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Genetic Analysis of the Glutamate Permease in *Escherichia coli* K-12[1]

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The glutamate permeation system in *Escherichia coli* K-12 consists of three genes: *gltC*, *gltS*, and *gltR*. The genes *gltC* and *gltS* are very closely linked, and are located between the *pyrE* and *tna* loci, in the following order: *tna*, *gltC*, *gltS*, *pyrE*; *gltR* is located near the *metA* gene. The three *glt* genes constitute a regulatory system in which *gltR* is the regulator gene responsible for the formation of a repressor, *gltS* is the structural gene of the glutamate permease, and *gltC* is most probably the operator locus. The synthesis of glutamate permease is partially repressed in wild-type K-12 strains, resulting in the inability of these strains to utilize glutamate as the sole source of carbon. Derepression due to mutation at the *gltC* locus enables growth on glutamate as a carbon source both at 30 C and at 42 C. Temperature-sensitive *gltR* mutants capable of utilizing glutamate for growth at 42 C but not at 30 C were found to be derepressed for glutamate permease when grown at 42 C and partially repressed (wild-type phenotype) upon growth at 30 C. These mutants produce an altered thermolabile repressor which can be inactivated by mild heat treatment (10 min at 44 C) in the absence of growth.

In previous papers from this laboratory (9, 10, 17), evidence was presented showing that a permeability barrier was responsible for the inability of wild-type strains of *Escherichia coli* K-12 to grow on glutamate as the sole source of carbon and energy. The location of a gene (*gltC*) determining or regulating glutamate permeation was established by conjugation (17). The mutated allele of this gene, *gltC*^{c} (previously designated *gltC*$^{+}$), enables the mutants to grow on glutamate, thanks to an increased rate of glutamate uptake and a higher capacity for glutamate accumulation, as compared with the wild-type strains carrying the *gltC*$^{+}$ allele (previously named *gltC*$^{-}$). On the basis of these data, *gltC* could be the structural gene of the glutamate permease or a gene which regulates the rate of synthesis of the glutamate permease.

The purpose of this report is to show that the glutamate permease system consists of at least three components: the structural gene, *gltS*, determining the synthesis of glutamate permease, and two regulatory genes, *gltR* and *gltC*. The *gltR* gene specifies the formation of a repressor which represses the synthesis of glutamate permease in wild-type strains; the *gltR* locus is not linked to the structural gene. The *gltC* locus determines the sensitivity of glutamate permease synthesis to the product of the *gltR* gene; *gltC* is very closely linked to the structural gene of glutamate permease.

[1] This work was part of a Ph.D. thesis submitted by the senior author to the Senate of the Hebrew University.

MATERIALS AND METHODS

Microorganisms. The strains used throughout this work were all derivatives of *E. coli* K-12. A list of the strains used is given in Table 1.

Growth media. The liquid minimal medium in which the bacteria were grown and the solid minimal medium used for the selection of recombinants have already been described (17). These media were supplemented with a carbon source as required (glucose, 0.2%; glutamate, 0.5%; lactose, 0.5%; mannitol, 0.5%; tryptophan, 0.3%; succinate, 0.5%; and xylose, 0.5%), amino acids (arginine, histidine, isoleucine, leucine, methionine, threonine, and valine, where required, 25 μg of the L isomer per ml, except where otherwise stated), thiamine hydrochloride (where required, 1 μg/ml), and thymine and uracil (where required, 20 μg/ml). Mating broth containing Casamino Acids and yeast extract was that described by Hayes (12). Transduction broth (T broth) contained, at *p*H 7.0: 1% tryptone (Difco), 0.5% yeast extract, 0.5% NaCl, and 5×10^{-3} M $CaCl_2$ (the $CaCl_2$ was autoclaved separately). Solid medium for phage P1kc titration contained transduction broth supplemented with 0.1% glucose and was solidified with 1% agar for the bottom layer and with 0.6% agar for the top layer. Stocks were maintained as described earlier (17).

Growth experiments. Young bacterial cultures from

TABLE 1. *Strains used*

Designation	Mating type	Growth on Glutamate		Relevant genotype[a]														
		30 C	42 C	gltR	gltC	gltS	arg	his	ilv	leu	met	thr	thi	pyrE	lac	mtl	tna	xyl	str
CS101	Hfr	−	−	+	+	+				+	−	+	+		+		+		S
CS7	Hfr	+	+	+	c	+			+	+	−	+	+	+	+	+	+	+	S
CS8	Hfr	+	+	+	c	+				+	−	+	+		+				S
CS2^{TC}	Hfr	−	+	TL	+	+	+				−		+		+				S
CS3^{TC}	Hfr	−	+	TL	+	+					−		+		+				S
CS4^{TC}	Hfr	−	+	TL	+	+					−		+		+				S
CS7/50	Hfr	−	−	+	c	−					−						+		S
P678	F$^-$	−	−	+	+	+				−	+	−	−		−	−		−	R
C600	F$^-$	−	−	+	+	+				−	+	−	−		−		+		R
GR103	F$^-$	−	−	+	+	+								+			−		R
DF1933	F$^-$	−	−	+	+	+	ECBH$^-$				A$^-$								R
AB1450	F$^-$	−	−	+	+	+		−	−										R
KL141	F$^-$	−	−		c	−								−					R

