glnA Mutations Conferring Resistance to Methylammonium in *Escherichia coli* K12

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(*Received 22 October 1986; revised 12 January 1987*)

Cells of *Escherichia coli* K12 were sensitive to 100 mM-methylammonium when cultured under nitrogen limitation, and resistant when grown with an excess of either NH_4Cl or glutamine. Glutamine synthetase activity was required for expression of the methylammonium-sensitive phenotype. Mutants were isolated which were resistant to 100 mM-methylammonium, even when grown under nitrogen limitation. P1 bacteriophage transduction and F′ complementation analysis revealed that the resistance-conferring mutations mapped either inside the *glnA* structural gene and/or elsewhere in the *E. coli* chromosome. Glutamine synthetase was purified from the wild-type and from some of the mutant strains. Strains carrying *glnA*-linked mutations that were solely responsible for the methylammonium-resistant phenotype yielded an altered enzyme, which was less active biosynthetically with either ammonium or methylammonium as substrate. Sensitivity to methylammonium appeared to be due to synthesis of γ-glutamylmethylamide by glutamine synthetase, which was synthesized poorly, if at all, by mutants carrying an altered glutamine synthetase enzyme.

INTRODUCTION

The ammonium ion analogue methylammonium has been used to determine the presence of ammonium transport systems in several prokaryotic and eukaryotic micro-organisms. In most cases, methylammonium enters the cells via specific ammonium transport systems (see Brown, 1980, and Kleiner, 1981, 1985, for reviews). In *Escherichia coli* and other bacteria, this system has a high affinity for ammonium (Stevenson & Silver, 1977; Kleiner, 1985; Jayakumar *et al.*, 1985). When the extracellular ammonium concentration is low, cells can generate intracellular concentrations which are 100-fold higher or more; at high external ammonium concentrations, cells grow efficiently without an active high affinity ammonium transport system, since the amount of ammonia that enters the cell by simple diffusion is sufficient for cellular growth (Kleiner, 1985).

In *E. coli* and other bacteria, synthesis of the ammonium carrier is under genetic control (Servín-González & Bastarrachea, 1984; Kleiner, 1985; Jayakumar *et al.*, 1986). The system that regulates ammonium transport in enterobacteria, known as Ntr, also regulates expression of several other systems involved in the transport and utilization of nitrogenous compounds such as arginine, proline, etc., and regulates synthesis of glutamine synthetase (see Magasanik, 1982, for a review).

Methylammonium can be used as a substrate by glutamine synthetase, even in strains that cannot use it as nitrogen source. In the presence of methylammonium, glutamate and ATP,

Abbreviations: GMAD, γ-glutamylmethylamide; MA^R, methylammonium resistant(ance).

glutamine synthetase catalyses synthesis of the glutamine analogue γ-glutamylmethylamide (GMAD) (Barnes & Zimniak, 1981; Barnes *et al.*, 1983; Kleiner, 1985).

In this paper we describe the methylammonium-sensitive (MA^S) phenotype of *E. coli*, and the role glutamine synthetase plays in it. This was possible through the isolation and characterization of MA^R mutants.

METHODS

Bacterial strains. All strains used were *Escherichia coli* K12 derivatives (Table 1).

Culture conditions. Cells were cultured at 37 °C, using NN minimal medium (Covarrubias *et al.*, 1980), with 0·2% (w/v) glucose as carbon source. All compounds added, i.e. carbohydrates, amino acids, vitamins, nitrogen sources and methylammonium, were sterilized by filtration. Liquid cultures were grown aerobically in a rotary shaker at 250 r.p.m., and growth was monitored in a Klett–Summerson colorimeter with a red filter (no. 66). For growth on minimal medium plates, 1·5% (w/v) Bacto-Agar (Difco) was added to NN medium. For mutagenesis, transductions and matings, cells were grown in LB broth (Miller, 1972).

Mutagenesis. MX614 cells were mutagenized with ethylmethane sulphonate according to Miller (1972), and plated directly on NN minimal medium plates with 0·2% (w/v) glucose as a carbon source, 0·2% (w/v) arginine as a nitrogen source, and 100 mM-methylammonium. Plates were incubated for 48 h at 37 °C; colonies from each plate were transferred with sterile wooden toothpicks to plates with either glutamine (1 mg ml^{-1}) or NH_4Cl (15 mM) as nitrogen source, and after overnight incubation growth patches were assayed for glutamine synthetase activity by a qualitative colony test (Pahel *et al.*, 1978). In this way, ten different mutants were selected from four different pools.

