasmid was introduced into *E. coli* C600, and the activities of PFK and TPI in the crude extracts of each transformant were assayed. Strains C600(pGE7) and C600(pGE42) showed a five- to sevenfold-higher level of activity for PFK than did strain C600, but they showed almost the same level of TPI activity (Table 1). On the other hand, strain C600(pGE51) showed a 16-fold-higher level of TPI activity than did strain C600, though the level of PFK activity remained constant. Strain C600(pBR322) showed almost the same level of PFK and TPI activities as did strain C600. These results clearly show the existence of the *pfkA* and *tpi* genes in each recombinant plasmid.

To ensure that the increase in levels of activities for PFK and TPI were due to gene dosage effects, crude extracts from strain C600 carrying each recombinant plasmid were analyzed by polyacrylamide gel electrophoresis. Strain C600(pLC16-4) showed two deep-stained bands for proteins corresponding to PFK and TPI (Fig. 3). In the same way, strains C600(pGE7) and C600(pGE42) both showed only one band corresponding to PFK. On the other hand, strain C600(pGE51) showed only one band for TPI.

Effect of gene-dosed cells on ATP formation. In the above sections, construction of the pBR322 hybrid plasmids carrying either *pfkA* or *tpi* genes and the gene dosage effect caused by each hybrid plasmid were described. Dried cells were prepared from each gene-dosed cell, with *E. coli* C600(pGE7) being used for *pfkA* and *E. coli* C600(pGE51) being used for *tpi*. These dried cells were used for the ATP formation reaction. The rate of AMP phosphorylation was accelerated in both reactions with dried cells of strains C600(pGE7) and C600(pGE51) (Fig. 4). In the reaction with dried cells of strains C600(pGE7) and C600(pGE51), about 70% of the initial AMP (50 mM) was phosphorylated to ATP after 1 h of incubation, but with dried cells of strain C600, only 10% of the initial AMP was phosphorylated to ATP. Dried cells of strain C600(pBR322) showed almost the same ATP formation activity as did strain C600. The changes in concentration of G6P, F6P, and FBP in the reaction mixture were measured. The total concentration of three hexosephosphates (G6P-F6P-FBP) is shown in Fig. 4. In reactions with dried cells of strains

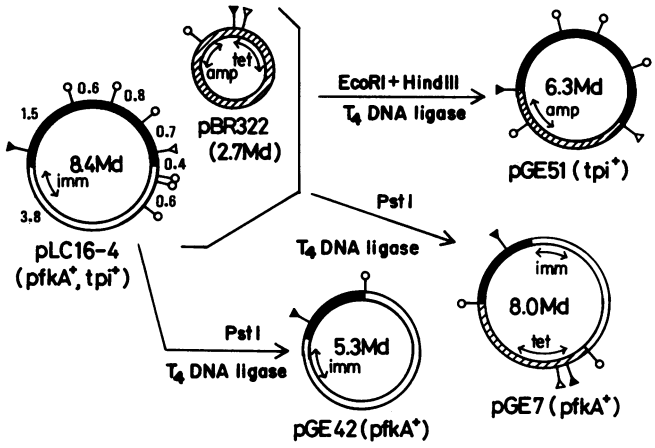

FIG. 2. Restriction cleavage map of pLC16-4 and schematic outline of construction of recombinant plasmids pGE7, pGE42, and pGE51. Plasmid pLC16-4 has a molecular mass of 8.4 Md and consists of *E. coli* chromosomal DNA (4.2 Md) and ColE1 DNA (4.2 Md). The numerals outside the circle show the molecular mass in Md of the DNA fragments between each two restriction endonuclease-susceptible sites of pLC16-4. Each recombinant plasmid was constructed as follows: pGE7, the ligation between *Pst*I-digested pBR322 DNA and the *Pst*I-digested DNA fragment (5.3 Md) containing the single *Eco*RI site of pLC16-4; pGE42, the self-ligation of the *Pst*I-digested 5.3-Md fragment of pLC16-4; pGE51, the ligation between *Eco*RI-*Hin*dIII double-digested pBR322 DNA and the 3.6-Md DNA fragment derived from *E. coli* chromosomal DNA of pLC16-4 with *Eco*RI-*Hin*dIII double digestion. Dark bands represent *E. coli* chromosomal DNA regions; white bands, ColE1 DNA regions; oblique line bands, pBR322 DNA regions. The molecular mass of each plasmid is indicated inside the circle. The marker gene of each plasmid is also indicated inside the circle: *amp* shows resistance to ampicillin; *tet*, resistance to tetracycline; *imm*, immunity to colicin E1. Symbols show restriction endonuclease-susceptible sites for *Eco*RI (▽), *Hin*dIII (▽), *Pst*I (▽).

C600(pGE7) or C600(pGE51), the consumption rate of the hexosephosphates was higher than that of strain C600.

