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Cloning of a Gene Responsible for the Biosynthesis of Glutathione in *Escherichia coli* B

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A gene (*gshI*) responsible for γ-glutamylcysteine synthetase (GSH-I) activity was cloned to construct an *Escherichia coli* B strain having high glutathione synthesizing activity. For this purpose, two *E. coli* B mutants (strains C912 and RC912) were used. C912 was deficient in GSH-I activity. RC912, a revertant of C912, had a GSH-I activity that was desensitized to feedback inhibition of reduced glutathione. To clone *gshI*, chromosomal DNAs of RC912 and plasmid vector pBR322 were digested with various restriction endonucleases and then ligated with T4 DNA ligase. The whole ligation mixture was used to transform C912, and the transformants were selected as tetramethylthiuramdisulfide-resistant colonies. Of about 20 resistant colonies, 2 or 3 became red when treated with nitroprusside and showed appreciably high GSH-I activities. The chimeric plasmid DNA, designated pBR322-*gshI*, was isolated from the strain having the highest GSH-I activity and transformed into RC912. The structure and molecular size of pBR322-*gshI* in RC912 were determined. The molecular size of this plasmid was 6.2 megadaltons, and the plasmid contained a 3.4-megadalton segment derived from RC912 chromosomal DNA, which included *gshI* gene. The GSH-I activity of RC912 cells containing pBR322-*gshI* was fourfold higher than that of RC912 cells without pBR322-*gshI*.

We have been studying the production of glutathione in a bioreactor system containing an ATP regenerating process. This tripeptide has been found to be continuously produced in a column packed with immobilized *Escherichia coli* B cells (7, 8) or immobilized *Saccharomyces cerevisiae* cells (6, 9, 11, 12), although the amount produced in these systems is relatively lower than the amounts produced either by organic synthesis or by extraction from yeast cells, both of which are now available industrially.

One reason for low production in our bioreactor systems was presumably feedback inhibition by reduced glutathione of γ-glutamylcysteine synthetase (GSH-I), the first enzyme in glutathione biosynthesis by *E. coli* B. To further improve our system, we tried cloning a gene responsible for GSH-I activity from a mutant insensitive to feedback inhibition by reduced glutathione. As a result, we succeeded in constructing an *E. coli* B strain with high glutathione synthesizing activity. In this paper, we discuss the results of these experiments.

MATERIALS AND METHODS

Chemicals. Restriction endonucleases (*Eco*RI, *Bam*HI, *Pst*I, and *Hin*dIII) and T4 DNA ligase were purchased from Takara Shuzo Co., Ltd., Kyoto, Japan. Tetramethylthiuramdisulfide (TMTD) and other reagents used for the assay of GSH-I activity were purchased from Sigma Chemical Co., St. Louis, Mo.

Strains. Mutants derived from *E. coli* B strain 355 are listed in Table 1.

DNAs. Chromosomal DNA of strain RC912 was purified by the method of Saito and Miura (15). Plasmid DNA was purified by the method of Tanaka and Weisblum (16).

Digestion and ligation of DNA. Digestion of DNA was carried out with *Eco*RI in 100 mM Tris-hydrochloride (pH 7.5)–50 mM NaCl–10 mM $MgCl_2$, *Bam*HI in 50 mM Tris-hydrochloride (pH 7.5)–0.2 mM EDTA–5.0 mM $MgCl_2$, and *Pst*I or *Hin*dIII in 6.0 mM Tris-hydrochloride (pH 7.5)–50 mM NaCl–6.0 mM $MgCl_2$. Digestion was carried out at 37°C with 0.1-ml volumes of the restriction enzyme solutions. Ligation with T4 DNA ligase was carried out by the method of Tanaka and Weisblum (16).

Electrophoresis in agarose gels. Agarose gels (slab type, 1.5%) were run in the same buffer as that described by Helling et al. (4).

