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Isolation, Genetic Mapping, and Characterization of *Escherichia coli* K-12 Mutants Lacking γ-Glutamyltranspeptidase

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***Escherichia coli* K-12 mutants lacking γ-glutamyltranspeptidase (EC 2.3.2.2) were isolated after mutagenesis of cells with ethyl methanesulfonate. They lost the enzyme activity to different extents. The mutations of two mutants that had lost the enzyme activity completely were mapped at 76 min of the *E. coli* K-12 linkage map. These mutations made the cells neither nutrient requiring nor cold sensitive. The mutants leaked much more glutathione into the medium than the wild type. We propose the symbol *ggt* for these mutations.**

γ-Glutamyltranspeptidase (GGT), which utilizes glutathione as the substrate for its transpeptidation and hydrolysis reactions, has been found in cells from bacteria to mammals (19, 23, 24, 36–39). Its physiological role, however, remains controversial (15, 26, 29–31). We (34) and Nakayama et al. (22) isolated GGTs from bacteria and studied their enzymatic properties to elucidate the physiological functions of bacterial GGTs. Nakayama et al. isolated GGT-less mutants of *Proteus mirabilis* and found that this bacterium does not require GGT activity for growth (R. Nakayama, doctoral thesis, Kyoto University, Kyoto, Japan, 1984). For an extensive genetic study, however, *Escherichia coli* K-12 is far more useful. Although Milbauer and Grossowicz (19), Szewczuk and Mulczuk (36), and Nakayama et al. (24) reported that *E. coli* shows only weak or negligible GGT activity, we found and reported that *E. coli* K-12, when grown at 20°C, shows higher GGT activity than the high-GGT-producing bacterium *P. mirabilis* (35). Schulman et al. found a patient with an inborn deficiency of GGT and glutathionuria (31). Meister and co-workers showed that administration of inhibitors of GGT causes glutathionuria (1, 9) and that there is some relationship between the GGT activity of lymphoid cells grown in a tissue culture and the amount of glutathione leaked into the medium (10). Nakayama et al. found that *P. mirabilis* leaked much more glutathione into the medium when its GGT was inhibited by inhibitors (21).

In this paper we report the isolation, genetic mapping, and characterization of GGT-less mutants of *E. coli* K-12.

MATERIALS AND METHODS

Reagents. Ethyl methanesulfonate (EMS) and nalidixic acid were purchased from Nakarai Chemical Co.; γ-glutamyl-*p*-nitroanilide and glycylglycine were from Wako Pure Chemical Co.; glutathione reductase from baker's yeast, disodium D, L-α-glycerophosphate, and streptomycin were from Sigma Chemical Co.; tetracycline was from Boehringer Mannheim Corp.; D-leucine was from Aldrich Chemical Co.; tryptone and MacConkey agar base were from Difco Laboratories; and yeast extracts were from Oriental Yeast Co. Reduced glutathione was a generous gift from Kirin Brewery Co. Other chemicals were the best reagent grade available from commercial sources.

Bacterial strains. The *E. coli* K-12 strains used in this study are listed in Table 1.

Growth media and cultivation of bacteria. M9 minimum medium (20) containing 0.2% glucose and 25 μg of tryptophan, 25 μg of threonine, 50 μg of leucine, 25 μg of proline, 50 μg of arginine, 10 μg of histidine, 20 μg of uracil, 20 μg of thymidine, and 1 μg of thiamine per ml was used when the concentration of glutathione in the medium was measured. Other media and growth conditions were as described previously (20, 35).

GGT activity assay. GTT activity was measured and calculated as described previously (34).

Determination of cell growth. Cell growth was expressed in terms of optical density at 610 nm as described previously (35).

Mutagenesis of cells. Strain MG1655 was grown overnight in 5 ml of LB broth at 37°C. One drop of EMS was added to 1 ml of the culture in a short test tube with a Pasteur pipette. The culture was vortexed and then incubated at 37°C for 150 min with reciprocal shaking. The mixture was then centrifuged at 2,000 × *g* for 10 min, and the supernatant was discarded. The cells were washed twice with M9 buffer and then suspended in 1 ml of LB broth. Then 0.2 ml of the suspension was transferred to 5 ml of LB broth in a 100-ml flask, and the cells were grown overnight at 37°C.