[a] The symbols used are based on the nomenclature of Demerec et al. (5). The symbols *arg*, *his*, *ilv*, *leu*, *met*, *thr*, *thi*, and *pyrE* designate genes which determine and regulate the synthesis of arginine, histidine, isoleucine-valine, leucine, methionine, threonine, thiamine, and uracil, respectively. Plus and minus signs indicate the ability or inability, respectively, to synthesize the amino acid, vitamin, or pyrimidine. A capital letter following the three-letter abbreviation indicates the specific gene or genes mutated among those sharing the same symbol. The genes *lac*, *mtl*, *tna*, and *xyl* determine and regulate the utilization of lactose, mannitol, tryptophan, and xylose, respectively. Plus and minus signs indicate, respectively, the ability and inability to utilize the given substrate. The genes *gltC gltR*, and *gltS* determine and regulate the synthesis of the glutamate permease. The gene *gltC* is most probably the operator gene in the glutamate permease system. Its *gltC*$^+$ allele is present in the wild-type strains which do not grow on glutamate (Glut$^-$ phenotype). The *gltC7* and *gltC8* alleles are present in mutants which grow on glutamate (Glut$^+$ phenotype). The gene *gltR* is the regulator gene determining the synthesis of repressor, and *gltR*$^+$ is the wild-type allele which determines the synthesis of an active repressor resulting in a Glut$^-$ phenotype in wild-type strains. The symbol *gltR*^{TL} represents the mutated allele. It determines the synthesis of a thermolabile repressor and thus gives a Glut$^+$ phenotype at high growth temperature (42 C) and a Glut$^-$ phenotype at low growth temperature (30 C). The gene *gltS* is the structural gene of glutamate permease; *gltS*$^+$ indicates the allele in wild-type strains which determines the synthesis of a permease with a high affinity for glutamate (K_m = 5 to 10 × 10^{-6} M), and *gltS*$^-$ indicates the mutated allele in mutant strains which do not grow on glutamate. This allele determines the synthesis of a permease with a low affinity for glutamate (K_m = 10^{-4} M). The *str* genes determine the response to streptomycin (S and R indicate susceptibility and resistance, respectively).

fresh agar slants were inoculated into media containing 0.5% glutamate as the sole source of carbon (enriched according to the requirements of the strain used) in conical flasks provided with side arms to allow direct turbidity measurements. The bacteria were incubated in a shaking water bath at the desired temperature. Growth was followed by measuring the changes in optical density in a Klett-Summerson photoelectric colorimeter, with the use of filter no. 42. Growth rates were determined graphically from semilogarithmic plots of optical density versus time of incubation.

Mutagenesis and selection of mutants. Mutations were induced by treatment with ultraviolet light (UV) or with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG; 1). The treated cultures were incubated overnight in test tubes containing nutrient broth in a shaking water bath at 37 C, to allow phenotypic expression. Glut^{TC} mutants (capable of growing on glutamate at 42 C but not at 30 C) were selected from strain CS101 treated with UV as follows. A 5-ml amount of the bacterial culture was centrifuged and washed with saline. The cells were suspended in 100-ml conical flasks to a density of 2 × 10^7 per ml in 40 ml of minimal medium which contained glutamate as the sole source of carbon and energy and was enriched with methionine. The flasks were incubated in a shaking water bath at 42 C until the bacterial density increased to 2 × 10^9 cells per ml. Only one mutant was selected from each culture. Forty independently isolated mutants were purified twice on plates of glutamate-minimal medium, and their ability to grow on glutamate at 42 C and at 30 C was tested by the replica method. Three mutants grew on glutamate at 42 C but not at 30 C.

Glut$^-$ mutants (unable to grow on glutamate at any temperature) were selected from strains CS7 and CS8 treated with UV or NG as follows. Samples (5 ml) from each of the two cultures were centrifuged, washed with saline, and resuspended in succinate-

minimal medium, enriched with methionine, to a density of 2×10^7 cells per ml. The cultures were grown in this medium to a density of 2×10^9 cells per ml to reduce the frequency of auxotrophic mutants. The bacteria were centrifuged, washed, and resuspended in glutamate-minimal medium enriched with methionine. The cultures were treated with penicillin according to the methods of Gorini and Kaufman (7) and Lubin (16), as described by Dar-Guttman et al. (3), to enrich for Glut$^-$ mutants. After this enrichment, the bacteria were grown in a succinate-minimal medium, enriched with methionine, to increase the total number of mutants, and another penicillin cycle was performed. Appropriate dilutions were spread on petri dishes with Tryptose Blood Agar Base. These plates served as master plates for replication onto minimal glucose, succinate, and glutamate media, enriched with methionine. Only colonies which grew on glucose and succinate media but did not grow on the glutamate medium were isolated and further studied.

Mating experiments. Matings were performed as described by us earlier (17).

Transduction experiments. Phage P1kc was used. Lysates were prepared as follows. Logarithmic cultures of the donor strains, at a density of 10^8 cells per ml in T broth containing 5×10^{-3} M $CaCl_2$ and 0.1% glucose, were infected with the phage at a multiplicity of 0.1 and incubated with aeration for 3 hr at 37 C. At that time, considerable lysis could be observed. A few drops of chloroform were added to the culture, and incubation was continued for 10 min more. The bacterial debris was removed by centrifugation at low speed (5,000 $\times$ *g*) for 10 min. The supernatant liquid was decanted and stored at 4 C in sterile test tubes to which one drop of chloroform was added. By this procedure, lysates with a titer of 5×10^{10} infective particles per ml were easily obtained. *E. coli* C600 served as the indicator strain. Transduction was performed as follows. The recipient strains were grown in T broth with aeration at 37 C to a density of 5×10^8 cells per ml. $CaCl_2$ (5×10^{-3} M) was then added, and incubation was continued for 5 min longer. Phage was added at a multiplicity of 1, and the suspension was incubated at 37 C without shaking for 20 min. The bacteria were then centrifuged in the cold and resuspended in sterile phosphate buffer (*p*H 7.0, 0.1 M). Samples (0.1 ml) of appropriate dilutions were spread on selective media. The recipient strain and the phage suspension were also spread separately on the same media to serve as controls. The plates were kept for 2 hr at room temperature and then incubated at the desired temperature as specified.

