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Construction of Various Recombinant Plasmids for the Enhancement of Glutathione Production in *E. coli*

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Abstract

In order to enhance glutathione production, various recombinant plasmids containing *gshI* and/or *gshII* genes isolated from *E. coli* K-12 were constructed and introduced into *E. coli*. Some plasmids contained one to three copies of *gshI* genes in pBR325 and others contained both *gshI* and genes for glutathione biosynthesis. γ-Glutamylcysteine synthetase activities of *E. coli* strains amplified tandem repeated *gshI* genes were dependent on the number of inserted *gshI* genes. The glutathione productivity of *E. coli* strains harboring various plasmids was investigated using an *E. coli* acetate kinase reaction as an ATP regenerating system. The glutathione productivity of *E. coli* strains harboring tandem repeated *gshI* genes was increased in proportion to the number of inserted *gshI* genes. By the introduction of *gshII* gene, the glutathione productivity of the *E. coli* was increased by two-fold compared with *E. coli* strain amplified *gshI* gene only. The enzymatic production of glutathione in *E. coli* was mainly affected by the increase of γ-glutamylcysteine synthetase activity. The highest glutathione productivity was obtained in *E. coli* strains harboring pGH-501 plasmid containing two copies of *gshI* and one copy of *gshII* genes in pUC8 vector.

Key words : *Glutathione production, tandem repeated gshI, gshII, E. coli*

Introduction

Glutathione (γ-glutamylcysteinylglycine), one of the major free thiols in most living cells, has great biological and pharmacological importance[1,2]. It has been commercially produced for hepatic medicine. Glutathione is produced either by extraction from yeast cells or by chemical synthesis[3]. But these methods have some limits due to the low contents of glutathione in yeast cells or to the difficulties in processing chemical synthesis. Therefore, the biological production of glutathione has been attempted with the glutathione synthesizing enzymes from microorganisms[4,5,6,7]. This tripeptide has been produced in a column packed with immobilized *E. coli* B cells or immobilized *Saccharomyces cerevisiae*. But the amount produced by these systems is relatively lower than that produced either by organic synthesis or by yeast cell extraction. Therefore a better system for industrial glutathione production is needed.

The low production of glutathione in bioreactor systems is caused by insufficient amounts of glutathione synthesizing enzymes, γ-glutamylcysteine synthetase and glutathione synthetase. Therefore, *gshI* and *gshII* genes responsible for glutathione biosynthesis were cloned from *E. coli* B[8,9] and *E. coli* K-12 strains[10,11], respectively. The amplification of only one of these two genes

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for glutathione biosynthesis in the *E. coli* strain was not sufficient for the enhancement of glutathione productivity. However, introducing a recombinant plasmid containing both genes, *gshI* and *gshII*, increased it[12].

Based on these results, the current study attempted to construct efficient *E. coli* strains which can produce a high level of glutathione. In order to increase glutathione production by maximizing the expression of genes for glutathione biosynthesis, the recombinant plasmids containing multiple copies of *gshI* genes or *gshI* and *gshII* genes required for glutathione biosynthesis were constructed. Their effects on the enzymatic production of glutathione were also examined.

Materials and Methods

Bacterial strains and plasmids

E. coli HB101 (*hsd*R *hsd*M *leu pro rec*A) was used for transformation and propagation of plasmids[13]. It was grown in LB medium (10 g of Bacto-Tryptone, 5 g of Yeast extract and 10 g of NaCl per liter) in the presence of ampicillin (50 μg/ul) at 37℃ for 16 hr with reciprocal shaking. The harvested cells were frozen immediately and kept at −20℃. *E. coli* cells were treated with toluene as previously described by Nam *et al.*[14]. Plasmids used in this study are presented in Table 1.

Chemicals and enzymes

ATP, acetylphosphate, amino acids, NADPH, NADH were purchased from Sigma (U. S. A). γ-Glutamylcysteine was purchased from Nakari Chemical LTD, in Japan. Glutathione reductase, pyruvate kinase (type II), and lactate dehydrogenase (type II) were purchased from Sigma. Restriction enzymes and T4 DNA ligase were from NEB (U. S. A).