Transformation and selection. Transformation of *E. coli* B strains was carried out as described by Norgard et al. (14). Selection of transformants was as follows. After incubation with the whole ligation mixture, about 10^8 cells were spread on Davis-Mingioli minimal agar medium (2) supplemented with 40 μg of TMTD per ml. Plates were incubated at 37°C for about 2 days, and the colonies were tested for their sensitivities to ampicillin (Amp) (20 μg/ml) and tetracycline (Tet) (20 μg/ml) in L-broth (1.0% polypeptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose [pH 7.2]). Colonies

TABLE 1. Strains used

Strain	Parent	Properties[a]	Reference(s)
M910	355 (wild type)	MG^r	8
C912	M910	*gshI*, MG^r	5, 13
RC912	C912	*gshI*$^+$, MG^r	5, 13

[a] MG^r, Ability to excrete glutathione into the growth medium; *gshI*$^+$, functional gene observed after back mutation of the C912 *gshI* gene.

resistant to TMTD and Amp, Tet, or both were further tested by the method of Apontoweil and Berends (1) for the ability to become red in the presence of nitroprusside.

Assay for GSH-I. Strains of *E. coli* B with or without the hybrid plasmid were grown to log phase at 37°C in Davis-Mingioli minimal medium. Preparation of cell extracts and assay for GSH-I were as described previously (5, 13).

Glutathione content. The intracellular glutathione level was determined as described previously (10).

Glutathione production. Glutathione production was measured by incubating toluol-treated cells (0.6 g [wet weight] in a 5.0-ml mixture containing 40 mM L-glutamate, 40 mM L-cysteine, 40 mM glycine, 25 mM $MgCl_2$, 30 mM acetyl phosphate, 20 mM ATP, and 50 mM potassium phosphate buffer (pH 7.0) at 37°C with shaking for several hours. At the prescribed time, 0.2 ml of the reaction mixture was taken, and the glutathione produced was determined by the method of Tietze (17). The preparation of toluol-treated cells and other reaction condition details were the same as those described previously (8).

RESULTS

Construction of the hybrid plasmid. Chromosomal DNA (1.2 μg) from RC912 was partially digested with appropriately diluted restriction endonucleases for either 1.5 or 6.0 h. Plasmid vector pBR322 (1.0 μg) was also digested completely with the same endonucleases. The products of the two kinds of digestion were ligated and used for the transformation of strain C912. After 2 to 3 days, several TMTD-resistant colonies appeared, although the number of colonies on the plate was dependent on both the restriction endonuclease used and the length of digestion (Table 2). TMTD-resistant colonies obtained by digestion with *Bam*HI or *Hin*dIII were resistant to Amp and sensitive to Tet, colonies obtained by digestion with *Pst*I were sensitive to Amp and resistant to Tet, and colonies obtained by digestion with *Eco*RI were resistant to both antibiotics (data not shown).

Colony color. The colonies that were resistant to TMTD and antibiotics were further tested for the ability to become red in the presence of nitroprusside. The number of resistant colonies reacting with nitroprusside was small (5 to 10%), and almost all colonies remained white-pink (Table 2). Figure 1 shows the typical colors of colonies after nitroprusside treatment.

Glutathione contents and GSH-I activities of transformants. The glutathione contents and GSH-I activities of strains that became red were measured (Table 3). An increased accumulation of glutathione and an elevated GSH-I activity were observed in strains H3 and P1, whereas in strains E3 and E11, both the glutathione content and the GSH-I activity remained identical to those of the parental strain (M910). H3 showed the highest GSH-I activity.

Transformation of RC912 with the hybrid plasmid pBR322-*gshI*. The hybrid plasmid, designated pBR322-*gshI*, carried by H3 was isolated and used for the transformation of RC912. Transformants of RC912 were selected on L-broth containing 20 μg of Amp per ml. Several Amp^r colonies were isolated on the plates, and these colonies were sensitive to Tet. The frequency of transformation of RC912 with pBR322-*gshI* was about 2×10^4/μg of plasmid DNA. One of the transformants was designated RC912(pBR322-*gshI*).