Isolation of recombinants and scoring for unselected markers. Recombinants were isolated and unselected markers were scored for as previously described (17).

Uptake of ^{14}C-glutamate. Glutamate uptake was studied by two different methods.

In the first method, incorporation of ^{14}C-L-glutamate (uniformly labeled, The Radiochemical Centre, Amersham, Bucks, England) into the proteins of logarithmically growing cultures was determined according to the method of Ames (2) as described by Halpern and Lupo (10), with some modifications. The bacteria were grown to the logarithmic phase at 30 or 42 C. Cultures grown at 42 C were transferred to a shaking water bath at 30 C 90 sec before the addition of the radioactive glutamate. The incorporation mixtures were incubated at 30 C for 3 min, at which time trichloroacetic acid was added and the tubes were heated at 90 C for 10 min. The bacteria were filtered, washed, and dried; the radioactivity was determined as previously described (10). Rates were expressed as micromoles of glutamate incorporated per milligram (dry weight) of bacteria per hour.

The second method involved measuring the accumulation of ^{14}C-glutamate by nongrowing cells. This was done according to the method of Kessel and Lubin (15) as earlier described (9, 10), with some modifications. The bacteria were grown to a density of 7×10^8 cells per ml in minimal medium containing 0.5% succinate (enriched as required) and were incubated in a shaking water bath at 30 or 42 C. Chloramphenicol was added to a final concentration of 200 μg/ml, and incubation was continued for 30 min longer. The uptake experiments were performed at 25 C. The cells were incubated in the presence of radioactive glutamate for 6 min. The incubation mixture was then filtered, washed, dried, and counted as described by Halpern and Even-Shoshan (9). The intracellular concentration of radioactive material was expressed as micromoles of glutamate per milliliter of intracellular water.

Determination of radioactive glutamate in the internal pool. Radioactive glutamate in the internal pool was determined according to the method of Halpern and Lupo (10). These authors found that about 75% of the radioactivity in the wild-type strain CS101 and in the glutamate-utilizing mutants tested was due to free glutamate. In the present work, we examined the radioactive pool in strain CS2^{TC}, which grows on glutamate at 42 C but not at 30 C, and we also found that more than 75% of the radioactive material was free glutamate.

Determination of the rate of exit of ^{14}C-glutamate. The rate of exit of radioactive glutamate from preloaded chloramphenicol-treated cells was determined according to the method described by Halpern (8) with some modifications. The cultures were grown in a succinate (0.5%)-minimal medium enriched as required, at 30 or at 42 C. The cells were preloaded with ^{14}C-L-glutamate at 25 C for 6 min. The exit reaction was also carried out at 25 C. The bacterial densities in the uptake mixtures varied with the different strains, as follows [mg (dry weight) per ml]: CS101, 3.2; CS7, 0.8; CS7/50, 3.2; CS2^{TC} grown at 30 C, 3.2; CS2^{TC} grown at 42 C, 0.8.

Enzyme assays. Cell-free extracts were used for the determination of enzyme activities. The procedures used for the preparation of extracts and for measuring the activities of glutamate dehydrogenase and glutamate-oxalacetate transaminase were as described by Halpern and Lupo (10). Aspartase activity was determined from the amount of ammonia formed from aspartate in the following reaction mixture: L-aspartate, 0.02 M, adjusted to *p*H 7.5; $MgSO_4 \cdot 7H_2O$, 0.001 M; potassium phosphate buffer, *p*H 7.5, 0.05 M; bacterial extract equivalent to 1 to 4 mg of protein;

total volume, 2 ml. The reaction mixture was incubated for 10 to 30 min at 30 C. The reaction was stopped by the addition of trichloroacetic acid to a final concentration of 3.3%. The precipitate was removed by centrifugation, and ammonia was determined on samples of the clear supernatant liquid by nesslerization according to the method of Halpern and Umbarger (11). Enzyme activities were expressed in micromoles of substrate transformed per milligram of protein per hour.

RESULTS

Isolation of temperature-conditional glutamate-utilizing mutants. We suggested earlier (17) that glutamate permease synthesis in *E. coli* is regulated by a genetically controlled mechanism of repression. Permease synthesis is partly repressed in the wild-type strains, resulting in their inability to utilize glutamate as the sole source of carbon, and is derepressed in the glutamate-utilizing mutants. On this assumption, one should be able to obtain mutants with lesions in the regulator gene specifying the synthesis of the repressor, which would produce a thermolabile repressor. Such mutants could be readily detected by their ability to grow on glutamate at high, but not at low, temperatures.

Logarithmic cultures of strain CS101 were treated with UV and mutants were selected capable of growing in a glutamate-minimal medium enriched with methionine, at 42 C. Forty independent mutants were isolated and tested for their ability to grow on glutamate at 30 C. Three of these mutants, $CS2^{TC}$, $CS3^{TC}$, and $CS4^{TC}$, were found to have acquired the $Glut^{TC}$ phenotype, i.e., the ability to utilize glutamate at 42 C but not at 30 C. The ability of these mutants to grow on glucose or succinate at 30 C was unimpaired.