DNA manipulation techniques

Most DNA manipulation techniques were carried out according to standard methods[15]. DNA fragments obtained from gel were recovered by the electroelution method followed by phenol extraction. The ligation reaction was performed either at 12℃ for 12hr (cohesive end ligation) or at 22℃ for 12 hr (blunt end ligation).

r-Glutamylcysteine synthetase assay

The activity of γ-glutamylcysteine synthetase was measured by Jackson's method[16] with a slight modification. The reaction mixture contained 30 μmol of

Table 1. Plasmids used in this study

Plasmids		Characteristics		Reference
pGH100	pBR322-*gshI*	Tc^r	7.9 Kb	Nam[11]
pGH200	pUC8-*gshI*	Ap^r	6.3 Kb	Nam[11]
pGH300	pBR322-*gshII*	Ap^r	5.4 Kb	Nam[11]
pGH325-100	pBR325-*gshI*	Tc^r, Cm^r	9.6 Kb	In this work
pGH325-101	pBR325-*gshI* · *II*	Cm^r	11.0 Kb	In this work
pGH325-200	pBR325-*gshI* · *I*	Tc^r, Cm^r	13.2 Kb	In this work
pGH325-201	pBR325-*gshI* · *I* · *II*	Cm^r	14.6 Kb	In this work
pGH325-300	pBR325-*gshI* · *I* · *I*	Tc^r, Cm^r	16.8 Kb	In this work
pGH325-301	pBR325-*gshI* · *I* · *I* · *II*	Cm^r	18.2 Kb	In this work
pGH400	pUC13-*gshI* · *II*	Ap^r	8.5 Kb	In this work
pGH500	pUC8-*gshI* · *I*	Ap^r	8.3 Kb	In this work
pGH501	pUC8-*gshI* · *I* · *II*	Ap^r	9.7 Kb	In this work

gshI : gene for γ-glutamylcysteine synthetase
gshII : gene for glutathione synthetase

L-glutamate, 30 μmol of L-cysteine, 30 μmol of ATP, 20 μmol of $MgSO_4 \cdot 7H_2O$, 200 μmol of KCl, 200 μmol of diethanolamine-HCl buffer (pH 9.15), and cell free extract in a final volume of 2.0 ml. They were incubated at 37℃ for 15 min. The reaction was terminated by adding 3.3 ml of ice cold 3.2% sulphosalicylic acid. The remaining cysteine in the reaction mixture was removed by Ball's method[17]. After standing in ice for 20 min, the reaction mixture was centrifuged at 1,000×g for 20 min. Then 1 ml of supernatant was transferred to a tube containing 4 ml of 1.0 M potassium phosphate buffer (pH 6.8), with 5 mM EDTA, 1.0 g/l ascorbic acid, and 3.0 g/l of glyoxylic acid monohydrate. The tubes were incubated at 60℃ for 5 min, rapidly cooled on ice , and then equilibrated at 25℃. Half milliliter of 3.8 mM DTNB[5,5'-dithiobis-(2-nitrobenzoic acid)] in 1.0 M potassium phosphate buffer (pH 6.8) was added. The absorbance was read at 412 nm for 7 min after adding DTNB. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the synthesis of 1 μmol of γ-glutamylcysteine per hour. The amount of protein was estimated by the method of Lowry *et al.*[18].

Glutathione synthetase assay

The reaction mixture contained 4.6 mM γ-glutamylcysteine, 12.5 mM glycine, 250 mM Tris-HCl buffer (pH 8.5), 1.25 mM ATP, 1.25 mM $MgCl_2$, 125 mM KCl, BSA (0.75 mg/ml), 0.125 mM NADH, 0.125 mM phosphoenolpyruvate, 7.4 units of pyruvate kinase, 12.7 units of lactate dehydrogenase, and cell free extract adjusted to a final volume of 0.8 ml. It was incubated at 37℃ for 2 min. The reaction was initiated in a 1 ml cuvette of 1 cm light path after adding γ-glutamylcysteine. The initial rate measurement was carried out by monitoring the change in the absorbance for 2 min. For calculation of NADH consumed, an extinction coefficient of 6.22 mM^{-1} cm^{-1} at 340 nm was used. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the synthesis of 1 μmol of glutathione per hour.

Glutathione estimation

The amount of glutathione was determined by Tietze's method[19]. The assay solution was composed of 0.2 umol NADPH, 1.6 mmol DTNB, and 0.12 units glutathione reductase. Its final volume was 1.0 ml. The rate of reaction was expressed as the change in absorbance per 1 min at 412 nm.

