Nitrogen Regulation of Synthesis of the High Affinity Methylammonium Transport System of *Escherichia coli*

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Uptake of $^{14}\text{CH}_3\text{NH}_3^+$ (methylammonium) was measured as a probe of NH_4^+ transport in intact *Escherichia coli* cells and derivatives impaired in the Ntr regulatory system. The results suggest that expression of the high affinity $^{14}\text{CH}_3\text{NH}_3^+$ transport system (a) requires *de novo* polypeptide synthesis, (b) is activated by the *glnG* and *glnF* regulatory products under nitrogen limitation, and (c) is repressed under nitrogen excess by the *glnL* product. Cells deficient in glutamate synthase activity by virtue of their harbouring the *gltB31* mutation were unable to activate synthesis of $^{14}\text{CH}_3\text{NH}_3^+$ transport. This could explain the inability of cells carrying *gltB* mutations to grow on low concentrations of NH_4^+ .

INTRODUCTION

Most micro-organisms assimilate NH_4^+ ions as a sole source of nitrogen and must have mechanisms that ensure penetration of these cations. Some bacteria and fungi have mechanisms that concentrate ammonium ions intracellularly to 100-fold or more across the cell envelope (see Brown, 1980, and Kleiner, 1981 for reviews). Stevenson & Silver (1977) used $^{14}\text{CH}_3\text{NH}_3^+$ uptake to demonstrate an NH_4^+ transport system in *Escherichia coli*. A genetical analysis of the NH_4^+ transport system has not been pursued in any prokaryote. We decided to determine whether the *E. coli* high affinity NH_4^+ transport system previously described by Stevenson & Silver (1977) was under the general genetic control of nitrogen metabolism, as suggested for *Klebsiella pneumoniae* (Kleiner, 1982). In enteric bacteria the latter system, known as Ntr, regulates the expression of the *glnALG* operon (Pahel *et al.*, 1982) and other genes or operons involved in the transport of several amino acids (Kustu *et al.*, 1979a; Wei & Kustu, 1981) and in the subsequent utilization of nitrogen sources such as arginine, proline or histidine (reviewed by Tyler, 1978; Magasanik, 1982). Ntr also regulates the expression of the complex nitrogenase system of *K. pneumoniae* (Streicher *et al.*, 1974; de Bruijn & Ausubel, 1981; Espin *et al.*, 1981; Ow & Ausubel, 1983) and *Rhizobium meliloti* (Sundaresan *et al.*, 1983).

The products of at least three genes, *glnF*, *glnG* and *glnL*, have been identified as the regulatory proteins involved in Ntr control (Kustu *et al.*, 1979b; McFarland *et al.*, 1981; Pahel *et al.*, 1982; Chen *et al.*, 1982). Under conditions of nitrogen limitation, the *glnG* product appears to act in concert with the *glnF* product to activate all genes or operons under Ntr control. Under conditions of nitrogen excess, the *glnG* product has been postulated to repress transcription of the same genes in concert with the *glnL* product. The evidence presented here indicates that synthesis of the high affinity NH_4^+ transport system of *E. coli* is also subject to Ntr regulation.

METHODS

All strains used were *E. coli* K12 derivatives. All mutations affecting the Gln and Ntr phenotypes were introduced into MX614 (F^- , *thi* $\Delta(\text{pro-lac})$ *ilv680 galE*) either by mutation or by genetic manipulation (Covarrubias *et al.*, 1980; Osorio *et al.*, 1984), except for MX705, which is derived from strain RR1 (Covarrubias *et al.*, 1980).

Strains were grown aerobically in liquid NN minimal medium (Covarrubias *et al.*, 1980) with 0.2% glucose as carbon source, and the indicated nitrogen source. $^{14}\text{CH}_3\text{NH}_3^+$ was obtained from New England Nuclear (50 mCi mmol⁻¹; 1.8 GBq mmol⁻¹); specific activity was lowered to 5.0 mCi mmol⁻¹ with nonradioactive CH_3NH_3^+ . $^{14}\text{CH}_3\text{NH}_3^+$ uptake assays were performed as described by Stevenson & Silver (1977) using 50 mM-Tris/HCl, 72 mM-NaCl, 0.2% glucose (pH 7.0) as assay buffer, and substituting M9 medium for NN minimal medium. ^{14}C radioactivity was counted in a Packard TriCarb liquid scintillation spectrometer using 10 ml Bray's scintillation mixture per filter (Peng, 1977). Specific activity determinations are expressed as nmol methylammonium accumulated min⁻¹ (g cell dry mass)⁻¹. These were highly reproducible for duplicates of a given culture but varied from ± 10 to 25% for independent cultures. Protein was determined by the method of Lowry. L-[U- ^{14}C]Glutamine was obtained from New England Nuclear; nonradioactive CH_3NH_3^+ and amino acids were from Sigma. NH_4^+ concentrations were measured with an ammonium electrode (Beckman model 39565). Glutamine synthetase [L-glutamate: ammonia ligase (ADP-forming), EC 6.3.1.2.] activity was measured by the γ -glutamyl transferase assay (Covarrubias *et al.*, 1980).