Screening for Ggt phenotype. Mutagenized cells were purified by single colony isolation twice on LB plates, patched onto LB plates in a grid pattern (6 × 8), and then allowed to grow for 48 h at 25°C. Cells were picked up from the plates with a metal block with 48 needles (6 × 8 pattern) and then transferred to 96-well microtiter dishes containing the GGT reaction mixture. The dishes were incubated at 37°C for 30 to 60 min. Ggt was determined on the basis of whether the reaction mixture became yellow or remained white.

Genetic mapping. (i) Mapping with Hfr::Tn*10*. Mapping of the mutations to a 20-min interval of the chromosome was achieved by using a series of Hfr strains which contained a Tn*10* inserted approximately 20 min from the point of origin. Recipient cells which became tetracycline resistant (Tet^r) were scored as to loss of the mutant phenotype. This mapping kit was constructed by Tania Baker and provided by Carol A. Gross. Derivatives of SH664 which were spontaneously streptomycin resistant (Str^r) or nalidixic acid resistant (Nal^r) were selected on LB plates containing 100 μg of streptomycin per ml or 20 μg of nalidixic acid per ml (SH594 or SH595, respectively). Since SH595 grew on LB plates containing 40 μg of nalidixic acid per ml, we use the

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TABLE 1. *E. coli* K-12 strains used in this study

Strain	Original designation	Genotype	Source or references
SH4	KL16	Hfr(PO45) *thi-1 relA1*	C. A. Gross
SH34	Hfr1	Hfr(PO1) *nadA*::Tn*10 thi-1 relA1 spoT1 supQ80*	C. A. Gross; *nadA*::Tn*10* mutant of HfrH
SH35	Hfr2	Hfr(PO3) *btuB*::Tn*10 metB1 relA1*	C. A. Gross; *btuB*::Tn*10* mutant of KL227
SH38	Hfr5	Hfr(PO45) *zed*::Tn*10 thi-1 relA1*	C. A. Gross; *zed*::Tn*10* mutant of KL16
SH39	Hfr6	Hfr(PO68) *thiA*::Tn*10 rpoD800 leu relA1*	C. A. Gross; *thiA*::Tn*10 leu rpoD800* mutant of KL14
SH46	Hfr13	Hfr(PO13) *malT*::Tn*10 thi-1 leu-6 gal-6 lacY1* or *Z4 supE44*	C. A. Gross; *malT*::Tn*10* mutant of KL228
SH50	MG1655	Prototrophic	13
SH61	MG1655-t40	F⁻ *malT*::Tn*10*	C. A. Gross
SH62	MG1655-t41	F⁻ *zhg*::Tn*10*	C. A. Gross
SH193	Lin8	Hfr(PO2A) *glpD3 glpR2 fhuA22 relA1 ΔphoA8 ompF627 fadL701 pit-10* T2ʳ *spoT1*	B. J. Bachmann (14, 33)
SH199	K165	F⁻ *htpR1*(Am) *malT66*(Am) *lacZ53*(Am) *phoA5*(Am) *supC47 supC91*(Ts) *trp-48*(Am) *relA1 rpsL150 spoT1*	B. J. Bachmann (7)
SH201	AT2455	Hfr(PO1) *thi-1 cysG44 mal-18 relA1 spoT1*	B. J. Bachmann (40)
SH222	K165	F⁻ *htpR*(Am) *lac*(Am) *trp*(Am) *pho*(Am) *supC*(Ts) *rpsL mal*(Am) *relA*	R. A. Van Bogelen (25)
SH224	AE4107	F⁻ *livH*::Mu *lstR leu-6 malA1 xyl-7 mtl-2 argG6 his-1 trp-31 str-104 nal*	D. L. Oxender (2, 3)
SH594		F⁻ *ggt-1 rpsL*	This work; spontaneous Strʳ mutant of SH664
SH595		F⁻ *ggt-1 nalA*	This work; spontaneous Nalʳ mutant of SH664
SH597	Hfr16	Hfr(PO45) *thiA*::Tn*10 relA1*	This work; SH4 × P1(SH39)
SH664		F⁻ *ggt-1*	SH50 mutagenized with EMS
SH673		F⁻ *ggt-2*	Same as SH664
SH675		F⁻ *ggt-1 rpsL malT*::Tn*10*	This work; SH594 × P1(SH61)
SH679		F⁻ *ggt-1 zhg*::Tn*10 rpsL*	This work; SH594 × P1(SH62)
SH682		F⁻ *ggt-2 zhg*::Tn*10*	This work; SH673 × P1(SH62)
SH683		F⁻ *zhg*::Tn*10*	Same as SH682
SH684		F⁻ *ggt-1 zhg*::Tn*10*	This work; SH50 × P1(SH679)
SH702		F⁻ *zhg*::Tn*10*	This work; SH50 × P1(SH682)
SH703		F⁻ *ggt-2 zhg*::Tn*10*	Same as SH702
SH723	SH309	F⁻ *araD139* Δ(*argF-lac*)*U169 rpsL150 deoC1 ptsF25 flbB5301 rbsR relA1* Δ(*malA-asd*)*3 ugpA704*::Tn*10*	H. Schweizer (32)
SH731		F⁻ *ggt-1 ugpA704*::Tn*10*	This work; SH664 × P1(SH723)