Determination of ^{14}C-L-glutamate uptake and exit in strains CS101, CS7, CS8, and $CS2^{TC}$ grown at 30 C and at 42 C. The uptake and exit of ^{14}C-L-glutamate were measured as described in Materials and Methods. The results shown in Table 2 clearly demonstrate that the rate of exit was similar in the three strains examined. The exit of glutamate was about 2.5 times faster in bacteria grown at 42 C than in those grown at 30 C. The affinity of the glutamate permease for glutamate was determined by measuring glutamate incorporation into the proteins of exponentially growing cultures and accumulation by nongrowing cells. The two methods gave similar results. The K_m for glutamate in the wild-type CS101, in the $Glut^+$ mutant CS8, and in the $Glut^{TC}$ mutant $CS2^{TC}$ grown at either 30 or 42 C was 10^{-5} M and differed only slightly in strain CS7 (5×10^{-6} M). However, the rate of uptake of glutamate and the maximal glutamate-concentrating capacity differed greatly in the four strains tested. The glutamate-utilizing mutants CS7 and CS8 showed a fivefold higher rate of glutamate uptake and accumulated four to six times more glutamate than did their $Glut^-$ parent strain CS101, regardless of the temperature of growth. On the other hand, glutamate uptake in strain $CS2^{TC}$ exhibited a dramatic dependence on growth temperature. When grown at 42 C, $CS2^{TC}$ took up glutamate at a fast rate similar to those of the $Glut^+$ mutants; however, $CS2^{TC}$ cultures grown at 30 C took up glutamate at a rate one-fifth of that of the $Glut^+$ mutant,

TABLE 2. *Determination of glutamate permease activity and glutamate exit in cultures grown at 30 C and at 42 C*

Strain	Growth temp	Uptake				Exit half-life
		Glutamate incorporated into proteins		Internal pool of glutamate		
		V_{max}[a]	K_m	MCC[b]	K_m	
	C		M		M	min
CS101	30	0.070	1×10^{-5}	4.0	1×10^{-5}	21
CS101	42	0.070	1×10^{-5}	4.0	1×10^{-5}	8
CS7	30	0.330	5×10^{-6}	16.0	5×10^{-6}	21
CS7	42	0.320	5×10^{-6}	13.5	5×10^{-6}	8
CS8	30	—	1×10^{-5}	25.0	1×10^{-5}	—
CS8	42	—	1×10^{-5}	15.0	1×10^{-5}	—
$CS2^{TC}$	30	0.075	1×10^{-5}	4.5	1×10^{-5}	21
$CS2^{TC}$	42	0.320	1×10^{-5}	14.6	1×10^{-5}	8

[a] V_{max} is expressed in micromoles of ^{14}C-L-glutamate per milligram (dry weight) of bacteria per hour.
[b] MCC (maximal concentrating capacity) is expressed in micromoles of ^{14}C-L-glutamate per milliliter of intracellular water.

similar to the rate characteristic of the Glut$^-$ parent strain CS101.

Growth of CS2^{TC} on glutamate at different temperatures. The growth rate of CS2^{TC} in a glutamate-minimal medium enriched with methionine, at 42 and 30 C, and the changes in the rate of growth after temperature shifts are given in Fig. 1. The results demonstrate a close correlation between the ability of CS2^{TC} to grow on glutamate at 42 C, its inability to grow at 30 C, and the rates of glutamate uptake and the maximal glutamate-concentrating capacities at these temperatures (*see* Table 2).

Mapping of the gene determining the Glut^{TC} phenotype in strain CS2^{TC} by interrupted mating. Preliminary experiments showed that it was possible to obtain Glut^{TC} recombinants with Hfr CS2^{TC} as the donor and F$^-$ C600 or F$^-$ P678 as the recipient. The results of an interrupted mating between Hfr CS2^{TC} and F$^-$ C600 are given in Fig. 2. These data clearly show that the mutation which enables growth on glutamate at a high temperature (*gltR*^{TL}) does not map at and is not even linked to the *gltC* locus, which maps near the *mtl* gene (17). Similar results were obtained in experiments in which CS3^{TC} and CS4^{TC} served as donors. Several recombinants were tested for their ability to take up ^{14}C-L-glutamate after growth at 30 and at 42 C. All the recombinants tested exhibited a pattern similar to that of the donor strain CS2^{TC}. The somewhat lower number of Glut^{TC} recombinants as compared with the number of Thi$^+$ recombinants probably resulted from the greater interference with integration on the minimal selective medium for Glut^{TC} recombinants (4, 17).

Mapping of the gltR^{TL} locus by recombination analysis. The results of the interrupted mating indicated that the *gltR*^{TL} locus entered before the *thi* locus. This observation was verified by an unselected-marker frequency analysis (Table 3), in which the difference in linkage within each pair of markers, depending on which of the two markers is selected for, is a consequence of the gradient of transmission (13).

The distance in time units between *gltR* and the *thi* locus as determined by the interrupted mating experiment was about 3 min (Fig. 2). This places the *gltR* gene on the *E. coli* K-12 chromosome map (19) near the *metA* locus (Fig. 3). A three-factor-cross analysis was therefore performed in which a prototroph derivative of CS2^{TC} served as the donor and F$^-$ DF1933

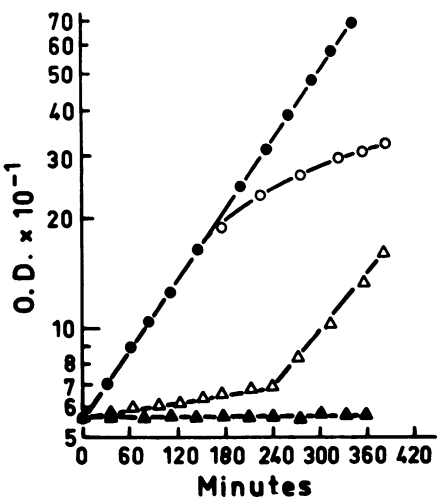

FIG. 1. *Growth of strain CS2^{TC} in a glutamate-minimal medium at 30 and 42 C. CS2^{TC} was grown for 10 generations in a glutamate-minimal medium at 42 C to attain steady-state conditions. The culture was then divided into two flasks; one was left at 42 C and the other was transferred to 30 C. The culture at 42 C continued to grow exponentially. At 30 C, growth slowed down after 140 min and stopped entirely after three generations. Part of this culture was then transferred from 30 to 42 C, and growth was followed turbidimetrically as described in Materials and Methods. Symbols: ●, culture grown at 42 C; ○, culture transferred at zero time from 42 to 30 C; △, culture transferred at zero time from 30 to 42 C; ▲, culture grown at 30 C.*

