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γ-Glutamyltranspeptidase from *Escherichia coli* K-12: Formation and Localization

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***Escherichia coli* cells showed maximum activity of γ-glutamyltranspeptidase (EC 2.3.2.2) when they were grown at 20°C, 14% of maximum activity at 37°C, and none at 43°C. The enzyme activity of intact cells grown at 20°C was stably maintained after the temperature was changed to 45°C. The activity increased during the exponential phase, and maximum activity was found at stationary phase. Its intracellular localization in the periplasmic space was confirmed.**

γ-Glutamyltranspeptidase (GGT) catalyzes the hydrolysis of γ-glutamyl compounds (hydrolyase activity glutaminase activity) and the transfer of their γ-glutamyl moieties to other amino acids and peptides (transferase activity) (24). Although a number of studies on GGT of mammalian cells have been performed and reviewed (1, 32, 33), the physiological roles of the enzyme are still controversial. GGT of yeasts was also investigated (15, 26).

Since Talalay (31) found GGT activity in *Proteus vulgaris*, several studies have been performed on GGT of bacteria, including an L-glutamate-fermenting bacterium (6) and *Bacillus natto* (2). Nakayama et al. in our laboratory reported that *P. mirabilis* exhibits high GGT activity (17), and then they purified and characterized it (18) and determined its localization in the cell (19). They also synthesized γ-glutamyl-L-3,4-dihydroxyphenylalanine (DOPA) enzymatically, using the GGT from *P. mirabilis* (16), and Togari et al. suggested that this γ-glutamyl-L-DOPA is useful as a precursor of L-DOPA in rat brain (manuscript in preparation). Kiuchi et al. (9) also showed that this γ-glutamyl-L-DOPA is useful as a substrate for measuring the enzymatic activity of GGT.

Milbauer and Grossowicz (12), Szewczuk and Mulczyk (29), and Nakayama et al. (20) found GGT activity in various bacteria, but they also found that *Escherichia coli*, the best-studied bacteria in laboratories, does not exhibit strong GGT activity.

In this study we found that the GGT activity of *E. coli* K-12 is greatly affected by growth temperature and growth phase. The localization of this enzyme was also determined by cell fractionation involving osmotic shock or lysozyme treatment.

MATERIALS AND METHODS

Reagents. γ-Glutamyl-*p*-nitroanilide, glycylglycine, and bovine serum albumin were purchased from Wako Pure Chemical Co., lysozyme, *o*-nitrophenyl-β-D-galactopyranoside, and 5′-AMP were from Sigma Chemical Co., tryptone was from Difco Laboratories, and yeast extract was from the Oriental Yeast Co. Other chemicals were the best reagents grade available from commercial sources.

Bacterial strains and cultures. *P. mirabilis* was the same strain that Nakayama et al. described (18). Prototrophic *E. coli* K-12 MG1655 (4) was donated by Carol A. Gross.

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LB broth (13) was used unless otherwise stated. A temperature gradient incubator, model TN-3 (Toyo Kagaku Sangyo Co.), was used in an air-conditioned room at 28°C to create temperature gradients for cultures. In other cases, cells were grown in a 20°C water bath with reciprocal shaking. Precultures were grown at 37°C unless otherwise stated.

Enzyme activity. (i) GGT activity. GGT activity was determined spectrophotometrically (23) by measuring the *p*-nitroaniline released from γ-glutamyl-*p*-nitroanilide with and without glycylglycine as described by Nakayama et al. (17). When whole cells were used, they were suspended in 20 mM Tris hydrochloride (pH 8.0).

(ii) 5′-Nucleotidase activity. 5′-Nucleotidase activity was measured as decribed by Neu (21); phosphate released from 5′-AMP was measured by the method of Fiske and Subbarow (3).