designation *nalA* for the mutation (20). Exponentially growing cultures (3 ml) of SH594 or SH595 were mixed with 0.3 ml of exponentially growing cultures of each of the Hfr strains. The mixtures were incubated without shaking in 100-ml flasks at 37°C. Samples of 0.2 ml were removed from the flasks after 0, 20, 25, 30, 35, and 40 min, mixed with 1.8 ml of M9 buffer in test tubes, and then vortexed at top speed for 2 min. Then 0.3 ml of the mixtures was added to 3 ml of LB soft agar containing 10 μg of tetracycline and 100 μg of streptomycin per ml or 10 μg of tetracycline and 20 μg of nalidixic acid per ml, and the resultant mixtures were poured onto LB plates containing the same antibiotics. The plates were incubated at 37°C. Exconjugants growing on the selective plates were purified by single colony isolation once on the same plates and once on LB plates, followed by screening for the Ggt phenotype.

(ii) Mapping with P1 *vir*. The mutants were transduced with P1 *vir* grown on strains with markers localized around 76 min of the *E. coli* K-12 linkage map (5), and the strains with markers were transduced with P1 *vir* grown on the mutant. Transductions were performed by the standard method (20).

Measurement of glutathione concentrations in the medium. Glutathione concentrations in the culture medium were measured with glutathione reductase by the method of Fahey et al. (8).

RESULTS

Isolation of GGT-less mutants. Prototrophic *E. coli* K-12 (MG1655) was mutagenized with EMS followed by screening for the GGT-less phenotype (Ggt⁻) as described in Materials and Methods. Mutagenesis was performed for two batches. Fourteen and nine Ggt⁻ mutants were isolated from batch 1 and batch 2, respectively. GGT activity per cell was measured. Two of the mutants, SH664 and SH673, were chosen as typical mutants which had lost the GGT activity completely from batch 1 and batch 2, respectively, and their mutations were named *ggt-1* and *ggt-2*, respectively. Periplasmic fractions of the strains bearing *ggt-1* (SH684) and *ggt-2* (SH703) did not give any precipitin line in Ouchterlony immunodiffusion test (27) with rabbit antiserum against purified *E. coli* GGT.

Genetic mapping of *ggt*. (i) Mapping with Hfr::Tn*10* strains. Hfr conjugation, selection, and screening of exconjugants were performed as described in Materials and Methods. The results of these conjugations (Table 2) suggest that *ggt* is localized between 75 and 84.5 min (Fig. 1).

(ii) Mapping with P1 *vir* transduction. Further mapping of *ggt* was carried out through a series of P1 *vir*-mediated transduction experiments. The results of the transductions are shown in Fig. 2, and those of three-factor crosses are shown in Table 3. The data show that the gene order around 76 min is *cysG-malT-glpD-ggt-ugpA-livH-htpR* (*rpoH*) (clockwise).

