Use of Homocysteic Acid for Selecting Mutants at the *gltS* Locus of *Escherichia coli* K12

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L-Homocysteic acid is toxic to *Escherichia coli* K12. Sensitivity to this compound is higher in cells which can utilize glutamate as sole carbon source via the Na^+-dependent glutamate transport system. Such cells become resistant by mutation at the *gltS* locus. Sensitivity of both wild-type and glutamate-utilizing strains is greater if cells are growing on acetate as compared with glucose as major carbon source.

INTRODUCTION

A genetic marker which can be selected for both active and inactive states can be very useful for genetic manipulations such as isolating deletions, making Hfr strains and isolating specialized transducing bacteriophages in nearby genes. The *gltS* gene of *Escherichia coli* K12 at minute 82 (Bachmann & Low, 1980) can be selected for the active state because wild-type cells are not capable of growth on glutamate as sole source of carbon, but mutants at *gltS* can grow on glutamate (Halpern & Lupo, 1965). However, selection for loss of ability to transport glutamate remains a problem. Halpern & Umbarger (1961) reported that α-methylglutamate was a specific inhibitor of this transport system in *E. coli* W and that growth of glutamate-utilizing bacteria was slowed by this compound. Subsequently, Miner & Frank (1974) used it to isolate mutants of *E. coli* B incapable of growth on glutamate. Quite high concentrations (200 mM) were required for mutant isolation. It seemed desirable to find an analogue effective at lower concentrations. This paper reports that L-homocysteic acid is toxic to glutamate-utilizing *E. coli* K12 at concentrations as low as 1 mM, and that resistance to homocysteic acid is acquired by mutation at the *gltS* locus.

METHODS

Bacterial strains and media. *Escherichia coli* strain AT2243 (*metB1 pyrE41*) was obtained from H. L. Kornberg, Department of Biochemistry, University of Cambridge, Cambridge, UK. Strain CS7 (*metB1 gltC14*, Marcus & Halpern, 1967) was obtained from Y. S. Halpern, Institute of Microbiology, the Hebrew University, Hadassah Medical School, Jerusalem, Israel, and prototroph 594 was obtained from R. W. Hendrix, Department of Biological Science, University of Pittsburgh, Pittsburgh, Pennsylvania 15213, USA. Strain RE74 (*metB1 pyrE41 gltC14 uhp-40 tna-6*) has been previously described (Essenberg & Kornberg, 1977). LB is the tryptone/yeast extract medium of Luria & Burrous (1957). Minimal medium A (Miller, 1972) was supplemented with thiamin at 1 μg ml⁻¹, amino acids and bases, as appropriate, at 100 μg ml⁻¹, and glutamate, acetate or succinate at 20 mM, glucose 6-phosphate at 5 mM or other carbon sources at 10 mM. For plates, media were solidified with 2% (w/v) Bacto-agar (Difco).

Materials. L-[U-¹⁴C]Glutamic acid was supplied by ICN Chemical and Nuclear Division; D- and L-homocysteic acid, L-methionine sulphone, L-methionine-DL-sulphoximine, L-methionine-*S*-methylsulphonium bromide and tetracycline were obtained from Sigma. DL-Homocysteic acid was obtained from Calbiochem and Sigma. It was neutralized with NaOH before use in media.

Growth curves. Cells were grown at 37 °C in a shaking water bath in 50 ml sidearm flasks in 5–10 ml of the appropriate medium. Cultures were inoculated from an overnight culture grown on minimal medium supplemented with 0·2% Casamino acids and were resuspended in minimal medium without supplements before

inoculation. Growth was followed by measuring turbidity with a Klett-Summerson photoelectric colorimeter, using a no. 42 (blue) or no. 66 (red) filter.