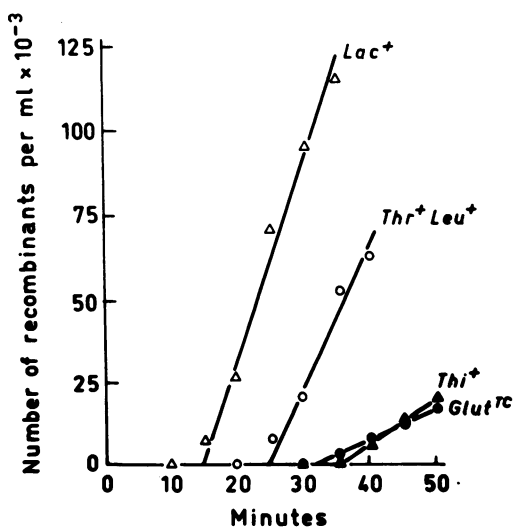

FIG. 2. *Mapping of the gltR gene by interrupted mating. A temperature-sensitive glutamate-utilizing mutant, Hfr CS2^{TC}, served as the donor and strain F$^-$ C600 was used as the recipient. Glut^{TC} recombinants were selected on a glutamate-minimal medium enriched with methionine at 42 C.*

TABLE 3. *Unselected-marker frequency analysis by conjugation of the linkage relationships between the thi and gltR loci in E. coli K-12*[a]

Selected marker	No. of recombinants	Unselected marker	No. of recombinants	Linkage
				%
$gltR^{TL}$	125	thi^+	45	36.0
thi^+	111	$gltR^{TL}$	106	95.5

[a] Hfr $CS2^{TC}$ and F^- C600 served as donor and recipient, respectively.

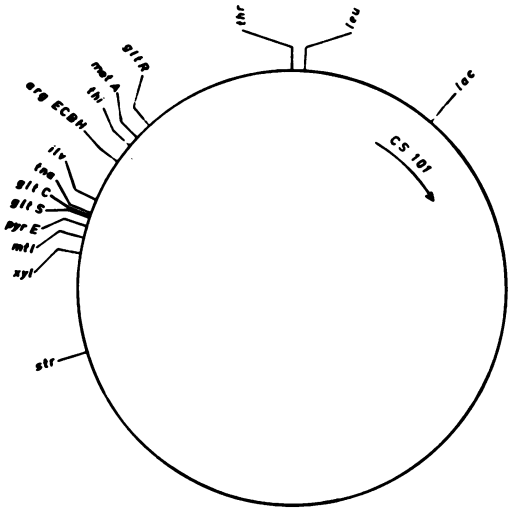

FIG. 3. *Escherichia coli K-12 chromosome drawn to scale according to Taylor and Trotter (19) showing the positions of gltC, gltS, and gltR.*

(*arg*$^-$, *met*$^-$) served as the recipient. Arg^+ recombinants were selected, and Met^+ and $Glut^{TC}$ recombinants were scored by the replica method. The results shown in Table 4 establish the following order of genes: *argECBH, metA, gltR.*

Linkage analysis by transduction between $gltR^{TL}$, argECBH, metA, and thi. Phage P1kc was grown on strain $CS2^{TC}$. The recipient strains were DF1933 and P678. The results shown in Table 5 confirm the order of genes established by conjugation.

Thermolability of the $gltR^{TL}$ product. The results described so far provide tentative support for our assumption that the glutamate permease synthesis is controlled by a mechanism of repression (17), in which *gltR* plays the role of the repressor-forming gene. In wild-type strains which do not grow on glutamate, the synthesis of the permease is partly repressed. The behavior of the $Glut^{TC}$ mutants would then be due to the production of a thermolabile repressor, which is destroyed at high growth temperatures, resulting in derepression of glutamate permease synthesis and in the acquisition of the ability to grow on glutamate at 42 C.

Evidence pointing to the *gltR* product as a thermolabile repressor was obtained in the following experiment. $CS2^{TC}$ and CS101 were grown in a succinate-minimal medium enriched with methionine, in a shaking water bath at 30 C. After 10 doublings (18), the cultures were diluted into fresh medium to a density of 1.5×10^7 cells per ml and further incubated for 90 min. The two cultures were then divided each in two equal portions and the four flasks were immediately placed in a shaking water bath at 30 C. Samples (8 ml) were withdrawn from each flask at the times indicated in Fig. 4. The rate of glutamate uptake was measured by mixing each sample with 2 ml of ^{14}C-L-glutamate (1.5 μc per μmole) to a final concentration of 5×10^{-6} M and incubating the mixture for 3 min at 30 C. Trichloroacetic acid was then added to a final concentration of 5%, and the amount of radioactive material incorporated into protein was determined. After two samples were taken from each of the four flasks, the remaining cultures were filtered on a membrane filter (47 mm in diameter, 0.45-μm pore size), and the filtered growth media were returned to the respective flasks. The cells were washed five times with 10 ml of 0.06 M phosphate buffer (*p*H 7.0), prewarmed to 30 C, and resuspended in 3 ml of buffer. One suspension of each strain was incubated at 44 C and the other at 30 C for 10 min and transferred back to its growth medium at 30 C (18). The cultures were incubated, sampled, and tested for glutamate uptake for a further 80 min. As shown in Fig. 4, heating of $CS2^{TC}$ for 10 min at 44 C in the absence of growth increased the differential rate of glutamate per-

TABLE 4. *Three-factor-cross analysis of the $gltR^{TL}$ gene in E. coli K-12 by conjugation*[a]

Selected marker	Unselected markers[b]	No. of recombinants	Percentage of recombinants
$argECBH^+$	$metA^+$, $gltR^{TL}$	89	68.5
$argECBH^+$	$metA^+$, $gltR^+$	15	11.5
$argECBH^+$	$metA^-$, $gltR^+$	26	20.0
$argECBH^+$	$metA^-$, $gltR^{TL}$	2	1.5

[a] Hfr $CS2^{TC}$ and F^- DF1933 served as donor and recipient, respectively.