(iii) β-Galactosidase activity. For determination of β-galactosidase activity, the reaction mixture contained 0.9 ml of Z buffer (13), pH 7.0, 0.1 ml of a cell suspension, and 0.2 ml of a 4-mg/ml concentration of *o*-nitrophenyl-β-D-galactopyranoside. The reaction was started by adding the *o*-nitrophenyl-β-D-galactopyranoside to the mixture. After incubation at 37°C for 20 min, the reaction was terminated by adding 0.5 ml of 1 M Na_2CO_3. The mixture was centrifuged at 10,000 × *g* (in a Kubota KM-15200 microfuge with an RA-150A rotor) for 5 min at 4°C. The A_{420} was measured to calculate the amount of *o*-nitrophenol released.

One unit of enzyme was defined as the amount of enzyme that released 1 μmol of product per min. Specific activities are expressed in units per milligram of protein.

Determination of cell growth. For determination of cell growth, a culture was diluted with saline and then the optical density was measured at 610 nm.

Cell fractionation. (i) Osmotic shock. *E. coli* K-12 was grown in LB broth with 0.2% galactose at 20°C for 23 h and then subjected to osmotic shock treatment (Fig. 1A) according to Neu and Heppel (22).

(ii) Lysozyme treatment. *E. coli* K-12 was grown in LB broth with 0.2% galactose at 20°C for 40 h and then treated with lysozyme (Fig. 1B) according to Mizushima and Yamada (14).

Protein determination. The protein concentration was determined by the method of Lowry et al. (10), with ovalbumin as a standard. Insoluble protein was determined by a modified method. A 0.01-ml portion of 10 N NaOH was added to 0.09 ml of a sample, and then the mixture was left to stand