DISCUSSION

Two kinds of pBR322 hybrid plasmids containing the *E. coli pfkA* gene (plasmid pGE7) and the *tpi* gene (plasmid pGE51) were constructed. Increases in the specific activities of PFK (7-fold) and TPI (16-fold) were observed when each plasmid was introduced into *E. coli* C600. As *E. coli* C600 carrying pBR322 showed almost the same level of PFK and TPI activity as did strain C600, it was confirmed that pBR322 used as a vector exerted no influence on the level of enzyme activities. The analysis of protein bands in polyacrylamide gel electrophoresis indicated that the increases in the levels of activities for PFK and TPI were presumably due to the gene dosage effects; that is, the amount of enzyme produced per cell was increased by the plasmid carrying the gene. The rate of ATP formation was greatly accelerated by using dried preparations of these gene-dosed cells. Since the growth rate of each transformant was almost the same as that of strain C600 (data not shown), it was improbable that the acceleration of AMP phosphorylation resulted from the altered cell growth caused by the hybrid plasmid. Thus, it was concluded that the increases of both PFK and TPI activities caused by the plasmids carrying the genes efficiently accelerated the rate of AMP phosphorylation to ATP in our reaction system.

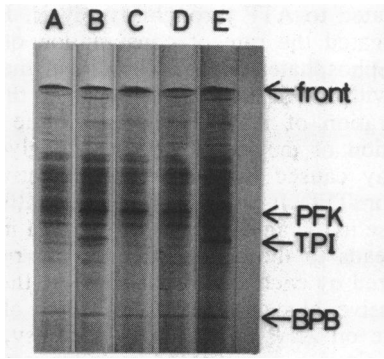

FIG. 3. Polyacrylamide gel electrophoresis of crude extracts from each transformant. Early-stationary-phase cells grown in LB broth were collected, and crude extracts were prepared as described in the text. A 50-μg amount of protein was applied for each gel, and the gel was stained for protein with Coomassie brilliant blue. Arrows show positions of the protein bands. BPB, Bromophenol blue. Lanes show results for strains C600 (A), C600(pLC16-4) (B), C600(pGE7) (C), C600(pGE42) (D), and C600(pGE51) (E).

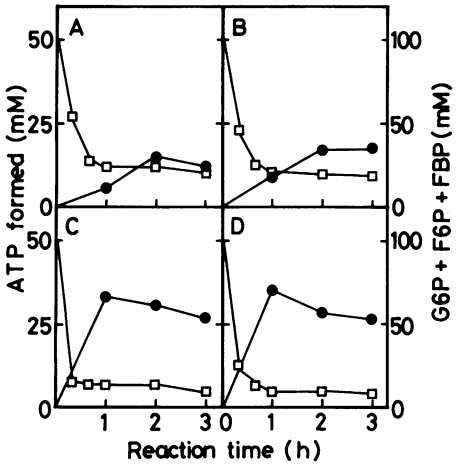

FIG. 4. ATP formation with dried cells of *E. coli* C600 (A), C600(pBR322) (B), C600(pGE7) (C), and C600(pGE51) (D). An ATP-forming reaction was carried out as described in the text. At the prescribed times, the reaction was terminated, and the concentrations of ATP (●) and three hexosephosphates (G6P-F6P-FBP) (□) were determined.

The mechanism of ATP formation in our reaction system is considered as follows. G6P added as an energy source is converted to FBP via F6P. FBP thus formed is one of the high-energy intermediates in glycolysis, and it can regenerate ATP by phosphorylating ADP during further metabolism through glycolysis. On the other hand, AMP added to the reaction mixture containing a high concentration of phosphate buffer is phosphorylated to ADP by adenylate kinase reaction with consumption of ATP (AMP + ATP → 2ADP). ADP thus formed is further phosphorylated to ATP through glycolysis. Having investigated the rate of consumption of three hexosephosphates (G6P-F6P-FBP) in the reaction with dried cells, we suppose that the acceleration of ATP formation is due to an activation of metabolism through a glycolytic pathway caused by the increased activity of PFK or TPI. It may be considered that the increase in the amount of PFK and TPI in dried cells leads to the acceleration of the reaction catalyzed by each enzyme. However, the comprehensive elucidation of the effect of each enzyme on ATP formation is not easy, since glycolysis is a complicated multienzyme pathway. For example, to elucidate the effect of PFK on ATP formation, it must be considered that PFK reaction proceeds with the consumption of ATP and ATP is an allosteric inhibitor for PFK. Investigation of the precise mechanism is now under way.

In this paper, the application of plasmids carrying either the *pfkA* or the *tpi* gene to an ATP-forming reaction was described. The results obtained here can be applicable to other bacteria and to yeasts. Application of hybrid plasmids carrying glycolysis genes to yeast cells such as *Saccharomyces cerevisiae* is worth notice in constructing a more efficient and practical ATP-regenerating system.

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