Characterization of pBR322-*gshI*. pBR322-*gshI* was purified from RC912(pBR322-*gshI*), and its structure was analyzed by restriction endonuclease digestions. The molecular sizes of restriction fragments of this plasmid are given in Table 4. The molecular size of this plasmid was estimated to be 6.2 megadaltons (Md), indicating that a 3.4-Md segment of RC912 chromosomal DNA was inserted into the *Hin*dIII restriction site of pBR322. On the basis of the data in Table 4, we determined the sites which were sensitive to restriction endonucleases (Fig. 2).

To determine the location of the *gshI* gene in the cloned fragment, pBR322-*gshI* was digested with *Pst*I or *Bam*HI, and the fragments produced were ligated again with pBR322 digested with *Pst*I or *Bam*HI (procedures not shown). The whole ligation mixture thus obtained was used for the transformation of C912, and the red

TABLE 2. Effect of digestion time and enzyme on the appearance of TMTD-resistant and red colonies

Enzyme	Digestion time (h)	No. of TMTD-resistant colonies	No. of red colonies
*Hin*dIII	1.5	1	0
	6.0	6	1 (H3)
*Bam*HI	1.5	0	0
	6.0	20	0
*Eco*RI	1.5	22	2 (E7 and E11)
	6.0	0	0
*Pst*I	1.5	2	1 (P1)
	6.0	9	0

FIG. 1. Violet-red color of transformants. Colonies were stained with nitroprusside by the method of Apontoweil et al. (1). Restriction enzymes used for the digestion of RC12 chromosomal DNA are indicated.

transformants were screened as described above. Several red colonies were obtained, and these colonies contained the hybrid plasmid, which had a molecular size of 4.4 Md. This result indicated that the *gshI* gene was in the fragment between the *Pst*I and *Hin*dIII sites (Fig. 2). On the other hand, no red colonies were obtained when pBR322-*gshI* was digested with *Bam*HI, suggesting that the *Bam*HI site was in the *gshI* gene. Detailed restriction analyses of pBR322-*gshI*, including determination of the base sequence of the *gshI* gene, are now under way.

TABLE 3. Glutathione contents and GSH-I activities of transformants

Strain	Glutathione (μmol/g [wet wt] of cells)	GSH-I activity (μmol/mg of protein per h)
H3	3.35	1.22
E7	1.42	0.43
E11	1.35	0.39
P1	2.32	0.68
M910	1.44	0.42

TABLE 4. Molecular sizes of restriction fragments of plasmid pBR322-*gshI*[a]

Digested with:	Molecular sizes (Md)	Total plasmid size (Md)
*Hin*dIII	2.8, 3.4	6.2
*Pst*I	1.8, 4.5	6.3
*Bam*HI	1.2, 2.0, 2.9	6.1
*Eco*RI	0.3, 0.6, 1.1, 1.8, 2.3	6.1
*Hin*dIII + *Pst*I	0.6, 1.2, 2.0, 2.6	6.4
*Pst*I + *Bam*HI	0.4, 0.6, 1.0, 1.8, 2.2	6.0
*Hin*dIII + *Bam*HI	0.3, 0.5, 1.2, 1.8, 2.5	6.3

[a] Digestion of plasmid DNA was carried out as described in the text. *Hin*dIII-*Pst*I double digestion was carried out under the conditions used for *Pst*I digestion. *Pst*I-*Bam*HI and *Hin*dIII-*Bam*HI double digestions were carried out under the conditions used for *Bam*HI digestion.

Stability of cloned fragments. A number of white-pink colonies were observed when the cells containing pBR322-*gshI* were grown on Davis-Mingioli minimal agar plates and treated with nitroprusside. When these white-pink colonies were tested on Amp-containing medium, they were unable to grow. Thus, it appeared that many colonies were losing pBR322-*gshI*. This was further substantiated by whole-colony lysis and agarose gel electrophoresis: no plasmid DNAs from Amp^s colonies were detected.

To determine the stability of the plasmid DNA, we grew host cells containing pBR322-*gshI* to mid-log phase (approximately three generations) in L-broth. The cells were then diluted 500-fold in broth with or without Amp and grown for 16 h, and the percentages of Amp^r cells in populations before and after cultivation were determined. In the presence of Amp, 98.5% of the cells were Amp^r, whereas in the absence of Amp, 68.7% of the cells were Amp^r.