Genetic procedures. P1*virA* bacteriophage was used for transductions. Preparation of phage lysates, and protocols for transductions and matings, were according to Miller (1972).

Enzyme assays. Glutamine synthetase [L-glutamate:ammonia ligase (ADP-forming); EC 6.3.1.2] was assayed by the γ-glutamyltransferase or by the biosynthetic, γ-glutamylhydroxamate-forming procedures, as described by Bender *et al.* (1977), with minor modifications (Covarrubias *et al.*, 1980); the average adenylylation state was determined as described by Bender *et al.* (1977). To measure biosynthetic activity of purified glutamine synthetase preparations, the phosphate-release method described by Shapiro & Stadtman (1970) was used. Protein was measured by the Lowry method.

Purification of glutamine synthetase. The polyethylene glycol precipitation procedure described by Streicher & Tyler (1980) was used, but with the final Sepharose 6B column step omitted.

Reagents. Amino acids, vitamins, methylamine.HCl and reagents used for assay or purification of glutamine synthetase were purchased from Sigma. All other reagents used were of analytical grade.

Amino acid analysis. Extracts for amino acid analysis were prepared as described in the legend to Table 7. The filtrates containing the free amino acids were lyophilized and the samples resuspended in lithium hydroxide/citric acid buffer (pH 2·88). Amino acids were separated with an Aminco amino acid analyser and quantified in an Aminco ratio fluorometer after coupling with *o*-phthaldialdehyde. Purified glutamine synthetase from the wild-type strain was used to obtain GMAD from methylammonium and glutamate in a standard biosynthetic reaction mixture. The amount of GMAD synthesized was calculated from the amount of phosphate liberated from ATP during the reaction. Both GMAD and glutamine (2·5 μmol ml^{-1}) were added to a Sigma amino acid standard solution. Using a Perkin-Elmer LC1-100 laboratory computing integrator, the GMAD response factor was calculated by the system from a calibration run. A full separation of glutamate, glutamine and GMAD was obtained with approximate retention times of 34, 36 and 41 min, respectively.

RESULTS

Effect of methylammonium on growth

Growth inhibition by methylammonium in *E. coli* was nitrogen-dependent. When cells were provided with a high (15 mM) NH_4Cl concentration as nitrogen source, growth was not affected by the presence of methylammonium in the medium. However, when the NH_4Cl concentration was low (0·5 mM) or when arginine, proline or glutamate were used as nitrogen source, growth was completely inhibited by the presence of 100 mM-methylammonium. All nitrogen sources under which methylammonium inhibited growth were limiting (i.e. high glutamine synthetase activity was required for growth under such conditions). Even though glutamine is nitrogen-limiting for growth in *E. coli*, 1 mg glutamine ml^{-1} allowed growth in the presence of methylammonium; however, with lower concentrations of glutamine (75 μg ml^{-1}), growth was inhibited by methylammonium.

Table 1. *E. coli strains*

Strain	Genotype	Source or derivation
MX614	Δ(*pro–lac*) *galE ilv-680 thi-1*	Bastarrachea *et al.* (1980)
MX734	Δ(*pro–lac*) *galE ilv-680 glnA71*::Tn*5 thi-1*	Osorio *et al.* (1984)
AB1206	*proA2 tfr-3 lacY1 supE44 galK2 his-4 rpsL8* ΔDE2(*ilvE–argH*) *thi-1*/F′14	B. Bachmann, *E. coli* Genetic Stock Center, Yale University, Conn., USA
MX821	*proA2 tfr-3 lacY1 supE44 galK2 his-4 rpsL8* ΔDE2(*ilvE–argH*) *thi-1*/F′14 *glnA71*::Tn*5*	Osorio *et al.* (1984)
MX881	Δ(*pro–lac*) *galE recA56 ilv-680 thi-1*	Our laboratories
MX913	Δ(*pro–lac*) *galE recA56 ilv-680 glnG74*::Tn*5 thi-1*	Our laboratories
MX944	Δ(*pro–lac*) Δ(*gal–attλ–bio*) *recA56 ilv-680 glnA71*::Tn*5 thi-1*	Our laboratories
MX961	Δ(*pro–lac*) *galE recA56 ilv-680 glnL82*::Tn*5 thi-1*	Our laboratories