RESULTS

General properties of the $^{14}\text{CH}_3\text{NH}_3^+$ transport system

Several characteristics of the $^{14}\text{CH}_3\text{NH}_3^+$ high affinity transport system described by Stevenson & Silver (1977) for *E. coli* strain ML308-225 were found in strain MX614 (Table 1). We confirmed: (a) uptake at pH values of 7 and 9, (b) energy dependence of the concentrative uptake as shown by its inhibition by cyanide, (c) inhibition of uptake by NH_4^+ ions but not by glutamate, and (d) variability in the levels of uptake (Table 2). In addition we found that glutamine at concentrations equivalent to those of NH_4^+ ions strongly inhibited $^{14}\text{CH}_3\text{NH}_3^+$ uptake (Table 1). Since we found that at equivalent concentrations neither CH_3NH_3^+ nor NH_4^+ ions inhibited L-[^{14}C]glutamine uptake (data not shown), the possibility that we were measuring $^{14}\text{CH}_3\text{NH}_3^+$ transport via the high affinity glutamine transport system (Weiner & Heppel, 1971; Willis *et al.*, 1975; Betteridge & Ayling, 1976) was ruled out. Contamination of our glutamine preparations with NH_4^+ ions was found to be of the order of less than 0.14% by mass, too low to account for the inhibitory effect of glutamine.

Activation and repression of $^{14}\text{CH}_3\text{NH}_3^+$ transport

MX614 cells grown under nitrogen limitation (0.5 mM- NH_4^+ or 6.8 mM-glutamine) developed a high capacity for $^{14}\text{CH}_3\text{NH}_3^+$ uptake (Table 2). By contrast, cells grown with an excess of nitrogen (15 mM) or with a combination of 15 mM- NH_4^+ plus 6.8 mM-glutamine took up background amounts of $^{14}\text{CH}_3\text{NH}_3^+$. These results suggest that synthesis of the $^{14}\text{CH}_3\text{NH}_3^+$ transport system is regulated by nitrogen availability in a manner similar to that of several amino acid transport systems (Kustu *et al.*, 1979a; Wei & Kustu, 1981). The kinetics of activation of $^{14}\text{CH}_3\text{NH}_3^+$ transport was measured by transferring MX614 cells previously grown on 15 mM- NH_4^+ to nitrogen limiting conditions. The $^{14}\text{CH}_3\text{NH}_3^+$ concentrative capacity increased significantly 60 min after the shift (Fig. 1a). The highest rate was observed when no nitrogen was added to the culture after the shift; a slightly lower rate was observed for cells grown on 0.5 mM- NH_4^+ and the lowest rate for those grown on glutamine. The highest rate obtained when no nitrogen was added following the shift cannot be taken as evidence to suggest that the lack of nitrogen is the event that triggers activation; these cells, even though they were washed after growth on 15 mM- NH_4^+ , probably contained intracellular amounts of NH_4^+ ions which could act as the effector. The *de novo* synthesis of the $^{14}\text{CH}_3\text{NH}_3^+$ carrier, and its polypeptide nature, were also suggested by the observation that chloramphenicol (34 $\mu\text{g ml}^{-1}$) completely inhibited its formation under otherwise derepressing conditions (Fig. 1a). A slow repression was observed when MX614 cells fully activated for $^{14}\text{CH}_3\text{NH}_3^+$ transport were shifted to a 15 mM- NH_4^+ medium (Fig. 1b). This slow onset of repression may be the consequence of a prolonged time required for cells to make active repressor, to a long half life of the previously synthesized carrier, or to both.

Ntr regulation of synthesis of the $^{14}\text{CH}_3\text{NH}_3^+$ transport system

$^{14}\text{CH}_3\text{NH}_3^+$ uptake assays were performed on strains harbouring mutations in several genes known to be involved in Ntr regulation. The strains were grown under nitrogen limiting or

Fig. 1. Kinetics of derepression (a) and repression (b) of the high affinity $^{14}\text{CH}_3\text{NH}_3^+$ transport system of *E. coli*. (a) *E. coli* MX614 cells were grown aerobically on glucose/15 mM- NH_4Cl NN medium. When they reached a turbidity of 80 Klett units they were washed three times and growth was continued in nitrogen limiting media, i.e. 0.5 mM- NH_4Cl (○), 6.8 mM-L-glutamine (■), or without nitrogen (□). Growth was also continued in the same 15 mM- NH_4Cl medium as a control and in the three nitrogen limiting media in the presence of 34 μg chloramphenicol ml^{-1} , all of which resulted in lack of derepression of $^{14}\text{CH}_3\text{NH}_3^+$ transport (●). (b) *E. coli* MX614 cells were grown aerobically on glucose/0.5 mM- NH_4Cl NN medium. When they reached a turbidity of 80 Klett units the culture was divided and growth was continued in the same 0.5 mM- NH_4Cl medium (○) or in 15 mM- NH_4Cl medium (●). Samples were taken at appropriate intervals, washed, and tested for 6 μM - $^{14}\text{CH}_3\text{NH}_3^+$ uptake.