Cold sensitivity as to growth and nutrient requirements of GGT-less mutants. Since our previous results (35) suggested that *E. coli* GGT is synthesized only at lower temperature, we expected that GGT-less mutants would be cold sensitive as to growth or nutrient requirement. The GGT-less mutants, however, grew at various temperatures. SH673 did not

TABLE 2. Mapping of the *ggt* gene by Hfr conjugation

Expt[a]	Hfr × recipient	Mating time (min)	No. of exconjugants	Selected marker	Counterselected marker	No. of *ggt*$^+$ mutants/total
A	Hfr1 × SH594	0	0	Tet^r	Str^r	
		30	7			0/7
		35	28			0/28
B	Hfr2 × SH595	0	0	Tet^r	Nal^r	
		35	32			0/32
C	Hfr5 × SH594	0	0	Tet^r	Str^r	
		25	0			
		30	7			0/7
		35	45			0/45
D	Hfr16 × SH595	0	0	Tet^r	Nal^r	
		30	0			
		35	39			14/39
		40	>100			22/48
E	Hfr13 × SH594	0	0	Tet^r	Str^r	
		20	0			
		25	1			0/1
		30	4			1/4
		36	24			10/24
		40	22			12/22

[a] In experiments A, C and E, the mating mixtures were spread on LB plates containing 10 μg of tetracycline and 100 μg of streptomycin per ml. In experiments B and D, the mating mixtures were spread on LB plates containing 10 μg of tetracycline and 20 μg of nalidixic acid per ml. As controls, the Hfr strains and recipients were plated on the selection plates separately, and it was confirmed that no colonies grew on the plates.

grow on M9 minimum medium plates at 37 or 42°C, but when *ggt-2* was transduced out into SH50 *ggt-2* did not cause any nutrient requirement; when the wild-type allele was transduced into SH673, the strain remained auxotrophic at high temperature. This shows that the *ggt-2* allele does not make the strain auxotrophic, but some other mutation in SH673 makes the strain auxotrophic.

Leakage of glutathione from GGT-less mutant cells into the medium. The concentrations of glutathione that had leaked into the medium were measured. GGT-less mutants leaked about a twofold amount of glutathione into the medium compared with wild-type cells. The amounts of glutathione leaked into the medium were compared between SH702 and SH703 at various temperatures. Both SH702 and SH703 leaked a lot of glutathione at around both 18 and 40°C, and at all temperatures tested SH703 leaked more glutathione into the medium than SH702 (Fig. 3). The amounts of glutathione leaked into the medium were also measured after various incubation times (Fig. 4). The amount of glutathione that SH702 leaked increased in the exponential phase and became maximum in the early stationary phase but thereafter decreased rapidly, whereas the amount of glutathione that

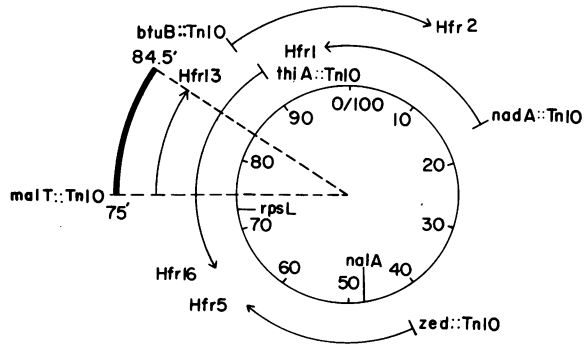

FIG. 1. Genetic map of *E. coli* K-12. The arrows show the extents to which the Hfr strains are thought to transfer into the recipients. The region in which the *ggt* gene is thought to be localized is shown by a heavy bar. The genetic symbols used are those of Bachmann (5), and the origins and directions of transfer of the Hfr strains are according to Bachmann and Low (6).