Effect of methylammonium on glutamine synthetase activity and adenylylation

Glutamine synthetase of *Escherichia coli* can utilize methylammonium as a substrate both *in vivo* and *in vitro*, leading to the formation of GMAD (Barnes *et al.*, 1983). We measured glutamine synthetase activity and adenylylation to determine whether addition of methylammonium, under conditions where growth is inhibited, had any apparent effect on glutamine synthetase. A 2 h exposure to 100 mM-methylammonium of a culture growing exponentially with arginine as nitrogen source had no apparent effect on either glutamine synthetase levels or adenylylation (data not shown). Apparently, growth inhibition by methylammonium could not be attributed to inhibition of glutamine synthetase, or to interference with the glutamine synthetase adenylylation system.

Isolation and characterization of MA^R mutants

Mutants which were no longer inhibited by methylammonium under nitrogen limitation were isolated. Cells of strain MX614 were treated with ethylmethane sulphonate, and plated on medium containing arginine as nitrogen source and 100 mM-methylammonium. Only 10 of the many independent MA^R mutants were further characterized. As can be seen in Table 2, most of the mutants had altered glutamine synthetase activity. P1*virA* phage was propagated in each of the mutant strains, and the lysates were used to transduce strain MX734 (Table 1) selecting for glutamine prototrophs. When strains MX1210, MX1211, MX1212, MX1213 and MX1214 were used as donors, more than 98% of transductants in each case were resistant to 100 mM-methylammonium (Table 3), indicating that the resistance-conferring mutations carried by these strains lay within the *glnA* gene, or very close to it. We therefore tentatively termed the alleles carried by the above strains *gln93*, *gln94*, *gln95*, *gln96* and *gln97*, respectively. Transductions were also done with lysates from strains carrying the *glnA*-linked MA^R-conferring mutations as donors and strain MX821 as recipient. This strain is a *glnA71*::Tn*5* derivative of the primary F′ strain AB1206. MX821 is therefore haploid for the entire *glnALG* operon carried on its F′14 merogenote (Osorio *et al.*, 1984). The results were similar to those obtained with MX734 as a recipient (Table 3). Glutamine synthetase activities of the transductants in either the MX614 or the AB1206 background were the same (Table 4); i.e. the MA^R phenotype was always accompanied by the altered glutamine synthetase activity which was characteristic of the donor strain. The F′14 derivatives carrying the *glnA*-linked mutations conferring methylammonium resistance could now be utilized for genetic complementation experiments (see below). Glutamine synthetase activity from strains carrying the *gln* mutations were also assayed by the biosynthetic γ-glutamylhydroxamate-forming procedure (see Methods). This activity was strongly diminished in the mutants as compared to the wild-type strain (Table 4). These data suggest that methylammonium resistance could be due to the presence of an altered glutamine synthetase enzyme, which was biosynthetically less active.

When P1 lysates from strains MX1215, MX1216, MX1217, MX1218 and MX1219 were used to transduce MX734, all the resulting Gln$^+$ transductants that were tested were MA^S (Table 3). These results could have been taken as an indication that methylammonium resistance in these strains was due to mutations unlinked to *glnA*. However, since such strains showed altered

Table 2. *Glutamine synthetase specific activities of wild-type and MA^R strains*

Cells were grown aerobically, until the cultures reached 100 ± 20 Klett units, and then assayed for γ-glutamyltransferase activity as described in Methods. Results are the means of two experiments. GN, glucose–NH_4Cl (15 mM)–NN medium; GGln, glucose–glutamine (1 mg ml^{-1})–NN medium; GArg, glucose–arginine (0·2%)–NN medium.