Properties of RC912(pBR322-*gshI*). The properties of RC912(pBR322-*gshI*) were investigated and compared with the properties of parental strains (Table 5). The glutathione content and GSH-I activity of RC912(pBR322-*gshI*) were respectively about 7- and 12-fold greater than those of strain 355. Furthermore, the GSH-I from RC912(pBR322-*gshI*) was insensitive to feedback inhibition by reduced glutathione, although the GSH-I of M910 was not (Fig. 3).

Glutathione formation by RC912(pBR322-*gshI*). To investigate the effect on glutathione formation of dosing cells with *gshI*, we investigated glutathione formed from L-glutamate, L-cysteine, and glycine by toluol-treated cells. The glutathione forming activity of RC912 cells

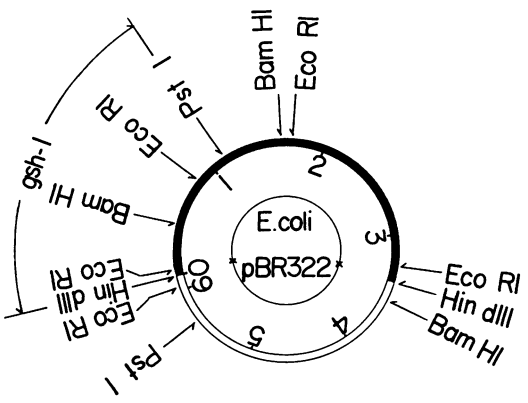

FIG. 2. Circular restriction map of pBR322-*gshI*, based on the relative sizes of restriction fragments (Table 4). The arrows indicate the points of restriction endonuclease attack. The numbers indicate the size of the fragments in Md. (×) Sites where the *Hin*dIII ends of pBR322 were ligated to a fragment of *E. coli* B (RC912) chromosomal DNA.

dosed with the *gshI* gene was much higher than that of undosed RC912 cells or RC912 cells carrying pBR322 (Fig. 4).

DISCUSSION

Glutathione is biosynthesized from L-glutamate, L-cysteine, and glycine by the sequential enzyme reactions catalyzed by GSH-I and glutathione synthetase. To produce glutathione efficiently from three constituent amino acids, we tried to increase the GSH-I activity, since a preliminary experiment showed that the reaction catalyzed by GSH-I limited the rate of glutathione production. For this purpose, the cloning of a gene for GSH-I (*gshI*) was first investigated, and a mutant strain deficient in GSH-I activity (C912) was constructed (11) for the selection of transformants carrying *gshI* cloned into pBR322.

However, C912 could grow on minimal medium without glutathione supplementation (5), indicating that the selection of transformants by manipulating required nutritives was impossible for the genes for glutathione biosynthetic enzymes. Although glutathione was not required for growth of C912, growth was severely inhibited by various compounds, such as sulfhydryl agents, metal-chelating agents, and others. Among these inhibitory compounds, TMTD most effectively arrested growth: the amount inhibiting the growth of C912 was much lower than that required for the inhibition of the parental strain of C912 (strain 355; 5). Colonies resistant to TMTD were selected as transformants harboring the *gshI* gene (Table 2).

Several TMTD-resistant colonies were obtained, although the appearance of colonies depended both on the restriction enzyme used for digestion of chromosomal DNA and on digestion time (Table 2). These differences may reflect differences in the sensitivity of *gshI* or the gene responsible for TMTD resistance (or both) to the enzymes used. As can be easily seen in the case of *Eco*RI digestion (Table 2), longer digestion completely suppressed the appearance of TMTD-resistant colonies, indicating that the *gshI* gene or the gene responsible for TMTD resistance (or both) was highly sensitive to *Eco*RI. In the cases of *Hin*dIII, *Bam*HI, and *Pst*I, on the other hand, longer digestions were effective in promoting the appearance of TMTD-resistant colonies. These results show that the appearance of TMTD-resistant colonies was also influenced by the size of the fragments generated by the enzyme digestions. The same observation was made by Harris-Warrick et al. (3), who showed that the transforming activity of a hybrid plasmid was dependent not only on the