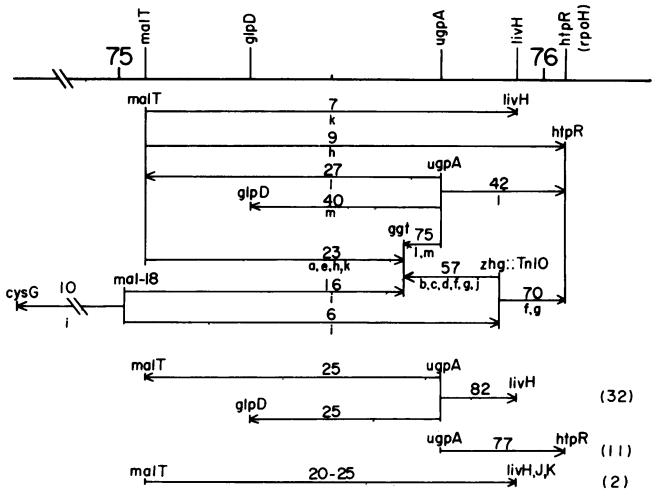

FIG. 2. Location of the *ggt* gene near 76 min. This figure summarizes the transduction frequencies as well as related data reported by other workers. The numbers above the lines are the cotransduction frequencies (percent), and the arrowheads indicate the unselected markers. The map is not drawn to scale. P1 *vir* transductions and number of transductants scored: (a) SH61 × SH594, 48; (b) SH62 × SH594, 48; (c) SH62 × SH673, 48; (d) SH679 × SH50, 203; (e) SH61 × SH664, 77; (f) SH679 × SH199, 200; (g) SH679 × SH222, 198; (h) SH675 × SH222, 200; (i) SH679 × SH201, 116; (j) SH682 × SH50, 88; (k) SH675 × SH224, 207; (l) SH731 × SH199, 199; (m) SH731 × SH193, 200. Media: (a through h, j, l) LB with 10 μg of tetracycline per ml and 10 mM sodium citrate; (i) E

TABLE 3. Three-factor analyses

P1 donor × recipient	Selected marker	Unselected marker	No. of cotransductants
SH675 × SH222	*malT*::Tn*10*	*ggt-1 htpR*	35
		ggt$^+$ *htpR*	148
		ggt-1 htpR$^+$	16
		ggt$^+$ *htpR*$^+$	1
SH679 × SH201	*mal*$^+$	*ggt-1 zhg*::Tn*10*	6
		ggt$^+$ *zhg*::Tn*10*	1
		ggt-1 Tet^s	12
		ggt$^+$ Tet^s	97
SH675 × SH224	*malT*::Tn*10*	*ggt-1 livH*	30
		ggt$^+$ *livH*	163
		ggt-1 livH$^+$	13
		ggt$^+$ *livH*$^+$	1
SH731 × SH199	*ugpA704*::Tn*10*	*ggt-1 htpRI malT66*	54
		ggt-1 htpRI malT$^+$	37
		ggt$^+$ *htpRI malT66*	25
		ggt$^+$ *htpRI malT*$^+$	0
		ggt-1 htpR$^+$ *malT66*	40
		ggt-1 htpR$^+$ *malT*$^+$	16
		ggt$^+$ *htpR*$^+$ *malT66*	27
		ggt$^+$ *htpR*$^+$ *malT*$^+$	0
SH731 × SH193	*ugpA704*::Tn*10*	*ggt-1 glpD3*	65
		ggt$^+$ *glpD3*	56
		ggt-1 glpD$^+$	77
		ggt$^+$ *glpD*$^+$	2

SH703 leaked became maximum in the early stationary phase and then decreased much more gradually than in the case of SH702.

DISCUSSION

A patient with an inborn deficiency of GGT and glutathionuria was found (31), and glutathionuria developed in mice whose GGT was inhibited by inhibitors (1, 9). Inoue et al. (16) showed that intracellular glutathione after being translocated out is cleaved by GGT and then reabsorbed via the membrane potential. Nakayama et al. (21) found that administration of serine with borate and 6-diazo-5-oxo-norleucine promoted the leakage of glutathione from *P. mirabilis* cells into the medium. In this study, we isolated GGT-less mutants of *E. coli* K-12 to elucidate the physiological role of GGT. After mutagenesis of cells with EMS and subsequent screening, 23 GGT-less mutants showing various levels of GGT activity were isolated. The mutations, *ggt-1* and *ggt-2*, were mapped by means of Hfr conjugation and P1 *vir*-mediated transduction. *ggt* was mapped between *glpD* and *ugpA* on the *E. coli* K-12 linkage map (Fig. 2), and it had become cotransducible with selective markers. Profound genetic studies are required to decide whether *ggt-1* and *ggt-2* are in the structural gene or in a possible regulator gene.