	Glutamine synthetase activity [nmol min^{-1} (mg protein)$^{-1}$]		
	Medium:		
Strain	GN	GGln	GArg
MX614	250	1770	2260
MX1210	760	890	1200
MX1211	140	100	60
MX1212	200	1090	1250
MX1213	120	200	280
MX1214	480	3060	2680
MX1215	760	1140	2080
MX1216	280	1930	2340
MX1217	830	1190	1700
MX1218	250	1960	1650
MX1219	960	1470	1990

Table 3. *Mapping of MA^R-conferring mutations by P1 transduction*

Transductants were selected on glucose–NH_4Cl (15 mM)–NN minimal medium plates. After growth, individual colonies were transferred as patches onto glucose–arginine (0·2%)–NN medium plates with and without 100 mM-methylammonium. MA^R transductants grew on plates of either medium after 24 h incubation, while MA^S transductants were unable to grow on plates with methylammonium, even after 48 h incubation.

P1 donor	Recipient	No. of Gln$^+$Kan^S transductants that inherited the MA^R phenotype
MX614 (*glnA*$^+$)		0/44
MX1210 (*gln93*)		24/25
MX1211 (*gln94*)		49/50
MX1212 (*gln95*)		21/21
MX1213 (*gln96*)		42/42
MX1214 (*gln97*)	MX734(*glnA71*::Tn*5*)	50/50
MX1215		0/16
MX1216		0/22
MX1217		0/21
MX1218		0/26
MX1219		0/50
MX1210 (*gln93*)		50/50
MX1211 (*gln94*)		50/50
MX1212 (*gln95*)	MX821(*glnA71*::Tn*5*)	50/50
MX1213 (*gln96*)		50/50
MX1214 (*gln97*)		50/50

glutamine synthetase activity, it was possible that they harboured more than one mutation. To distinguish between these options, the *glnALG* region of the mutants was replaced by a wild-type *glnALG* region in the following way. P1*virA* phage propagated on strain MX734 (*glnA71*::Tn*5*) was used to transduce strains MX1215, MX1216, MX1217, MX1218 and MX1219 to glutamine auxotrophy (Kan^R selection). These transductants were then used as recipients of P1 phage grown on either MX614 or on each original parental mutant strain (MX1215 through MX1219), selecting in all cases for glutamine prototrophy. All strains except one (MX1215) yielded MA^S transductants with MX614 phage, and MA^R transductants when their original *glnA* chromosomal region was re-introduced (data not shown). Therefore, the MA^R phenotype of strains MX1216, MX1217, MX1218 and MX1219 seems to be due to more than one mutation, one of them being within the *glnALG* region. Only the MA^R phenotype of strain MX1215 appears to be associated with one or more mutations unlinked to *glnA*.

Table 4. *Glutamine synthetase specific activities of E. coli strains carrying glnA-linked, MA^R-conferring mutations, and of transductants derived from them*

Strains MX1220 to MX1224 are transductants obtained using MX734 as recipient of P1 lysates of strains MX1210 to MX1214. Strains MX1225 to MX1229 are transductants obtained with the same lysates, but with MX821 as recipient (see text for a detailed explanation). Cells were grown aerobically as described in Table 2, and assayed for γ-glutamyltransferase activity. Cells grown in GGln medium were also assayed for biosynthetic γ-glutamylhydroxamate-forming activity (results given in parentheses). Results are the means of three experiments. GN; GGln, see Table 2.

		Glutamine synthetase activity [nmol min^{-1} (mg protein)$^{-1}$]			Glutamine synthetase activity [nmol min^{-1} (mg protein)$^{-1}$]			Glutamine synthetase activity [nmol min^{-1} (mg protein)$^{-1}$]	
Allele	Strain	GN	GGln	Strain	GN	GGln	Strain	GN	GGln
glnALG$^+$	MX614	240	1960 (890)	–	–	–	AB1206	260	1670 (1200)
gln93	MX1210	520	1380 (120)	MX1220	610	1110	MX1225	440	1090 (190)
gln94	MX1211	140	140 (100)	MX1221	120	160	MX1226	130	160 (150)
gln95	MX1212	160	1110 (150)	MX1222	220	950	MX1227	210	1520 (200)
gln96	MX1213	90	170 (100)	MX1223	80	140	MX1228	180	470 (380)
gln97	MX1214	490	2480 (250)	MX1224	610	2150	MX1229	730	1840 (390)

Table 5. *Complementation analysis with wild-type F'14 and derivatives of strains carrying Tn5 insertions in the glnA, glnL and glnG genes*

The MA^R phenotype of exconjugants from each mating was scored as described in Table 3. One exconjugant from each cross was purified; cells were grown until the cultures reached 100 ± 20 Klett units, and then assayed for γ-glutamyltransferase activity. Representative results from several experiments are shown. MA, resistance (R) or sensitivity (S) to 100 mM-methylammonium. GN, GGln, see Table 2. NG, No growth.