Glutamate transport assay. Cells were grown overnight on succinate/minimal medium. They were washed twice and resuspended at room temperature in the same volume of medium A made without citrate, containing 15 mM-NaCl (Halpern & Evan-Shoshan, 1967). To 1·0 ml samples, L-[U-^{14}C]glutamate (5 mCi mmol^{-1}, 185 MBq mmol^{-1}) was added to 0·1 mM. Samples of 0·2 ml were removed at intervals and filtered through 0·45 μm pore size membrane filters (Gelman Instruments) which were washed with 5 ml of the same medium without glutamate. The filters were immediately placed in 95% ethanol/toluene (20:80, v/v) containing 0·4% 2,5-diphenyloxazole and 0·02% *p-bis*(*O*-methylstyryl)benzene for liquid scintillation counting.

Insertion of Tn10 in gltS. Transposon insertion followed the procedure of Kleckner *et al.* (1977). Strain CS7 was grown overnight in LB and resuspended in an equal volume of 10 mM-MgSO$_4$. A 0·5 ml volume was infected at 37 °C with λ561 (b221 cI857 cI171::Tn10 Oam29 P80, obtained from Nancy Kleckner, Harvard University, Cambridge, Massachusetts 02138, USA) at a multiplicity of infection of 7. After 20 min, 5 ml LB was added and the cells shaken at 37 °C. After 1–2 h, tetracycline was added to 10 μg ml^{-1}. After the cells grew up (1–2 d), they were plated on succinate/minimal medium containing 10 μg tetracycline ml^{-1}. A small amount of solid DL-homocysteic acid was put on the plate. After 3–4 d there was a clear zone around the homocysteic acid with revertant colonies in it. Several of these were picked and tested for growth on glutamate. All were incapable of growth on glutamate and were further tested for cotransduction of *gltS* and Tn10 using bacteriophage P1 grown on strain CS7. Mutant RE175 showed 100% cotransduction.

RESULTS AND DISCUSSION

Identification of toxic glutamate analogues

L-Methionine sulphone, L-methionine-DL-sulphoximine, L-methionine *S*-methylsulphonium bromide and DL-homocysteic acid were tested for inhibition against strain CS7, which can grow on glutamate, and strain 594, which cannot, after plating on minimal medium with fructose, glycerol or succinate as carbon source by placing a small amount of the solid on the agar which had been spread with bacteria. After 2 d growth at 37 °C, zones of inhibition were apparent around methionine sulphone, methionine sulphoximine, and homocysteic acid, but not methionine *S*-methylsulphonium bromide. Zones of inhibition were larger for the glutamate-utilizing strain CS7 than for wild-type 594, and tended to be larger on glycerol than fructose, with those on succinate intermediate. Revertant colonies appeared in the zones of inhibition for all three inhibitory compounds for strain CS7, but only around methionine sulphoximine for strain 594. Those appearing around methionine sulphone were small, while those appearing around methionine sulphoximine and homocysteic acid were large. Several of the revertants from strain CS7 were picked and tested for growth on glutamate. Only those from homocysteic acid failed to grow on glutamate as carbon source, but all those from homocysteic acid were unable to grow on glutamate.

One of these glutamate-negative, homocysteic acid-resistant strains was selected for further tests. Its growth in liquid medium was compared with that of the parent CS7 on glutamate and fructose. The mutant grew very slowly on glutamate (doubling time 620 min, compared to 115 min for strain CS7) while growth on fructose was normal. Transport of glutamate by washed cell suspensions was also deficient in the mutant as compared to strain CS7 [0·057 ± 0·013 and 0·164 ± 0·008 nmol min^{-1} (mg dry weight)$^{-1}$, respectively].