In the previous paper (35) we reported that *E. coli* showed maximum GGT activity when it was grown at 20°C but no activity above 43°C. So we assumed that if GGT is essential for growth at a lower temperature, GGT-less mutants must become cold sensitive as to growth. We checked two mutants which had completely lost the GGT activity, but neither of them had become cold sensitive as to growth. We also imagined that GGT might synthesize some γ-glutamyl compounds which are essential for cell growth, but the GGT-less mutations did not make *E. coli* nutrient requiring.

The GGT-less mutants did not show any phenotypic difference from the wild type, except that they leaked much more glutathione into the medium. Both the wild-type and Ggt$^-$ mutants leaked more glutathione into the medium at around 18 and 40°C than at other temperatures (Fig. 3). The specific activities of the glutathione biosynthetic enzymes (γ-glutamylcysteine synthetase and glutathione synthetase) in *E. coli* K-12 cells grown at 20, 30, and 45°C were not significantly different (data not shown), whereas both glutathione biosynthetic enzymes of *E. coli* B show optimum reaction temperatures of 45°C (12, 42), and those of *P. mirabilis* show optimum temperatures of 37 to 40°C (17; T. Maruyama, Master thesis, Kyoto University, Kyoto, Japan, 1982). *E. coli* K-12 is thought to synthesize glutathione at a high rate at around 40°C, and this may be the reason why *E. coli* K-12 leaks a lot of glutathione at around 40°C. *E. coli* K-12 also leaks a lot of glutathione at around 18°C. This may be because the activity of an enzyme other than GGT which cleaves or which promotes the uptake of glutathione in the medium is low at around 18°C. Apontoweil and Berends (4), and Owens and Hartman (28) reported that the concentration

(41) with 0.2% maltose, 40 μg of cysteine per ml, 1 μg of thiamine per ml, and 10 mM sodium citrate; (k) LB with 10 μg of tetracycline per ml, 50 μg of thymine per ml, and 10 mM sodium citrate; (m) LB with 0.4% glucose, 10 μg of tetracycline per ml, and 10 mM sodium citrate. Phenotypes: growth (Htp$^+$) or no growth (Htp$^-$) at 42°C on LB plates (for experiment k, on LB plates with 50 μg of thymine per ml); growth (CysG$^+$) or no growth (CysG$^-$) on M9 minimum plates with and without cysteine; utilization (GlpD$^+$) or nonutilization (GlpD$^-$) of glycerol-3-Pi as a sole carbon source (see Hayashi et al. [14]); utilization (LivH$^+$) or nonutilization (LivH$^-$) of 100 μg of D-leucine per ml as an L-leucine source (see Anderson and Oxender [2]); red (MalT$^+$) or white (MalT$^-$) colonies formed on MacConkey-maltose plates.