	MX881(*glnALG*$^+$)			MX944(*glnA71*::Tn*5*)			MX961(*glnL82*::Tn*5*)			MX913(*glnG74*::Tn*5*)		
		Glutamine synthetase activity [nmol min^{-1} (mg protein)$^{-1}$]			Glutamine synthetase activity [nmol min^{-1} (mg protein)$^{-1}$]			Glutamine synthetase activity [nmol min^{-1} (mg protein)$^{-1}$]			Glutamine synthetase activity [nmol min^{-1} (mg protein)$^{-1}$]	
Episome	MA	GN	GGln	MA	GN	GGln	MA	GN	GGln	MA	GN	GGln
None	S	200	1590	NG	NG	< 10	NG	80	150	NG	30	60
F'14 *glnALG*$^+$	S	300	1080	S	180	2220	S	380	3770	S	390	3980
F'14 *gln93*	S	190	1440	R	780	1030	S	400	1080	S	370	2060
F'14 *gln94*	S	370	1410	R	160	260	S	430	1230	S	470	1980
F'14 *gln95*	S	210	1330	R	400	1300	S	240	2600	S	550	2280
F'14 *gln96*	S	270	1790	R	180	70	S	200	1770	S	260	1380
F'14 *gln97*	S	550	2550	R	1750	1370	S	1040	2220	S	620	2400

Complementation analysis of glnA-linked, MA^R-conferring mutations

Since the MA^R-conferring mutations carried by strains MX1210 to MX1214 appeared to be due to mutational events within the *glnALG* operon, we used F'14 derivatives carrying these alleles to complement a set of strains carrying chromosomal Tn*5* insertion mutations in either of the three operon genes. Matings were done using strains MX1225 through MX1229 (carrying alleles *gln93* to *gln97*, respectively) as donors. As a control donor we used strain AB1206, which transfers the wild-type F'14 episome. All recipients used carried the *recA56* allele, and were wild-type for the *glnALG* region (MX881), or carried Tn*5* insertions in either the *glnA* (MX944), *glnL* (MX961) or *glnG* (MX913) genes (see also Table 1). Ilv$^+$ His$^+$ exconjugants were selected, and tested for methylammonium resistance on plates containing arginine as sole nitrogen source. One exconjugant selected from each cross was purified and assayed for glutamine synthetase activity (Table 5). The MA^R-conferring mutations were all recessive to wild-type, since strain MX881 (*glnALG*$^+$) carrying any of the F'14 derivatives (*gln93* to *gln97*) retained its sensitivity to methylammonium, and had normally regulated glutamine synthetase levels. The F'14 derivatives were able to complement the *glnL*::Tn*5* and *glnG*::Tn*5* insertions, since the resulting merodiploids had normal glutamine synthetase activity and were MA^S. When the same F'14 derivatives were introduced into strain MX944 (carrying a *glnA*::Tn*5* insertion), altered glutamine synthetase activities, corresponding to the original mutant strains, were obtained; these merodiploids were MA^R, as compared to MX944 carrying the wild-type F'14 episome, which was MA^S and had normal glutamine synthetase activities. We conclude that the MA^R conferring mutations are *trans*-recessive and lie inside the *glnA* gene. They were, therefore, renamed *glnA93*, *glnA94*, *glnA95*, *glnA96* and *glnA97*.