[b] The $gltR^{TL}$ recombinants were selected at 38 C because DF1933 did not grow well at 42 C. At 38 C, growth of $Glut^{TC}$ mutants on glutamate is slower than at 42 C and recombinant colonies appeared only after 4 days of incubation.

TABLE 5. *Determination of linkage between gltR, metA, and argECBH by transduction*

Expt no.	Donor strain	Recipient strain	Selected marker	No. of recombinants	Unselected markers	No. of recombinants	Linkage
1	CS2^{TC}	DF1933	*argECBH*$^+$	156	*metA*$^+$	25	16.0
2	CS2^{TC}	DF1933	*argECBH*$^+$	156	*gltR*^{TL}	0	<0.6
3	CS2^{TC}	DF1933	*metA*$^+$	469	*argECBH*$^+$	140	29.8
4	CS2^{TC}	DF1933	*metA*$^+$	469	*gltR*^{TL}	12	2.6
5	CS2^{TC}	DF1933	*metA*$^+$	469	*argECBH*$^+$, *gltR*^{TL}	0	<0.2
6	CS2^{TC}	P678	*thi*$^+$	150	*gltR*^{TL}	0	<0.6
7	CS2^{TC}	P678	*gltR*^{TL}	150	*thi*$^+$	0	<0.6

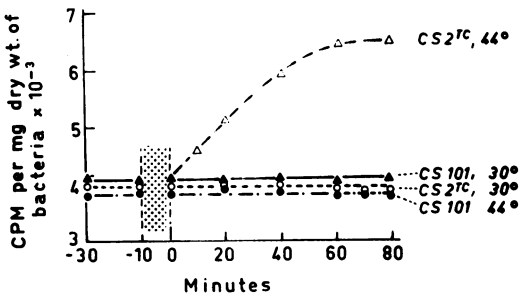

FIG. 4. *Thermolability of the glutamate permease repressor in the temperature-sensitive glutamate-utilizing mutant CS2^{TC}.*

mease synthesis during subsequent growth at 30 C. This was manifested by the continuous increase, for about 60 min, in the specific activity of the permease in CS2^{TC} heated at 44 C. In contrast, a CS2^{TC} culture heated at 30 C and CS101 cultures heated at either 30 or 44 C exhibited low specific activities which remained constant throughout the entire experiment. It is of interest to point out that the highest specific activity of glutamate permease in CS2^{TC} grown at 30 C after a brief treatment at 44 C was only about 1.5-fold higher than that of the control cultures, whereas CS2^{TC} cells grown for 10 generations at 44 C exhibited a rate of glutamate uptake four times as high as that of cells grown at 30 C (*see* Table 2). These results can be best explained by the production of a thermolabile repressor by the *gltR*^{TL} allele of the repressor-forming *gltR* gene. Destruction of the repressor by brief heating results in a burst of permease synthesis at a high, derepressed rate. Accumulation of new repressor molecules following the resumption of growth at the lower temperature brings about a gradual reestablishment of repression.

Isolation of Glut$^-$ mutants from the Glut$^+$ strain CS7 (gltC7). The *gltC* locus had been mapped by interrupted mating with HfrC Glut$^+$ mutants serving as donors and the P678 wild-type strain as the recipient (17). Since no Glut$^+$ recombinants appeared until 6 to 7 min after the time of entry of the *gltR* locus, it would seem that the *gltC* mutants examined carried a normal wild-type *gltR* allele. Moreover, as shown in Table 9, mutant CS7 (*gltC*^{c}) as the donor in a transduction experiment yielded Glut$^+$ transductants at a high frequency (1.6×10^{-5}). Since *gltR* and *gltC* are too far apart to be cotransduced, these Glut$^+$ transductants must have inherited the *gltR* allele of the recipient, which was wild type. The fact that the *gltC* mutants and recombinants are derepressed in spite of the presence of a normal *gltR* gene suggests that the *gltC* mutations may be of the operator-constitutive type (14). That the *gltC* mutants carry mutations in a regulatory rather than in the structural gene of glutamate permease is also supported by the finding that the affinity of the permease of these strains for glutamate is the same as in the wild-type parent. One should be able to find structural gene mutants among the apparent Glut$^-$ revertants of any Glut$^+$ mutant. If indeed *gltC* is the operator gene, then the mutations in the structural gene of glutamate permease should be closely linked to the *gltC* locus.