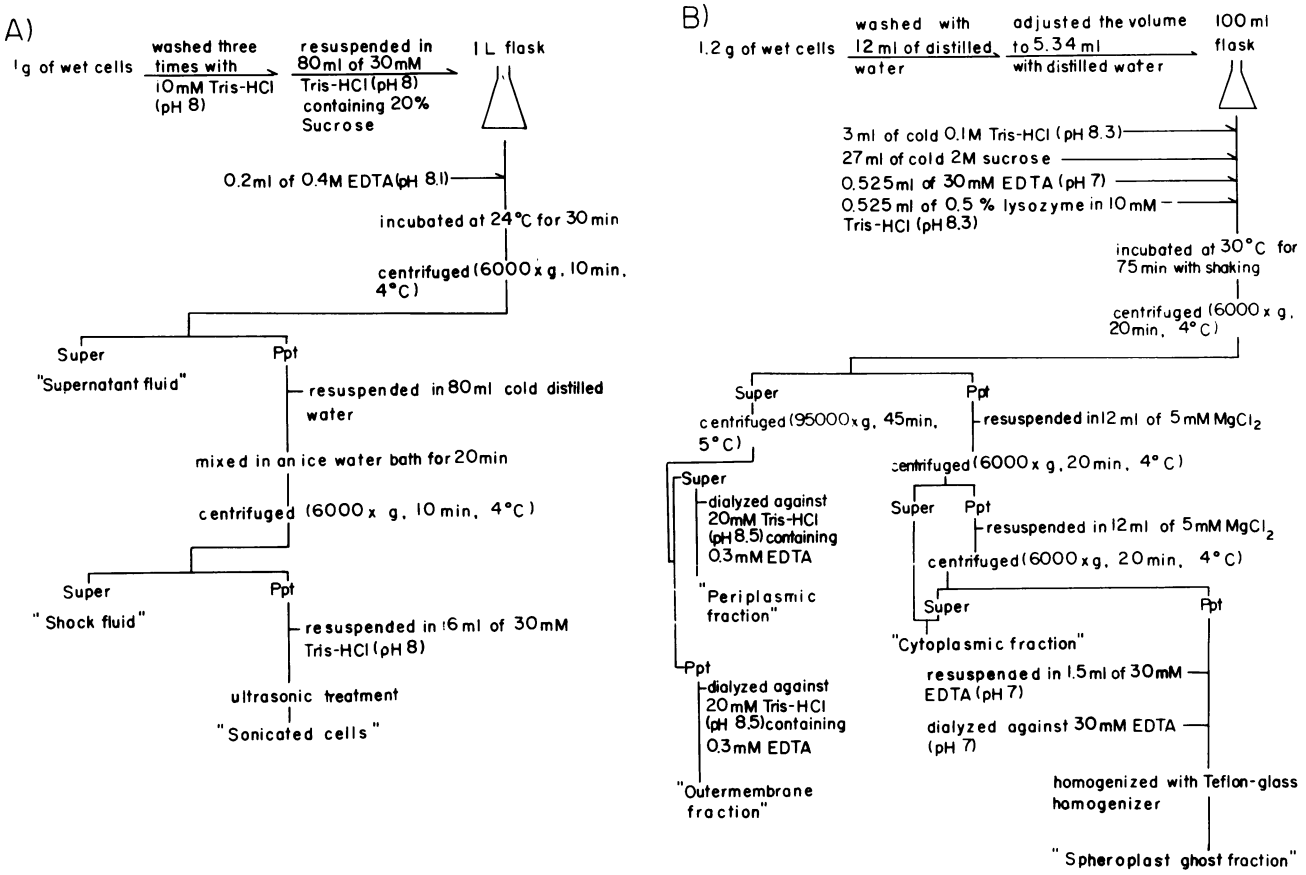

FIG. 1. Schemes of cell fractination. (A) Osmotic shock treatment; (B) lysozyme treatment.

for 30 min at room temperature for solubilization. Next, 0.83 ml of 2% Na_2CO_3 and 0.17 ml of Folin B reagent were added to the mixture, followed by incubation at 37°C for 10 min. A 0.1-ml amount of phenol reagent was added to the mixture, followed by incubation at 37°C for 20 min. The A_{610} was measured.

RESULTS

Effect of growth temperature on GGT activities. *E. coli* K-12 and *P. mirabilis* were grown at various temperatures in a temperature gradient incubator, and both the transferase and hydrolyase activities were measured. In the case of *P. mirabilis*, GGT activity did not differ so much with the growth temperature from 20 to 40°C (Fig. 2B). In the case of *E. coli*, however, GGT activity was greatly affected by the growth temperature, although cell growth was not (Fig. 2A). The maximum activity was observed at 20°C, 14% of which was found at 37°C; little and none were found at 40 and 43°C, respectively.

GGT activities after incubation at 45°C. A culture of *E. coli* that had been grown at 20°C for 24 h was incubated at 20 and 45°C after chloramphenicol (20 μg/ml) had been added. Both activities were quite stable at 20°C (85 and 89% of the transferase and hydrolyase activities remained after 42 h), but they decreased gradually at 45°C. Even after 42 h, 55% of the transferase activity remained (Fig. 3).

Growth phase and GGT activities. GGT activities increased exponentially during the exponential phase and then became constant at a maximum level in stationary phase (data not shown).

Distribution of GGT in *E. coli* cells. *E. coli* cells were fractionated by osmotic shock treatment followed by ultrasonic treatment. Most of the transferase activity was found in the shock fluid and was well coincident with the distribution of periplasmic marker enzyme 5'-nucleotidase (Table 1). *E. coli* cells were also fractionated by lysozyme treatment followed by homogenization of the spheroplasts with a Teflon-glass homogenizer. Most of the transferase activity was found in the periplasmic fraction (Table 2). These results show that GGT is a periplasmic enzyme.

DISCUSSION

Although several studies on GGT from bacteria have been reported (2, 6, 18, 19, 30, 31), only GGT from *P. mirabilis* was purified to a homogeneous state and studied extensively (18). To perform further physiological and genetic studies on GGT and to elucidate its physiological role, *E. coli* K-12 is much more convenient and useful. All previous studies on the GGT distribution in bacteria, however, showed that *E. coli* shows only weak or negligible GGT activity (12, 20, 29).

In this study, we found that the GGT activities of *E. coli* are greatly affected by the growth temperature, while those of *P. mirabilis* are not. Also, the maximum transferase activity per cell found in *E. coli* at 20°C is more than twice that found in *P. mirabilis*. This finding enabled us to start a study on the GGT of *E. coli* K-12 from various angles.