Table 1. Effect of inhibitors on $^{14}\text{CH}_3\text{NH}_3^+$ uptake by *E. coli*

E. coli MX614 cells were grown aerobically on glucose/glutamine NN medium. When they reached a turbidity of 100 Klett units they were washed twice and assayed for 6 μM - $^{14}\text{CH}_3\text{NH}_3^+$ uptake, as described in Methods. Inhibitors were added 5 min before reactions were started by addition of $^{14}\text{CH}_3\text{NH}_3^+$. Results are the means of two experiments. ND, Not determined.

Addition	Uptake [nmol min^{-1} (g cell dry mass) $^{-1}$]		Percentage inhibition at pH 7.0
	pH 7.0	pH 9.0	
None	13.8	19.7	—
1 mM-KCN	0.9	ND	93.5
50 μM - NH_4Cl	0.4	ND	97.1
50 μM -L-Glutamate	12.8	ND	7.2
50 μM -L-Glutamine	0.3	ND	97.8

Table 2. Effect of nitrogen availability on the capacity of *E. coli* to synthesize a CH_3NH_3^+ transport system

See Table 1 for growth and assay conditions. Results are the means of three experiments $\pm$ SE.

Nitrogen source	$^{14}\text{CH}_3\text{NH}_3^+$ uptake [nmol min^{-1} (g cell dry mass) $^{-1}$]
15 mM- NH_4Cl	0.6 $\pm$ 0.1
0.5 mM- NH_4Cl	61.6 $\pm$ 6.3
6.8 mM-L-Glutamine	16.6 $\pm$ 3.9
15 mM- NH_4Cl + 6.8 mM-L-Glutamine	1.1 $\pm$ 0.3

Table 3. *Glutamine synthetase activity and $^{14}\text{CH}_3\text{NH}_3^+$ uptake by E. coli strains grown under nitrogen limiting or excess conditions*

Strains were grown under nitrogen limiting (6.8 mM-glutamine) or excess (15 mM- NH_4Cl) conditions. See Table 1 and Methods for growth and assay conditions. Representative results from several experiments are shown.

Strain	Relevant genotype	Glutamine synthetase activity [nmol min ⁻¹ (mg protein) ⁻¹]		$^{14}\text{CH}_3\text{NH}_3^+$ uptake [nmol min ⁻¹ (g cell dry mass) ⁻¹]	
		15 mM- NH_4Cl	6.8 mM-L-Glutamine	15 mM- NH_4Cl	6.8 mM-L-Glutamine
MX614	Wild-type	150	1270	0.6	16.6
MX705	<i>glnA70</i>	NG	< 10	NG	0.2
MX727	<i>glnA71::Tn5</i>	NG	< 10	NG	0.4
MX852	$\Delta(glnA)$	NG	< 10	NG	0.6
MX856	<i>glnD84</i>	NG	50	NG	0.5
MX902	<i>glnG74::Tn5</i>	50	50	ND	0.6
MX960	<i>glnL82::Tn5</i>	80	230	0.5	0.3
MX1019	<i>glnL83</i>	1190	1910	3.7	8.2
MX1029	<i>glnL82::Tn5 glnG85</i>	1170	2030	5.0	6.0
MX848	<i>glnF73::Tn5</i>	NG	< 10	NG	0.5
MX988	<i>gltB31</i>	110	260	ND	0.5

ND, Not done; NG, no growth.

excess conditions. None of the strains carrying mutations that lead either to the Gln^- or the Ntr^- phenotypes, or both, were able to activate synthesis of the $^{14}\text{CH}_3\text{NH}_3^+$ transport system. The strains tested carried mutations in either *glnA*, *glnF*, *glnG* or *glnD*. This last mutation, by affecting the ability of *E. coli* cells to synthesize uridylyltransferase, leads to low levels of highly adenylylated glutamine synthetase and to the Gln^- Ntr^- phenotypes (Bloom *et al.*, 1978). Strains MX705 and MX727 probably owe their Ntr^- phenotype to polarity effects exerted by their *glnA* mutations on *glnG* expression.