Glutathione production

The reaction mixture for glutathione production contained 60 mM L-glutamate, 20 mM L-cysteine, 20 mM glycine, 20 mM $MgCl_2$, 1 mM ATP, 10 mM acetylphosphate, 50 mM potassium phosphate buffer (pH 7.5), and toluene treated *E. coli cells* (100 mg wet wt/ml). The reaction was carried out at 37℃ with shaking for 2 hr and terminated after immersing the reaction tube in boiling water for 3 min.

Results and Discussion

Construction of recombinant plasmids

The γ-glutamylcysteine synthetase catalyzes the first reaction, known as the rate limiting step, of glutathione biosynthesis. To evaluate the effect of the expression level of *gshI* gene for glutathione production in *E. coli*, several recombinant plasmids containing from one to three copies of *gshI* gene were constructed by tandem polymerization of DNA fragments possessing a whole transcriptional unit of *gshI* gene. The construction scheme of various recombinant plasmids is shown in Fig. 1. Two sequential ligation reactions led to the fusing of the *gshI* genes in tandem. The first ligation was carried out to polymerize 3.6 kb *PstI* fragments containing the entire *gshI* gene at 4℃ for 12 h. The resulting ligation mixture was transferred into the second ligation mixture containing pBR325 vector DNAs digested with *PstI*. This mixture was then used for the transformation of *E. coli* HB 101 after 12 h at 4℃. The repeated number of *gshI* genes in recombinant plasmid from each transformant

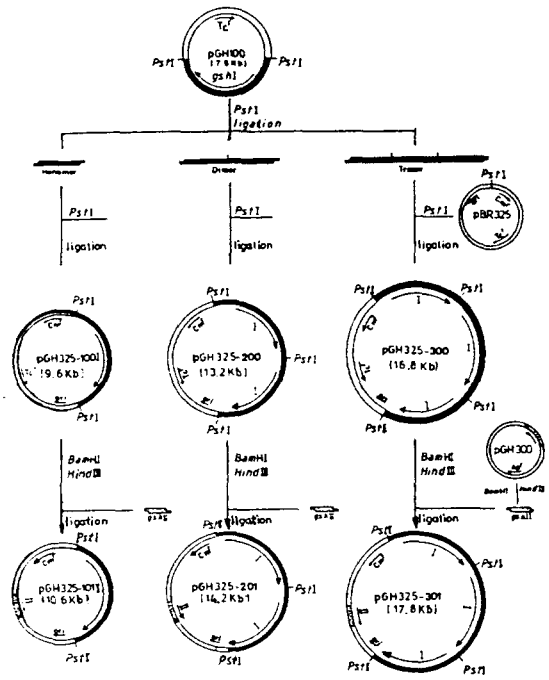

Fig. 1. Scheme for the construction of various recombinant plasmids containing *gshI* and *gshII* genes on pBR325 vector.

was estimated by the size of DNA fragments digested with *SalI* restriction enzyme (Fig. 2). The size of each plasmid was 9.6 kb, 13.2 kb and 16.8 kb, and it corresponded to one, two and three copies of the *gshI* gene containing *PstI* DNA fragments in pBR325 plasmid DNA, respectively. It has been reported that cloned polymers were unstable in *vivo* if all copies of fragments made a secondary structure by inverted repeats[13,20]. The orientation of polymerized *gshI* gene in pBR325 was examined by *Bgl*II and *Hin*dIII digestion[10]. Two different orientations with respect to the *Cm* gene were observed by size differences of fragments generated by digestion. The transcriptional direction of polymerized *gshI* genes was also confirmed by digesting with the same restriction enzymes. One type contained one, two or three copies of *gshI* gene in the reverse orientation with respect to the *Cm* gene, which were designated as pGH325-100, pGH325-200 and pGH325-300, respectively. The other type contained one or two copies of the *gshI* gene in the same orientation with respect to the *Cm* gene, and they were designated as pGH325-110 and pGH325-210. Two or three copies of *gshI* genes in recombinant plasmids were arranged in the same transcriptional direction[10].