E. coli cells grown at 45°C for 25 h showed no GGT activities. But when *E. coli* cells grown at 20°C were incubated at 20 and 45°C for 42 h, 85 and 55%, respectively,

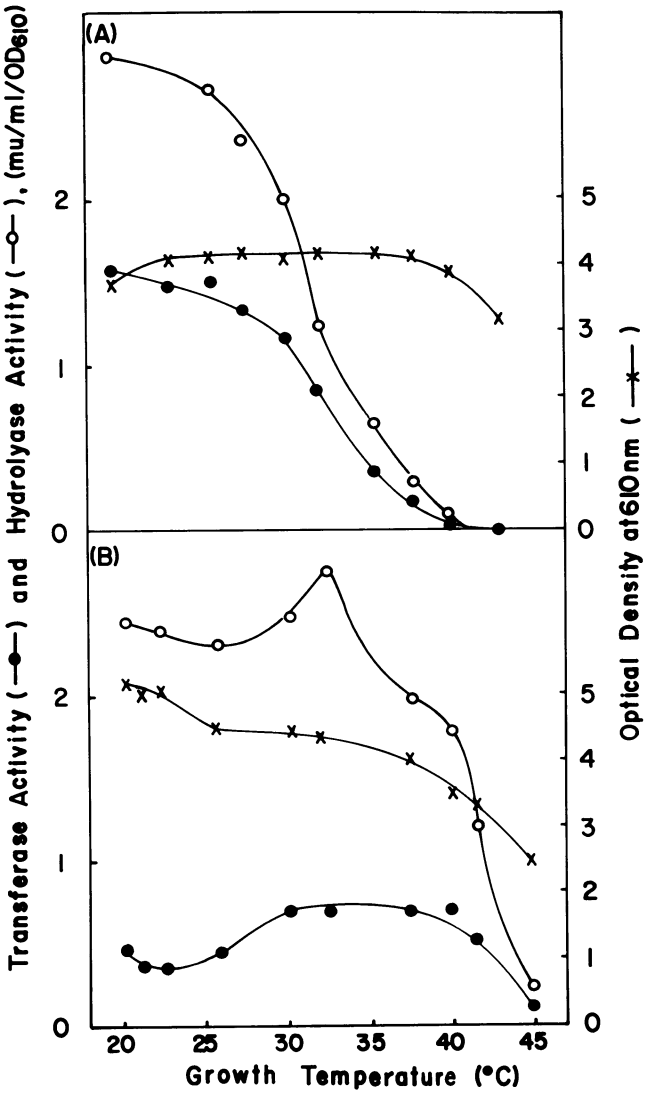

FIG. 2. Effect of growth temperature on GGT activities. (A) *E. coli* K-12; (B) *P. mirabilis*. Cells were grown in 10 ml of LB medium in L-shaped test tubes set in a temperature gradient incubator for 25 h with reciprocal shaking (60 rpm). The cells were collected by centrifugation, suspended in 20 mM Tris hydrochloride (pH 8.0), and then subjected to enzyme assay and optical density (OD) measurement at 610 nm.

of the transferase activity was stably maintained in the cells. These results suggest that GGT is synthesized only at a lower temperature, rather than that the synthesized GGT is degraded at a higher temperature.

The genetic mechanisms and physiological importance, if any, of this temperature-dependent GGT biosynthesis would

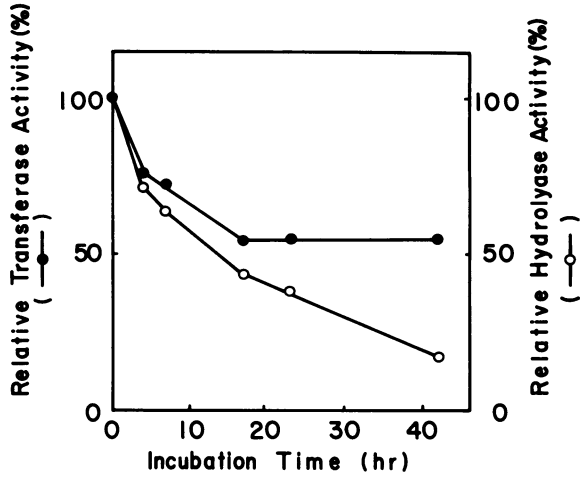

FIG. 3. GGT activities remaining after incubation at 45°C. A culture of *E. coli* grown at 20°C for 24 h was transferred to a 45°C incubator with reciprocal shaking (50 rpm), and the remaining GGT activity was followed. The activity was measured in cells. Transferase activity of *E. coli* cells of 1.03 mU/ml per optical density at 610 nm (OD_{610}) and hydrolylase activity of 2.84 mU/ml per OD_{610} are each indicated as 100%.

