Note

γ -Glutamylcysteine Production by *Escherichia coli* B Cells Dosed with the γ -Glutamylcysteine Synthetase Gene

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Previously,¹⁾ we presented a method for the cloning of a gene responsible for γ -glutamylcysteine synthetase (GSH-I), the first enzyme involved in the glutathione biosynthetic pathway in *Escherichia coli* B. The gene (*gsh I*) for GSH-I was cloned onto vector plasmid pBR322 using the chromosomal DNA partially digested with various restriction endonucleases such as *Hind*III, *Eco*RI and *Pst*I. Analysis of one of the hybrid plasmids constructed with the *Hind*III digest showed that the gene *gsh I* had no susceptible sites for *Pst*I, suggesting that the gene would be cloned by the use of chromosomal DNA completely digested with *Pst*I.

So, we tried again to isolate this gene using the complete digest of chromosomal DNA with *Pst*I and could obtain such fragment carrying *gsh I* as expected. This note deals with the properties of hybrid plasmids obtained and the application of these plasmids to the production of γ -glutamylcysteine (γ -GC).

The conditions used for the cloning of *gsh I* were the same as those described previously.¹⁾ Chromosomal DNA extracted from strain RC912, an *E. coli* B mutant having the GSH-I desensitized from feedback inhibition of reduced glutathione,¹⁾ was completely digested with *Pst*I, and the digest was annealed with vector plasmid pBR322 pre-treated with *Pst*I. After ligation with T₄ DNA ligase, the whole ligation mixture was used for the transformation of strain C912, a GSH-I activity deficient mutant derived from *E. coli* B.²⁾ Transformation was carried out according to the method given by Kushner³⁾ and transformants harbouring plasmids with the *gsh I* gene were selected as the colonies resistant to both 10 μ g/ml tetramethylthiuram disulfide⁴⁾ and 4.0 μ g/ml tetracycline on Davis-Mingioli minimal agar plates.⁵⁾ The methods used for the preparation of hybrid plasmids and for the restriction mapping were the same as those described previously.¹⁾ The cell

extracts were prepared from cells growing exponentially on Davis-Mingioli minimal medium as described previously.⁶⁾ The assays for GSH-I and glutathione synthetase (GSH-II), the second enzyme in the glutathione biosynthetic pathway in *E. coli* B, were carried out as described previously.²⁾ γ -GC-producing reactions were carried out in 1.0 ml of a mixture containing 20 mM L-glutamate, 20 mM L-cysteine, 20 mM MgCl₂, 20 mM adenosine 5'-triphosphate (ATP), 50 mM potassium phosphate buffer (pH 7.1) and 120 mg (wet wt.)/ml of toluene-treated cells at 37°C for several hours with shaking. The conditions for cultivation of *E. coli* B and for toluene treatment were also given in our previous papers.^{7,8)} The reaction was terminated by immersing the test tube in boiling water for 2 min and γ -GC produced in the reaction mixture was determined by High Pressure Liquid Chromatography with a reverse phase column packed with Zorbax^{BP}-NH₂ resin.

Two kinds of hybrid plasmids with the *gsh I* gene were obtained and designated as pGS100-1 and pGS100-2, respectively. Restriction analysis of pGS100-1 was carried out and the molecular sizes of the fragments generated are summarized in Table 1. Based on these results, the circular restriction map of pGS100-1 was determined as shown in

TABLE I. SIZES OF FRAGMENTS OF pGS100-1 DIGESTED WITH VARIOUS RESTRICTION ENDONUCLEASES

*Mlu*I, *Bam*HI, *Sal*I and *Hind*III gave one fragment having a molecular weight of 4.7 Md. The digestion conditions employed were: 10 mM Tris-HCl (pH 7.5), 7.0 mM MgCl₂, 175 mM NaCl, 0.2 mM EDTA, 7.0 mM 2-mercaptoethanol (2-ME), 0.01% bovine serum albumin (for *Sal*I); 10 mM Tris-HCl (pH 7.5), 7.0 mM MgCl₂, 60 mM NaCl, 7.0 mM 2-ME (for *Pvu*II); 10 mM Tris-HCl (pH 7.5), 7.0 mM MgCl₂, 150 mM NaCl, 7.0 mM 2-ME (for *Mlu*I). The double digestions of *Pst*I-*Eco*RI, *Sal*I-*Eco*RI, *Mlu*I-*Eco*RI and *Hind*III-*Mlu*I were carried out under the conditions for *Pst*I, *Sal*I, *Mlu*I and *Hind*III, respectively. Other digestion conditions were given in our previous paper.¹⁾