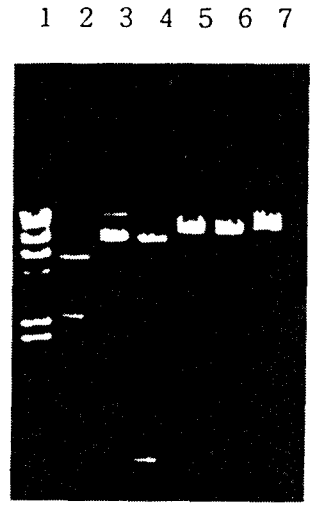

Fig. 2. Restriction pattern of the recombinant plasmids containing tandem repeated *gshI* genes. Lane 1 : λ DNA+*Hin*dIII, Lane 2 : pBR325+*Sal*I, Lane 3 : pGH325−100+*Sal*I, Lane 4 : pGH325−110+*Sal*I, Lane 5 : pGH325−200+*Sal*I, Lane 6 : pGH325−210+*al*I, Lane 7 : pGH325−300+*Sal*I

It was suggested that coexpression of two glutathione synthesizing genes in a cell could be useful for glutathione production[9,12]. Therefore, the 1.4 kb *Bam*HI-*Hin*dIII DNA fragments possessing a whole transcriptional unit of the *gshII* gene were subcloned into pGH325-100, pGH325-200 and pGH325-300 plasmids. The recombinant plasmids containing *gshI* and *gshII* genes were designated as pGH325-101, pGH325-201, and

pGH325-301, respectively. The restriction enzyme cleavage patterns of the recombinant plasmids containing both *gshI* and *gshII* genes are shown in Fig. 3.

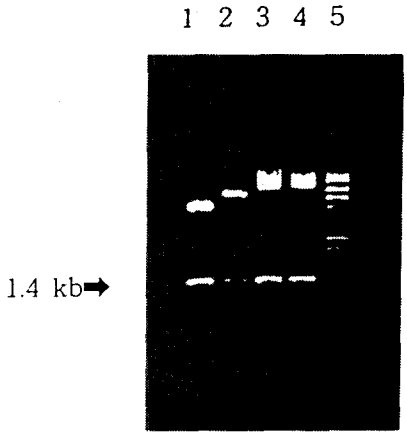

Fig. 3. Restriction pattern of the recombinant plasmids containing tandem repeated *gshI* and *gshII* genes. Lane 1 : pGH300+*Bam*HI+*Hin*dIII, Lane 2 : pGH325−101+*Bam*HI+*Hin*dIII, Lane 3 : pGH325−201+*Bam*HI+*Hin*dIII, Lane 4 : pGH325−301+*Bam*HI+*Hin*dIII, Lane 5 : λ DNA+*Hin*dIII

To compare the gene dosage effect between increasing the copy number of gene in the plasmid and increasing the copy number of the plasmid containing gene, pUC8 vector with more copy numbers than pBR325 was used for the expression of *gsh* genes. A 1.95 kb *Alu*I DNA fragment containing a whole transcriptional unit of *gshI* gene was subcloned into the *Sma*I site of the pGH200 (pUC8-*gshI*), and the recombinant plasmid was designated as pGH500 (Fig. 4). The 1.4 kb *Bam*HI-*Hin*dIII DNA fragment containing *gshII* gene was filled-in with Klenow fragment and annealed with pGH500 (pUC8-*gshI* · *I*). This recombinant plasmid, pGH501 (pUC8-*gshI* · *I* · *II*), was designed with two copies of *gshI* gene in the same transcriptional direction and one copy of the *gshII* gene in the pUC8 vector.

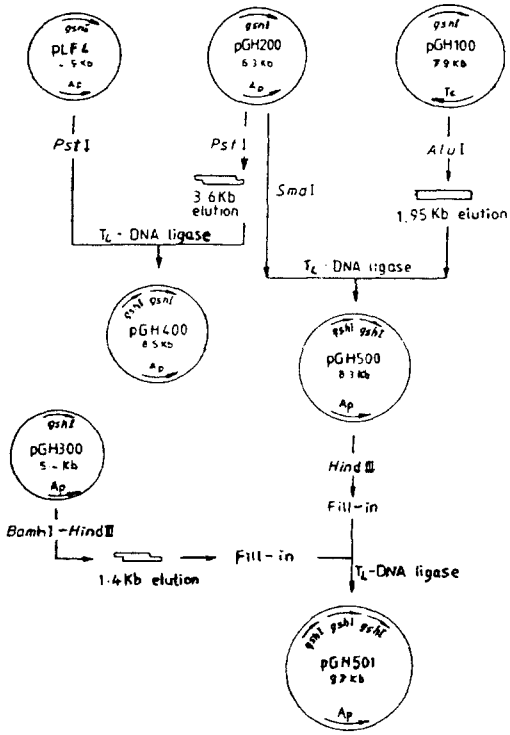

Fig. 4. Schematic representation of various recombinant plasmids containing *gshI* and *gshII* genes on pUC 8 vector

Gene dosage effect on activities of glutathione synthesizing enzymes.