be very interesting. To obtain information on these, we purified GGT as a crystalline preparation and we report its properties in the accompanying paper (28). Furthermore, we isolated GGT-less mutants of *E. coli* and performed genetic mapping and characterization of them (manuscript in preparation).

Orlowski and Meister (25) proposed that the γ-glutamyl cycle plays a role in the amino acid uptake through the cell membrane into mammalian cells and that GGT is a key enzyme in this system, but Pellefigue et al. (27) showed that normal amino acid transport does not require GGT. Amino acid auxotrophs of *E. coli* are usually not heat sensitive and normally grow at higher temperature if the required amino acids are added to the media. This shows that GGT is not essential for amino acid uptake by *E. coli*, at least at higher temperatures.

In mammalian cells, GGT was found to be localized at the external surface of the plasma membrane, anchoring in the membrane (7, 32, 35). Nakayama et al. (19) studied the intracellular distribution of GGT in bacterial cells for the first time, and they found that GGT is localized in the cell wall or periplasmic space or both. We fractionated *E. coli* cells by osmotic shock and lysozyme treatment, and transferase activity was found to be localized in the periplasmic space in both cases. Hydrolyase activity was distributed differently by the two methods. This may be because glutaminase (5), which is different from GGT, might be measured by our method in disrupted cells, and this glutaminase activity may be different depending on growth phase or the method of cell

TABLE 1. Distribution of enzyme activities in *E. coli* K-12[a]

Sample	Protein		γ-Glutamyltransferase		γ-Glutamyl hydrolyase		5′-Nucleotidase		β-Galactosidase	
	mg	%	mU/mg	%	mU/mg	%	mU/mg	%	mU/mg	%
Supernatant fluid	8.64	9.3	0.663	3.4	0.220	3.1	0.131	7.7	0	0
Shock fluid	9.33	10.0	15.7	85.7	3.38	50.8	1.33	84.8	4.49	4.1
Sonicated cells	75.4	80.7	0.246	10.9	0.381	46.1	0.015	7.5	12.9	95.9

[a] Cells were grown in LB with 0.2% galactose at 20°C for 23 h and then subjected to osmotic shock treatment.

TABLE 2. Distribution of enzyme activities in *E. coli* K-12[a]

Sample	Protein		γ-Glutamyltransferase		γ-Glutamyl hydrolyase		5'-Nucleotidase		β-Galactosidase	
	mg	%	mU/mg	%	mU/mg	%	mU/mg	%	mU/mg	%
Periplasmic space	3.15	4.1	31.4	98.8	6.32	96.7	51.1	99.1	0.0121	0.7
Outer membrane	0.956	1.2	0.041	0	0.121	0.6	1.07	0.6	0.0020	0
Cytoplasm	27.3	35.2	0.042	1.2	0	0	0.019	0.3	0.0884	70.9
Spheroplast ghost	46.2	59.5	0	0	0.017	2.7	0	0	0.0583	28.4

[a] Cells were grown in LB broth with 0.2% galactose at 20°C for 40 h and then treated with lysozyme.

disruption. In mammalian cells, alkaline phosphatase (8, 34) and 5'-nucleotidase (34, 36) are localized in the plasma membrane, while in *E. coli* cells both are localized in the periplasmic space (11, 21). We found that this is also true in the case of GGT, and this is very interesting from the viewpoint of evolution. Also, since GGT activities can be easily measured with γ-glutamyl-*p*-nitroanilide, GGT could be useful as a periplasmic marker enzyme in *E. coli*.

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