Endonucleases	Sizes of fragments (Md)				Total size (Md)
<i>Pst</i> I	2.6	2.1			4.70
<i>Pvu</i> II	3.0	1.8			4.80
<i>Eco</i> RI	2.2	1.6	1.0		4.80
<i>Pst</i> I- <i>Eco</i> RI	2.2	1.3	0.78	0.46	4.74
<i>Sal</i> I- <i>Eco</i> RI	1.9	1.6	0.78	0.42	4.70
<i>Mlu</i> I- <i>Eco</i> RI	2.3	1.7	0.44	0.32	4.74
<i>Hind</i> III- <i>Mlu</i> I	2.6	1.6	0.38		4.58

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FIG. 1. Circular Restriction Maps of pGS100-1 (Left) and pGS100-2 (Right).

The restriction sites are drawn relative to their scale on circular maps. The numbers in the circles represent megadaltons (Md). P* in the map of pGS100-2 indicates the site resistant to *Pst*I attack. ■: *E. coli* B (RC912) chromosomal DNA; □: Vector plasmid pBR322. Abbreviated symbols: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; M, *Mlu*I, P, *Pst*I; Pv, *Pvu*II; S, *Sal*I

Fig. 1 (left). pGS100-1 was 4.7 megadaltons (Md) in size and contained a 2.1 Md fragment of chromosomal DNA of strain RC912 in the *Pst*I site of pBR322. Restriction analysis of pGS100-2 was also carried out, although the data are not shown here, and the circular restriction map was constructed as shown in Fig. 1 (right). pGS100-2 was 7.3 Md in size and contained the same fragment of chromosomal DNA as that found in pGS100-1. Restriction analysis of this hybrid plasmid indicated that pGS100-2 consisted of pBR322 welded at the *Pst*I site, which is indicated by P* in Fig. 1 (right), and the chromosomal DNA fragment was inserted between the *Pst*I sites originating from two molecules of pBR322. The *Pst*I site (P*) welding two molecules of pBR322 was resistant to *Pst*I attack, though the reason for this resistance is unknown.

Previously,¹⁾ we showed that *E. coli* B cells dosed with the hybrid plasmid carrying the *gsh* I gene had the ability to produce a large amount of glutathione from the constituent three amino acids (L-glutamate, L-cysteine and glycine) in the presence of ATP regeneration system that consisted of the acetate kinase reaction in *E. coli* B. This suggested that a large amount of γ -GC would be also produced by *E. coli* B cells dosed with hybrid plasmid carrying *gsh* I, since this dipeptide is an intermediate of glutathione biosynthesis. So, the production of γ -GC from L-glutamate and L-cysteine was investigated using toluene-treated cells of strain C1001/pGS100-1.

Figure 2 shows the time courses of γ -GC production. This dipeptide was efficiently produced by the cells of strain C1001/pGS100-1 and 20 mM L-cysteine was completely converted to γ -GC in the presence of a stoichiometrically excess concentration of L-glutamate (80 mM). This unusual requirement for L-glutamate was presumably due to the utilization of L-glutamate for the unwanted side reactions. On the other hand, γ -GC production by the cells of strain C1001 was very low being

FIG. 2. γ -Glutamylcysteine (γ -GC) Production by Toluene-treated Cells of Strain C1001 with (A) or without (B) Hybrid Plasmid pGS100-1.

γ -GC-producing reactions were carried out as described in the text, except for the concentrations of L-glutamate. L-Glutamate concentrations used were: -○-, 20 mM; -◐-, 50 mM; -●-, 80 mM. Conversion in the figure indicates the percentage of L-cysteine incorporated into γ -GC.

independent of the L-glutamate concentration. This low activity of C1001 was partly due to the feedback inhibition of GSH-I by L-cysteine.¹⁰⁾

The results presented here may be applicable to the production of various γ -glutamyl derivatives other than γ -GC by utilizing the substrate specificity of GSH-I.

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