TABLE 5. Construction of *E. coli* B strains with high glutathione synthesizing activities

Strain	Properties	Glutathione (μmol/g [wet wt] of cells)	GSH-I activity (μmol/mg of protein per h)
355	Wild type	0.71	0.13
M910	MG^r	1.48	0.38
C912	MG^r, *gshI*	0	0
RC912	MG^r, *gshI*$^+$	2.45	0.43
RC912 (pBR322-*gshI*)	MG^r, *gshI*$^+$	4.81	1.76

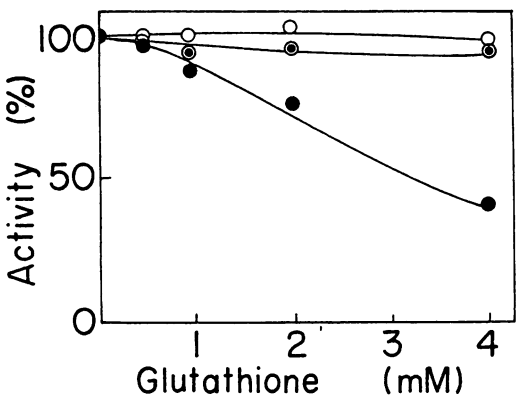

FIG. 3. Desensitization of GSH-I to feedback inhibition by reduced glutathione. GSH-I activities in the presence or absence of reduced glutathione at various concentrations were determined (see the text). Symbols: ○, RC912(pBR322-*gshI*); (●), M910; (⊙), RC912.

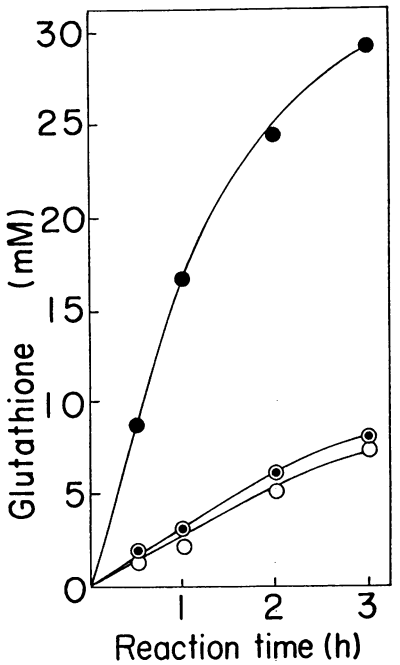

FIG. 4. Glutathione formation by RC912 cells. Symbols: ⊙, RC912; ○, RC912(pBR322); ●, RC912(pBR322-*gshI*).

molecular sizes of the fragments but also on the distances of the genes from a cleaved site.

However, the number of colonies that became red after reaction with nitroprusside, a reagent which reacts with sulfhydryl compounds in cells, was small, and almost all of the TMTD-resistant colonies remained white-pink (Fig. 1). This result suggested that sensitivity to TMTD was not a specific character of the glutathione biosynthesis-deficient mutant (C912) and that this sensitivity was neutralized by factors other than glutathione, although such factors were not elucidated in this study. The GSH-I activity in red transformants was determined (Table 3). Only two strains (H3 and P1) showed appreciably high GSH-I activity, and the other two (E7 and E11) showed the same levels as shown by the parental strain. Therefore, the color produced by E7 and E11 colonies seemed to be due to the accumulation of sulfur compounds other than glutathione.

Hybrid plasmid pBR322-*gshI* in strain H3, which showed the highest GSH-I activity, was isolated and introduced into RC912 with GSH-I insensitive to feedback inhibition by reduced glutathione. With the plasmid, RC912 showed appreciably higher glutathione synthesizing activity than did RC912 without the plasmid or RC912 with pBR322. Using cells dosed with the *gshI* gene, we are now studying glutathione production in bioreactor systems described in our previous papers (6–9, 11, 12). We are also studying the detailed restriction map of *gshI* in combination with the *gshI* base sequence.

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