Cultures of *E. coli* K-12 CS7 were treated with nitrosoguanidine. Mutants that lost the ability to grow on glutamate, but still grew normally on glucose and succinate, were isolated from the mutagenized cultures after two cycles of penicillin treatment. The mutants were tested for the activities of aspartase, glutamate dehydrogenase, and glutamate-oxalacetate transaminase, for the rate of ^{14}C-L-glutamate uptake, for the affinity of the glutamate permease for glutamate, and for the rate of glutamate exit from preloaded cells. Several mutants of independent origin were found to have an altered glutamate permease, as manifested by a decrease in its affinity for glutamate and by the lower rate of glutamate uptake. One such mutant, CS7/50, is described in Table 6. One can see that the activity of the glutamate permease in this mutant is about one-

TABLE 6. *Activities of aspartase, glutamate dehydrogenase (GDH), glutamate-oxalacetate transaminase (GOT), and glutamate permease in the Glut⁻ mutant CS7/50 and in the Glut⁺ parent strain*

Strain	Growth temp	Entry				Exit, half-life	GDH[c]	GOT[c]	Aspartase[d]
		Glutamate incorporation into proteins[a]		Accumulation of glutamate[b]					
		V_{max}	K_m	MCC	K_m				
	C		M		M	min			
CS7	30	0.33	5×10^{-6}	16.5	5×10^{-6}	21	25.0	38.0	2.8
CS7	42	0.32	5×10^{-6}	13.5	5×10^{-6}	8	25.0	39.0	3.0
CS7/50	30	0.11	1×10^{-4}	5.5	1×10^{-4}	25	26.0	40.0	2.7
CS7/50	42	0.12	1×10^{-4}	5.0	1×10^{-4}	8	25.0	40.0	3.2

[a] The rate of glutamate uptake was expressed in micromoles of ^{14}C-L-glutamate per milligram (dry weight) of bacteria per hour.

[b] Maximal glutamate-concentrating capacity (MCC) was expressed in micromoles of ^{14}C-L-glutamate per milliliter of intracellular water.

[c] GDH and GOT were determined on extracts prepared from cultures grown in a glucose-minimal medium enriched as required.

[d] Aspartase activity was determined on extracts prepared from cultures grown in a succinate-minimal medium enriched as required.

third of that of the parent strain and the K_m for glutamate of the mutant permease is 20-fold higher than that of the parent CS7 strain. The altered affinity of the permease suggests that the mutation in CS7/50 occurred in the structural gene of the glutamate permease.

Mapping of the CS7/50 mutation by transduction. The donor strains were prototroph (Met⁺) derivatives of CS101, CS2^{TC}, and CS7. The recipient was CS7/50. Glut⁺ and Met⁺ transductants were selected. The *met* locus served as an outside marker for comparison of transduction frequencies. If *gltC* and *gltS* were closely linked genes, CS7 as the donor should give Glut⁺ transductants at a much higher frequency than would CS101 or CS2^{TC}. On the other hand, if there were no linkage between the two genes, all three of the transductions should give similar results. The results presented in Table 7 show that *gltC* and *gltS* are very closely linked.

Further mapping of *gltC* and *gltS* by an unselected-marker frequency analysis by transduction, involving known adjacent markers, was performed. CS7 served as the donor, and P678, GR103, AB1450, and KL141 were the recipients. The results shown in Table 8 are in agreement with those of our previous mapping experiments (Table 7; *see also* reference 17). On the basis of the known linkage of the *tna*, *pyrE*, and *ilv* loci (6, 19) and the linkage of *tna*, *gltC*, *gltS*, and *pyrE* obtained here, we concluded that *gltC* and *gltS* were located between *pyrE* and *tna*, very close to the *tna* locus.

The order of *gltC* and *gltS* with reference to the *tna* locus was established by transduction between CS7/50 (*gltS*⁻, *gltC*^{c}) as the donor and GR103 (*tna*⁻) as the recipient. A two-point-cross experiment with CS7 (*gltS*⁺, *gltC*^{c}) as the donor and GR103 as the recipient was performed for comparison of the frequencies of Glut⁺ transductants. The results presented in Table 9 show that the order is *gltS*, *gltC*, *tna*, since this arrangement requires a double crossing-over in order to obtain Tna⁻ Glut⁺ transductants in experiment 1, whereas the alternative order, *gltC*, *gltS*, *tna*, would necessitate a quadruple crossing-over event, which is by far too rare to account for the high percentage of cotransduction observed between *gltC* and *tna* (72.2%). Another point of significance is the 40-fold higher frequency of Glut⁺ transductants obtained with strain CS7 as the donor, as compared with the cross in which CS7/50 served as the donor. The lower frequency observed in the latter case was obviously due to the forced crossing-over between the very close *gltS* and *gltC* loci.

DISCUSSION

Wild-type strains of *E. coli* K-12 are unable to grow on glutamate as the sole source of carbon. However, glutamate-utilizing mutants of this organism have been readily isolated, after treatment with different mutagenic agents (10, 17). Earlier studies from this laboratory showed that the transport of glutamate into the cell was the limiting factor in the utilization of this compound as an energy and carbon source for growth. Both wild-type and glutamate-utilizing *E. coli* strains were shown to be capable of actively transporting

TABLE 7. *Determination of linkage between gltC and gltS by transduction*

Donor strain	Recipient strain	A Frequency of transduction to Met+[a]	B Frequency of transduction to Glut+[a, b]	B/A
CS101	CS7/50	8×10^{-6} (450)	4×10^{-8} (40)	0.005
$CS2^{TC}$	CS7/50	1.1×10^{-5} (500)	6.6×10^{-8} (40)	0.006
CS7	CS7/50	1×10^{-5} (500)	4×10^{-6} (600)	0.400

[a] The frequency of transduction was determined by relating the number of transductants obtained to the number of infective particles in the transduction mixture. The numbers in parentheses indicate the total number of transductant colonies found.

[b] A number of Glut+ transductants were isolated and tested for glutamate uptake and permease affinity for glutamate. All of the transductants tested had a high affinity for glutamate and exhibited a high rate of uptake, similar to that of strain CS7.