Homocysteic acid sensitivity in strains able or unable to grow on glutamate

An isogenic set of strains able or unable to grow on glutamate was made by mutagenizing strain AT2243 with ethylmethane sulphonate (Miller, 1972) and selecting for growth on glutamate. These strains were then screened for sensitivity to 20 mM-DL-homocysteic acid on glucose as carbon source. The majority of strains capable of utilizing glutamate were sensitive to homocysteic acid under these conditions, but 28 out of 101 isolates tested were resistant, as was AT2243. Strain RE241 was selected as representative of the sensitive class. Since activation of the *gltS* system to enable growth on glutamate as carbon source involves alteration of control rather than of the carrier protein (Marcus & Halpern, 1969), it is not clear how some strains could be sensitive and others not. The most likely explanation is that one of the other glutamate transport systems (Marcus & Halpern, 1967; Schellenberg & Furlong, 1977) has been activated, and that homocysteic acid is not a substrate of this other system.
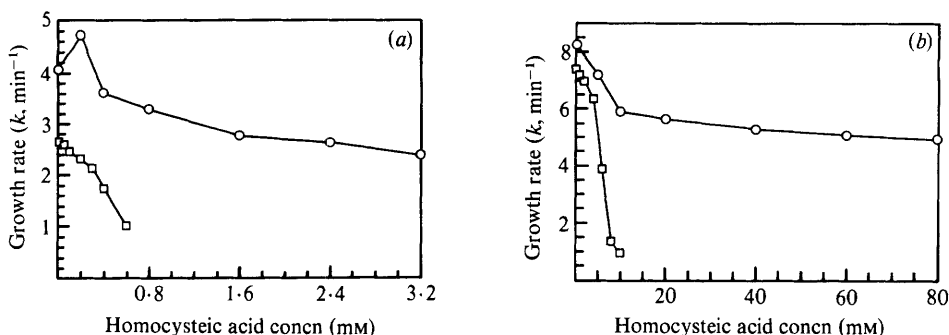
Fig. 1. Effect of DL-homocysteic acid on growth of *E. coli* strains AT2243 and RE241 on acetate. Strains AT2243 (○) and RE241 (□) were inoculated into medium A containing (*a*) 20 mM-sodium acetate or (*b*) 10 mM-glucose as carbon source. At a Klett value of about 50, neutralized homocysteic acid was added to the indicated concentration. The growth rates are the slopes of the linear portions of plots of ln(Klett reading) vs time.

The response of strains AT2243 and RE241 to various concentrations of DL-homocysteic acid was tested by measuring growth rates in liquid medium with acetate or glucose as carbon source. As shown in Fig. 1, on both carbon sources growth of strain RE241 is affected more by the inhibitor, and lower concentrations of the inhibitor cause an effect. Much lower inhibitor concentrations were effective if acetate was the carbon source: for strain RE241 the concentration causing the growth rate to fall to half its original value was 0·5 mM for acetate and 5 mM for glucose. This difference suggests that the transport system is under control by catabolite repression. At slightly higher concentrations than those used for the experiments shown in Fig. 1, e.g. 1 mM for acetate or 20 mM for glucose, growth was not detectable after 24 h for strain RE241. For parent AT2243, there appears to be a maximum inhibition such that even very high concentrations of DL-homocysteic acid cause only about 50% inhibition of growth rate. When D- and L-homocysteic acids were tested separately, only the L isomer was inhibitory.

If cells were spread on plates containing various concentrations of DL-homocysteic acid, the response was somewhat different from that in liquid media. Parent AT2243 failed to grow on either carbon source if the inhibitor concentration was high enough (80 mM for glucose, 40 mM for acetate). However, strain RE241 failed to grow at inhibitor concentrations similar to those that prevented growth in liquid medium. Also, the plating efficiency decreased, rather than the colony size, as the inhibitor concentration increased, suggesting that the cells within the population had different sensitivities to the inhibitor. That this was a physiological rather than genetic phenomenon is suggested by the fact that the colonies appearing at higher inhibitor levels showed the same response to the inhibitor as the original strain when retested.

Location of a homocysteic acid-resistance mutation at gltS

Several experiments have been done in which a culture of a glutamate-utilizing, homocysteic acid-sensitive strain was mutagenized and plated on media containing a concentration of homocysteic acid sufficient to prevent growth of the parent strain. Colonies grew up and in each case all tested failed to grow on glutamate.

To demonstrate that this mutation was in the locus responsible for the Na⁺-dependent glutamate transport system (*gltS*) the transposon in strain RE175 was mapped. As indicated above, transduction of strain RE175 by P1 bacteriophage grown on strain CS7 gave glutamate-utilizing strains all of which had lost tetracycline resistance. When bacteriophage P1 grown on strain RE175 were used to transduce strain RE74 to tetracycline resistance, the results in Table 1 were obtained. The cotransduction frequencies of the transposon to *pyrE* and *uhp* are consistent with those obtained earlier (Essenberg & Kornberg, 1977) and the lack of linkage implies the order *pyrE gltS*::Tn*10 uhp*, as expected.