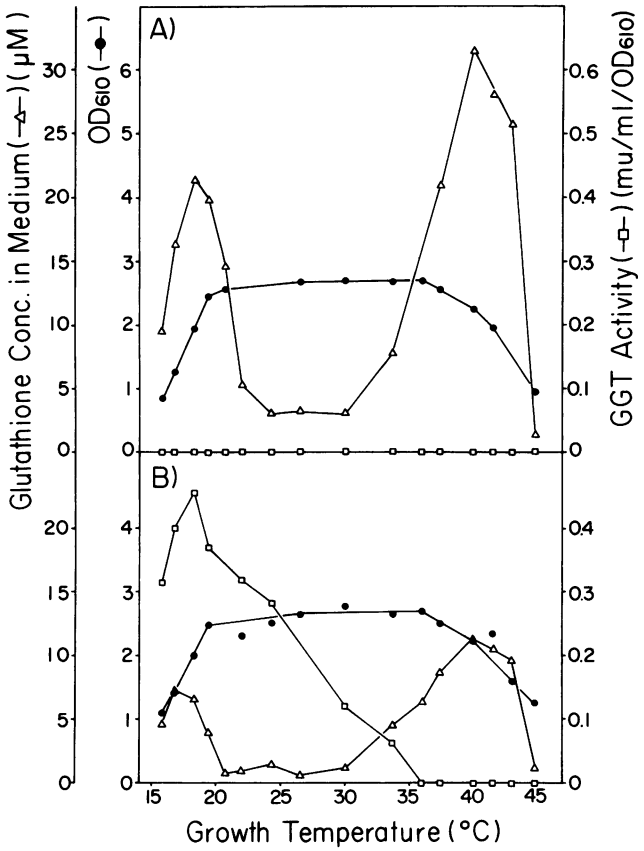

FIG. 3. Effect of the growth temperature on glutathione leakage into the medium. Strains SH703 (A) and SH702 (B) were grown in 10 ml of medium in L-shaped test tubes, in a temperature gradient incubator, for 48 h with reciprocal shaking (60 rpm).

of intracellular glutathione in *E. coli* increases throughout the exponential phase and becomes maximum in the stationary phase, whereas Loewen (18) reported that it only increases after the stationary phase. Owens and Hartman also reported that the concentration of extracellular glutathione in *E. coli* increases during the exponential phase and becomes maximum in the stationary phase sometime after the concentration of the intracellular glutathione has become maximum (28). So we compared the concentrations of glutathione in the culture media of the wild type and the Ggt$^-$ mutant. The wild-type *E. coli* leaks the maximum amount of glutathione into the medium in the early stationary phase, but the concentration of it in the medium decreases rapidly during the stationary phase, whereas in GGT-less mutants the concentration of glutathione in the medium decreased much more gradually during the stationary phase (Fig. 4). We think that this is because GGT does not participate in the secretion of glutathione, but glutathione that has leaked into the medium is utilized by GGT, and GGT is not the only enzyme that utilizes glutathione in the medium or that promotes its uptake from the medium. Hence, even in GGT-less mutants, the concentration of glutathione in the medium decreased during the stationary phase, albeit gradually. Nakayama et al. (21) used 6-diazo-5-oro-norleucine to inhibit the GGT of *P. mirabilis* and found that although GGT was completely inhibited, the concentration of glutathione in the medium decreased gradually during the stationary phase. This also indicates that enteric bacteria

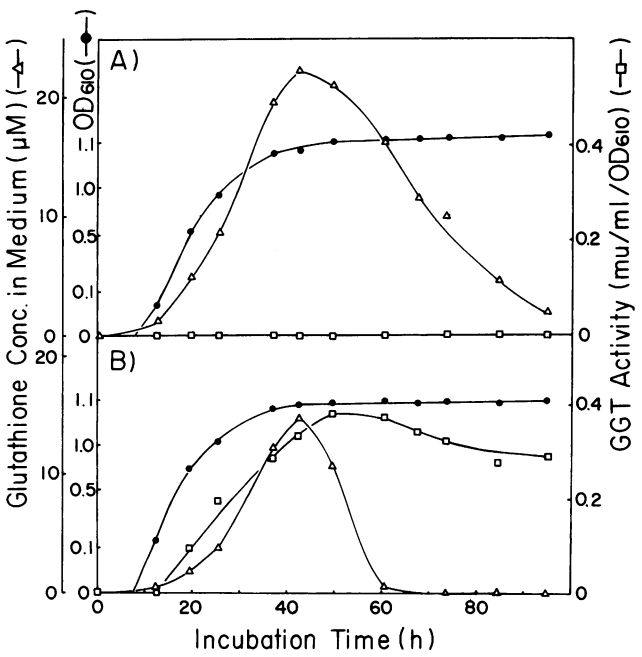

FIG. 4. Glutathione leakage into the medium during cultivation. Strains SH703 (A) and SH702 (B) were grown in 100 ml of medium in 1-liter flasks at 20°C with reciprocal shaking (60 rpm). Samples of 1 ml were removed from the flasks at appropriate times and then subjected to the analyses.

possess another mechanism besides a GGT-mediated one for utilizing glutathione in the medium.

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