γ-Glutamylcysteine synthetase activities of strains harboring recombinant plasmids increased in accordance with the number of inserted *gshI* genes (Table 2). The γ-glutamylcysteine synthetase activity of *E. coli* strain harboring pGH325-300 was approximately three times higher than that of the strain containing pGH325-100 plasmid. On the other hand, the γ-glutamylcysteine synthetase activity of strain harboring pGH500 with two copies of *gshI* in the pUC8 vector was higher than that of pGH325-300 with three copies of the *gshI* gene. These results imply that using a high copy number vector system is more useful for maximizing the expression of a gene than by tandem repetition of the gene.

Table 2. Activities of -glutamylcysteine synthetase and glutathione synthetase in *E. coli* HB101 strains harboring recombinant plasmids

Strains	Properties	Glutathione contents in culture broth(mg/ml)[a*]	Activities[b*]	
			GSH-I	GSH-II[c*]
HB101		0.01	0.16	0.38
HB101/pGH325-100	pBR325-*gshI*	0.08	2.45	0.38
HB101/pGH325-101	pBR325-*gshI* · *II*	0.08	2.45	2.70
HB101/pGH325-200	pBR325-*gshI* · *I*	0.08	5.20	0.38
HB101/pGH325-201	pBR325-*gshI* · *I* · *II*	0.08	5.00	2.60
HB101/pGH325-300	pBR325-*gshI* · *I* · *I*	0.08	6.30	0.38
HB101/pGH325-301	pBR325-*gshI* · *I* · *I* · *II*	0.08	6.30	2.50
HB101/pGH200	pUC8-*gshI*	0.08	4.23	0.38
HB101/pGH400	pUC8-gsh*I* · *II*	0.08	4.10	3.80
HB101/pGH500	pUC8-gsh*I* · *I*	0.08	8.12	0.38
HB101/pGH501	pUC8-*gshI* · *I* · *II*	0.08	8.10	3.90

a* : Gluthathione content (mg/ml) was determined as described in Material and Methods.

b* : μmol/h/mg protein

c* : GSH-I : γ-glutamylcysteine synthetase

GSH-II : glutathione synthetase

Introduction of recombinant plasmids containing various sets of two genes for glutathione biosynthesis into *E. coli* strains resulted in an increase in the activities of γ-glutamylcysteine synthetase and glutathione synthetase (Table 2). The increase of their activities was in proportion to the copy number of each gene. The strain possessing pGH501 plasmid containing two *gshI genes* and one *gshII* gene showed the highest activities of the two enzymes.

Despite the increases of enzyme activity for glutathione biosynthesis in cells, the amount of glutathione produced was not changed to significantly. It indicated that glutathione biosynthesis might be affected by various factors, such as supplies of substrates and ATP, feedback inhibition, side reactions rather than just enzyme amounts. Therefore, glutathione production by an enzymatic method is more feasible than by direct fermentation.

Because There was reported that sequences which are present in multiple copies within the same plasmid may be gradually lost and cloned polymers are unstable in vivo unless all copies of segments are present in the same orientation[13,21], the stability of the plasmids was examined. All plasmids were stably maintained in cells regardless of the presence or absence of antibiotics for 20 generations[10]. It is accountable that the tandem repeated *gshI* genes in plasmids are in the same transcriptional direction and the host strain is *rec*A$^-$. In addition the transcriptional orientation of inserted *gshI* gene with respect to *Cm* gene did not influence to express the gene (Data not shown).