L-Homocysteic acid is toxic to cells possessing derepressed activities of the Na⁺-dependent glutamate transport system coded by the *gltS* locus (Marcus & Halpern, 1967, 1969;

Table 1. *Transduction of E. coli strain RE74 by bacteriophage P1 grown on RE175*

Transduction was performed as described by Miller (1972). Tetracycline-resistant transductants were tested by replica plating for ability to grow on glucose 6-phosphate and glutamate and to grow in the absence of uracil. All were unable to grow on glutamate. The value of χ^2 estimates the effect of inheritance of one gene on that of a different one (Essenberg & Kornberg, 1977).

Unselected marker:		No. of
pyrE	*uhp*	recombinants
+	+	48
+	−	16
−	+	73
−	−	26

Total no. of tetracycline-resistant transductants: 163
Cotransduction (%): Tn*10–pyrE*   39
Tn*10–uhp*   74

χ^2 0·032 (0·9 > P > 0·8)

Schellenberg & Furlong, 1977) at lower concentrations than repressed cells. Derepressed cells gain resistance to this analogue by losing or repressing the *gltS* transport system and the ability to use glutamate as sole carbon source. Homocysteic acid is toxic to glutamate-utilizing cells at lower concentrations (10 mM on glucose, 2 mM on acetate) than is α-methylglutamate, which was used at 200 mM to find mutants (Miner & Frank, 1974). Thus, one can select mutants at *gltS* by growth on glutamate as carbon source and can select for loss of this ability by resistance to homocysteic acid.

REFERENCES

BACHMANN, B. J. & LOW, K. B. (1980). Linkage map of *Escherichia coli* K-12, edition 6. *Microbiological Reviews* **44**, 1–56.

ESSENBERG, R. C. & KORNBERG, H. L. (1977). Location of the gene specifying hexose phosphate transport (*uhp*) on the chromosome of *Escherichia coli*. *Journal of General Microbiology* **99**, 157–169.

HALPERN, Y. S. & EVEN-SHOSHAN, A. (1967). Properties of the glutamate transport system in *Escherichia coli*. *Journal of Bacteriology* **93**, 1009–1016.

HALPERN, Y. S. & LUPO, M. (1965). Glutamate transport in wild-type and mutant strains of *Escherichia coli*. *Journal of Bacteriology* **90**, 1288–1295.

HALPERN, Y. S. & UMBARGER, H. E. (1961). Utilization of L-glutamic and 2-oxoglutaric acid as sole sources of carbon by *Escherichia coli*. *Journal of General Microbiology* **26**, 175–183.

KLECKNER, N., ROTH, J. & BOTSTEIN, D. (1977). Genetic engineering *in vivo* using translocatable drug-resistance elements. New methods in bacterial genetics. *Journal of Molecular Biology* **116**, 125–159.

LURIA, S. E. & BURROUS, J. W. (1957). Hybridization between *Escherichia coli* and *Shigella*. *Journal of Bacteriology* **97**, 461–476.

MARCUS, M. & HALPERN, Y. S. (1967). Genetic analysis of glutamate transport and glutamate decarboxylase in *Escherichia coli*. *Journal of Bacteriology* **93**, 1409–1415.

MARCUS, M. & HALPERN, Y. S. (1969). Genetic analysis of the glutamate permease in *Escherichia coli* K12. *Journal of Bacteriology* **97**, 1118–1128.

MILLER, J. H. (1972). *Experiments in Molecular Genetics*. New York: Cold Spring Harbor Laboratory.

MINER, K. M. & FRANK, L. (1974). Sodium-stimulated glutamate transport in osmotically shocked cells and membrane vesicles of *Escherichia coli*. *Journal of Bacteriology* **117**, 1093–1098.

SCHELLENBERG, G. D. & FURLONG, C. E. (1977). Resolution of the multiplicity of the glutamate and aspartate transport systems of *Escherichia coli*. *Journal of Biological Chemistry* **252**, 9055–9064.