Properties of purified glutamine synthetase from MA^R strains

To ascertain the structural nature of the *glnA*-linked, MA^R-conferring mutations, the glutamine synthetases from the wild-type (AB1206) and from the mutants MX1225 to MX1229 were purified as described in Methods. The glutamine synthetase preparations so obtained had only some minor contaminants, as evidenced by SDS-polyacrylamide disc gel electrophoresis. The major polypeptide observed in all preparations was a single band with the same mobility as that of the wild-type enzyme (data not shown). This implies that the resistance-conferring mutations probably are single-site mutations which do not grossly alter the molecular mass of the glutamine synthetase polypeptide. Glutamine synthetase biosynthetic activity was measured by the phosphate-release assay (see Methods), using either ammonium or methylammonium as substrates (Table 6). It is evident that alleles *glnA93* to *glnA97* are structural mutations, since they produced altered glutamine synthetase enzymes, which were less active biosynthetically with either ammonium or methylammonium as substrates. We tried to determine the K_m values of the wild-type and mutant glutamine synthetases for ammonium. This was not possible for the enzymes from strain MX1225 (*glnA93*), which became inactive during purification (Table 6), or that from strain MX1226 (*glnA94* allele), which yielded low and variable results. The K_m values for ammonium for the wild-type and three of the altered enzymes (Table 6) show that the enzymes encoded by alleles *glnA95* and *glnA97* had much higher K_m values than the wild-type, while that encoded by the *glnA96* allele had approximately the same K_m as the wild-type.

GMAD accumulation by wild-type and mutant strains

The low affinity for ammonium (methylammonium) found in some of the mutant glutamine synthetases could result in a poor ability to convert methylammonium into GMAD, which in turn could be the cause of the MA^R phenotype. We therefore measured intracellular pools of glutamate, glutamine and GMAD in wild-type cells and in one of the mutants after exposure to 100 mM-methylammonium in glucose–arginine medium (Table 7). Cells of strain MX614 accumulated large amounts of GMAD; by contrast, cells of strain MX1210 synthesized very small amounts, consistent with their having a low glutamine synthetase biosynthetic activity (Table 4). Under such conditions, glutamine pools were very small in both strains [< 2 nmol (mg protein)$^{-1}$; data not shown].

Table 6. *Specific activity of purified glutamine synthetase from wild-type and MA^R strains*

Glutamine synthetase was purified from cells grown on glucose–arginine (0·2%)–NN minimal medium. Biosynthetic activity was assayed as described in Methods. Standard reaction mixtures were prepared with either NH_4Cl or methylamine.HCl. For K_m determinations reaction mixtures were prepared in order to assay ammonium concentrations ranging from 0·01 mM to 1 M. The K_m values were calculated from Lineweaver–Burk double reciprocal plots (not shown). Results are the mean of two experiments. ND, Not determined.

Strain	Glutamine synthetase activity [μmol min^{-1} (mg protein)$^{-1}$]		K_m (mM) for ammonium
	Ammonium	Methylammonium	
AB1206 (*glnA*$^+$)	67·32	3·06	1
MX1225 (*glnA93*)	<0·01	<0·01	ND
MX1226 (*glnA94*)	0·33	0·08	ND
MX1227 (*glnA95*)	4·53	0·14	83
MX1228 (*glnA96*)	1·54	0·16	1
MX1229 (*glnA97*)	1·15	<0·01	100

Table 7. *GMAD accumulation by cells of strains MX614 (glnA$^+$) and MX1210 (glnA93)*

Cells were grown aerobically in glucose–arginine (0·2%)–NN minimal medium. When cultures reached 80 Klett units they were divided and growth was continued in the same medium with and without 100 mM-methylammonium (MA). Samples were taken at appropriate intervals; cells were immediately centrifuged, resuspended in 80% (v/v) ethanol, and disrupted in a MSE Soniprep 150 sonic oscillator. The extracts were then heated in boiling water for 10 min, filtered and lyophilized. Amino acid analysis was done as described in Methods, ND, None detected.

Time (min)	MX614 (MA^S)				MX1210 (MA^R)			
	−MA		+MA (100 mM)		−MA		+MA (100 mM)	
	Glutamate [nmol (mg protein)$^{-1}$]	GMAD [nmol (mg protein)$^{-1}$]	Glutamate [nmol (mg protein)$^{-1}$]	GMAD [nmol (mg protein)$^{-1}$]	Glutamate [nmol (mg protein)$^{-1}$]	GMAD [nmol (mg protein)$^{-1}$]	Glutamate [nmol (mg protein)$^{-1}$]	GMAD [nmol (mg protein)$^{-1}$]
0	130	ND	130	ND	84	ND	84	ND
30	–	–	43	99	–	–	97	1
60	90	ND	76	390	90	ND	129	1
120	130	ND	77	395	130	ND	150	<1