Glutathione production coupled with ATP regenerating system by acetate kinase reaction in *E. coli*

Glutathione productivity of *E. coli* strains harboring various recombinant plasmids was investigated in a bioreactor system coupled with an ATP regeneration reaction catalyzed by acetate kinase of *E. coli*[22]. Glutathione

productivity of one strain harboring pGH100 was 18 times higher than that of the wild type strain (Table 3). In contrast, introduction of pGH300 containing only the *gshII* gene into *E. coli* showed little effect on the glutathione productivity. This seems to be caused by the low concentration of γ-glutamylcysteine synthesized by γ-glutamylcysteine synthetase.

Table 3. Glutathione production in *E. coli* HB101 strains harboring recombinant plasmids.

Strains	Properties	Glutathione Production (mg/ml)[a*]
HB101		0.05
HB101/pGH325-100	pBR325-*gshI*	0.90
HB101/pGH300	pBR322-*gshII*	0.05
HB101/pGH325-101	pBR325-*gshI* · *II*	1.50
HB101/pGH325-200	pBR325-*gshI* · *I*	1.20
HB101/pGH325-201	pBR325-*gshI* · *I* · *II*	2.10
HB101/pGH325-300	pBR325-*gshI* · *I* · *I*	1.30
HB101/pGH325-301	pBR325-*gshI* · *I* · *I* · *II*	2.30
HB101/pGH200	pUC8-*gshI*	1.50
HB101/pGH400	pUC13-*gshI* · *II*	2.40
HB101/pGH500	pUC8-*gshI* · *I*	1.80
HB101/pGH501	pUC8-*gshI* · *I* · *II*	2.80

a* : Gluthathione content (mg/ml) was determined as described in Material and Methods.
Glutathione production was carried out as described in Material and Methods.

The glutathione productivities of strains harboring recombinant plasmids such as pGH325-100, pGH325-200, and pGH325-300 were gradually enhanced according to the number of inserted *gshI* genes. In other words, glutathione productivity of strains harboring recombinant plasmids were enhanced by the increase of γ-glutamylcysteine synthetase activity even though they did not get the gene dosage effect of *gshII*. These results seem to indicate that the increase of γ-glutamylcysteine synthetase activity is crucial in the enzymatic production of glutathione.

Glutathione productivity of *E. coli* strains which were simultaneously amplified with both *gshI* and *gshII* genes was about twice higher than that of those strains amplified only *gshI* genes (Table 3). It indicates that the overproduction of the two enzymes is essential for the efficient glutathione production in *E. coli* system using acetate kinase reaction as the ATP regenerating system. The strain harboring pGH501 plasmid, which had the highest activity in γ-glutamylcysteine synthetase and glutathione synthetase, showed the highest glutathione productivity. From these results, we can suggest that the strain harboring pGH501 may be used for the mass production of glutathione in a bioreactor system coupled with an economic ATP-generating system[22,23].

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Construction of Various Recombinant Plasmids for the Enhancement of Glutathione Production in *E. coli*

초록 : *E. coli* 에서 글루타치온 생산 증가를 위한 재조합 플라스미드의 구성

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E. coli 에서 글루타치온 생산 증가를 위해서 *E. coli* 에서 분리한 *gshI* 과 *gshII* 유전자를 함유하고 있는 여러 재조합 플라스미드를 구성하여 도입하였다. pBR325 벡터에 *gshI* 유전자를 각각 1-3개를 포함한 재조합 플라스미드 및 *gshI*과 *gshII* 유전자를 동시에 갖는 재조합 플라스미드를 구성하였다. 계속적으로 반복된 *gshI* 유전자가 증폭된 *E. coli*의 γ-glutamylcysteine synthetase의 효소활성은 삽입된 *gshI* 유전자의 수에 따라 증가하였다. 구성된 재조합 플라스미드를 함유한 *E. coli*의 글루타치온 생산능을 acetate kinase 반응을 ATP 재생계로 사용하여 조사한 결과 반복된 *gshI* 유전자를 함유한 *E. coli*의 글루타치온 생산능력은 삽입된 *gshII* 유전자의 수에 비례하여 증가하였으며, *gshI* 유전자의 추가적인 도입에 의해 글루타치온 생산능력은 2배 증가하였다. *E. coli*에서 글루타치온의 효소적 생산은 주로 γ-glutamylcysteine synthetase의 효소활성에 의해 영향을 받았다. 가장 높은 글루타치온 생산능은 pGH501 (pUC8-*gshI.I.II*) 플라스미드를 갖는 균주에서 관찰되었다.