TABLE 8. *Unselected-marker frequency analysis by transduction of the linkage between mtl, xyl, gltC, gltS, tna, and ilv*[a]

Donor strain	Recipient strain	Selected marker	No. of transductants	Unselected marker	No. of transductants	Linkage
						%
CS7	P678	*xyl*+	150	*gltC7*	0	<0.7
CS7	P678	*mtl*+	150	*gltC7*	0	<0.7
CS7	GR103	*gltC7*	140	*tna*+	100	71.4
CS7	AB1450	*gltC7*	100	*ilv*+	0	<1.0
CS7	KL141	PyrE+	175	*gltS*+	31	17.5

[a] All of the Glut+ transductants tested showed a high glutamate permease activity and a high permease affinity for glutamate.

TABLE 9. *Ordering of the gltC, gltS, and tna loci by transduction*

Expt. no.	Donor strain	Recipient strain	Frequency of transduction to Glut+[a]	No. of *tna*+/no. of transductants examined (cotransduction of *tna*+)	*gltC*- *tna* linkage
					%
1	CS7/50	GR103	4×10^{-7} (180)	130/180	72.2
2	CS7	GR103	1.6×10^{-5} (500)	100/140	71.4

[a] The frequency of transduction was determined by relating the number of transductants to the number of infective particles in the transduction mixture. The numbers in parentheses indicate the number of transductants counted. All of the Glut+ transductants tested showed a high glutamate permease activity and a high permease affinity for glutamate.

the amino acid across the cell membrane. However, the rate of uptake and the capacity for accumulating glutamate were several-fold higher in the mutants as compared with wild-type strains which were unable to grow on glutamate (9, 10, 17).

Interrupted-mating experiments performed on four glutamate-utilizing mutants independently isolated from *E. coli* K-12 CS101 and one mutant of the *E. coli* K-12 Hayes strain showed that in all these cases the mutation mapped near the *mtl* locus (17). The wild-type allele of Hfr CS101 (Glut−) was also mapped near the mannitol gene.

Although these studies did not provide any direct evidence on the nature of the glutamate permeation gene affected by these mutations, we tentatively assumed it to be a control gene rather than the structural gene of glutamate permease. Our assumption was based on the observation that the affinity of the permease for glutamate in all of the mutants examined was practically the same as in the wild-type parent strains.

On this assumption, the low level of permease activity in the wild-type strains would be due to partial repression of glutamate permease synthesis. Mutations in the control gene (designated as *gltC*) result in derepression of glutamate permease synthesis and thus enable the cell to take up glutamate at a rate sufficient to support growth.

If this interpretation is correct, one should be

able to find mutations in the repressor-forming gene that lead to temperature-sensitive regulation of glutamate permease synthesis. Mutants producing a thermolabile repressor, or in which the synthesis of active repressor is prevented at high temperatures (18), should be able to grow on glutamate only at high temperatures. One would also expect to find Glut$^-$ mutants in which the mutation occurred in the structural gene of glutamate permease. In at least some of these mutants, a change in the affinity of the permease for glutamate would be expected.

The two types of mutants predicted by the repression hypothesis were indeed found, as described in the Results section (Tables 2 and 6, and Fig. 1). Three temperature-sensitive glutamate-utilizing mutants were independently isolated from the wild-type CS101 strain. As predicted, these mutants grew readily on glutamate at 42 C but could not utilize glutamate for growth at 30 C. That the mutation indeed affected glutamate uptake was clearly demonstrated by the fact that cultures of the temperature-sensitive (CS2^{TC}) mutant grown at 42 C exhibited a high rate of glutamate uptake and a high capacity for glutamate accumulation, similar to those of the temperature-insensitive Glut$^+$ mutants discussed above. However, when grown at 30 C, CS2^{TC} showed low glutamate permease activity similar to that of the wild-type parent, which does not grow on glutamate at all. It is significant that the affinity of the CS2^{TC} permease for glutamate remained the same as in the parent strain, and was not affected by the growth temperature. The rates of glutamate exit from preloaded cells were the same in the temperature-sensitive mutants as in the wild type and in the temperature-insensitive mutants (Table 2).

Experiments in which CS2^{TC} cells grown at 30 C were exposed to brief heat treatment in the absence of growth, and then were allowed to resume growth at 30 C and were tested periodically for glutamate permease activity (Fig. 4), showed that the mutation resulted in the formation of a thermolabile repressor, rather than in a temperature-sensitive synthesis of repressor (18). The possibility that the heating inactivated a permease inhibitor rather than a repressor is extremely unlikely in view of the gradual increase in the specific activity of the permease following the heat treatment. If an inhibitor of permease activity were removed, one would expect an immediate relief from inhibition, not followed by any further increase in permease activity during subsequent growth at 30 C. We therefore conclude that the mutation occurred in the repressor-forming gene (designated as *gltR*), resulting in the synthesis of a thermolabile repressor and thus leading to temperature-sensitive regulation of glutamate permease synthesis.

Genetic analysis of the *gltR*^{TL} mutation unequivocally places the *gltR* gene close to the *metA* locus, that is, at a distance of about 6 to 7 min [in time of chromosome transfer according to Taylor and Trotter (19)] from the *gltC* locus (*see* Fig. 3).

In view of these findings, it seems reasonable to assume that the control gene *gltC* may be the operator of the glutamate permease operon. This assumption is supported by the isolation of Glut$^-$ mutants with a lesion in the structural gene of glutamate permease, in which both the transport activity and the affinity for glutamate are greatly reduced (*see* Table 6). Mapping experiments revealed that the structural gene, *gltS*, is very closely linked to the *gltC* locus (*see* Tables 7 and 9). *Cis-trans* tests are now in progress to establish the dominance relationships among the different genes of this system. Preliminary results indicate that the *gltC*^{c} allele, when in a *cis* position to *gltS*$^+$, is dominant over *gltC*$^+$, as indeed would be expected if *gltC* were the operator gene.

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