DISCUSSION

We found that under nitrogen limitation, aerobic growth of *E. coli* was inhibited by high (100 mM) extracellular concentrations of methylammonium. Our results suggest that GMAD, and not methylammonium, is responsible for growth inhibition. This is implied by the fact that extracellular methylammonium has a growth inhibitory effect on cells only under conditions of nitrogen limitation, when ammonium is limiting and glutamine synthetase is derepressed and biosynthetically active (deadenylylated). Under such conditions, synthesis of GMAD from glutamate and methylammonium would be favoured by reduced competition with ammonium for glutamine synthetase. Apparently, excess ammonium and low levels of adenylylated enzyme prevent accumulation of GMAD, at least in sufficient amounts to be growth inhibitory.

Mutants resistant to methylammonium under conditions of nitrogen limitation were isolated. Some of these showed altered γ-glutamyltransferase specific activities (Table 2). It therefore seemed possible that they carried mutations in *glnA*, the structural gene for glutamine synthetase. Genetic characterization of the mutants was accomplished by P1 transductions and F'14 complementations (Tables 3, 4 and 5). Four out of ten independent strains turned out to carry more than one mutation, at least one of them being linked to *glnA*; both *glnA*-linked and unlinked mutations were required for these strains to display the MA^R phenotype; another strain (MX1215) carried mutation(s) unlinked to *glnA*. Further mapping and characterization of

the *glnA*-unlinked mutations has not yet been done. The remaining five mutant strains owe their MA^R phenotype to mutations that lie inside the *glnA* gene. These mutants were characterized more thoroughly. Even though they seemed quite diverse with regard to their γ-glutamyltransferase activity, it is clear that the relevant glutamine synthetase biosynthetic activity was reduced in all of them. It cannot yet be ruled out that strains with high transferase activity carry more than one *glnA*-linked mutation, with one mutation reducing the biosynthetic activity and another allowing high constitutive levels of the altered enzyme; this seems to be the case for the *glnA97* allele, which confers to cells *cis*-dominant constitutive levels of a glutamine synthetase with a high K_m for ammonium (Tables 5 and 6).

Some of the altered glutamine synthetases of the MA^R mutants are less active, at least in part, because of a reduced affinity for ammonium or methylammonium. It was interesting to find, however, that MX1228, carrying the *glnA96* allele, had an enzyme with the same affinity as the wild-type, but with altered catalytic properties; therefore, insensitivity to methylammonium is not necessarily due to a reduced affinity for it by the altered glutamine synthetases, indicating again that this molecule could not be the real inhibitor. Whether the *glnA96* mutation carried by MX1228 is associated with a diminished affinity of its glutamine synthetase for glutamate or ATP remains to be elucidated.

Since a wild-type strain is able to synthesize growth-inhibitory amounts of GMAD only under nitrogen limitation, it seems that those intracellular concentrations are not easy to attain. In fact, extracellular methylammonium concentrations which are 5- or 10-fold lower than the 100 mM concentration used throughout this study still allowed growth to proceed at a slightly diminished rate (data not shown). It therefore seems obvious that the mutant strains carrying the biosynthetically inefficient glutamine synthetases are unable to synthesize inhibitory amounts of GMAD. When we measured conversion of methylammonium into GMAD in the wild-type strain and in one of the mutant strains, we found a considerably higher concentration of GMAD in the former than in the latter.

It is noteworthy that among the MA^R mutants we have isolated none was found affected in ammonium (methylammonium) transport. Since methylammonium concentrations required to inhibit growth are high, access into cells could not be limited to the high affinity transport system; as in the case of excess ammonium, sufficient methylammonium could also diffuse through the membrane.

Our results show that GMAD accumulation is responsible for growth inhibition in *E. coli*, but further experimentation is required to elucidate the mechanism of inhibition. It will also be interesting to explore the prevalence of a similar phenotype in other micro-organisms, and whether it might provide a general way for the isolation of mutations affecting glutamine synthetase.

We are grateful to A. V. Osorio and J. C. Urbina for their gift of some of the strains used in this work.

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