We also examined two strains with mutations in the *glnL* gene. One of these, MX1019, carries the *glnL83* mutation which we assume is non-polar to *glnG* due to its ability to exhibit the GlnC and NtrC phenotypes. The other strain, MX960, carries the *glnL82::Tn5* mutation which seems to be polar to *glnG* as suggested by its GlnR and Ntr^- phenotypes. MX960 cells were unable to activate synthesis of the $^{14}\text{CH}_3\text{NH}_3^+$ transport system whereas MX1019 cells did so (Table 3). Moreover, MX1019 cells built up a capacity to transport $^{14}\text{CH}_3\text{NH}_3^+$ under both nitrogen limitation or excess, i.e. they were constitutive for $^{14}\text{CH}_3\text{NH}_3^+$ transport as well as for glutamine synthetase (Table 3).

A mutation tightly linked to *glnL82::Tn5* was isolated in strain MX960. The new strain, MX1029, carried in addition to *glnL82::Tn5* a secondary mutation tentatively termed *glnG85* which probably acts by relieving the polarity exerted by *glnL82::Tn5* on *glnG* (J. C. Urbina & F. Bastarrachea, unpublished results). MX1029 turned out to be GlnC and NtrC , and thus constitutive for both $^{14}\text{CH}_3\text{NH}_3^+$ transport and glutamine synthetase (Table 3). Whether *glnG85* affects a *glnG* regulatory region or creates a strong promoter within *Tn5* in MX1029 cells, has not been determined.

In *E. coli*, the *gltB* gene which maps at minute 68 of the standard map (Pahel *et al.*, 1978) is said to code for glutamate synthase [L-glutamate : NADP⁺ oxidoreductase (transaminating), EC 1.4.1.13]. Cells carrying the *gltB* mutation were also unable to activate synthesis of their $^{14}\text{CH}_3\text{NH}_3^+$ transport system. These results are in agreement with previous ones (Pahel *et al.*, 1978) showing that *gltB* mutants of *E. coli* fail to grow or grow poorly on NH_4^+ ion concentrations lower than 0.1 mM or on nitrogen sources such as arginine or proline, i.e. they are Ntr^- .

It is known that *gltB* mutations are suppressed to Ntr^+ by *glnL* secondary mutations (Pahel *et al.*, 1978). Interestingly, while the *glnL82::Tn5* mutation alone was unable to suppress the Ntr^- phenotype conferred by the *gltB31* mutation, it did so in combination with *glnG85* as well as did *glnL83* (data not shown). The *glnG85* mutation apparently allows synthesis of the *glnG* product in a constitutive manner (J. C. Urbina & F. Bastarrachea, unpublished results). Mutations

leading to *glnG* constitutivity provide *E. coli* cells with a potential constitutivity for all Ntr regulated systems (NtrC), since the other product required for activation, the *glnF* product, is naturally constitutive (Castaño & Bastarrachea, 1984).

All the $^{14}\text{CH}_3\text{NH}_3^+$ uptake assays described in Table 3 were carried out at pH 7.0. The fact that strains MX902 (*glnG74::Tn5*) and MX988 (*gltB31*) failed to take up $^{14}\text{CH}_3\text{NH}_3^+$ at pH 9.0 (specific activities of 0.8 and 1.7, respectively; data not shown), apparently indicates that the same carrier is responsible for the transport of $^{14}\text{CH}_3\text{NH}_3^+$ at either pH value.

Collectively these results provide good evidence that the products of *glnG* and *glnF* are required to activate expression of the $^{14}\text{CH}_3\text{NH}_3^+$ carrier system, and that a functional *glnL* product is necessary to achieve its repression. They also suggest that a functional *glnL* product is not necessary for its activation.

DISCUSSION

Our results confirm and extend those of a previous report by Stevenson & Silver (1977) on the capacity of *E. coli* cells to express an energy-dependent concentrative uptake system for $^{14}\text{CH}_3\text{NH}_3^+$. Moreover, the results show that this system is activated by growth of cells under nitrogen limitation and repressed under conditions of nitrogen excess. Synthesis of the $^{14}\text{CH}_3\text{NH}_3^+$ carrier showed a strict dependence for *de novo* polypeptide synthesis, as demonstrated by its inhibition by chloramphenicol. Once activated, $^{14}\text{CH}_3\text{NH}_3^+$ uptake could be inhibited by NH_4^+ ions but not by glutamate. Surprisingly, $^{14}\text{CH}_3\text{NH}_3^+$ uptake was also inhibited by glutamine. Further evidence suggested that $^{14}\text{CH}_3\text{NH}_3^+$ does not enter the cells via the high affinity glutamine transport system. Glutamine inhibition of $^{14}\text{CH}_3\text{NH}_3^+$ uptake is difficult to explain on the basis of a structural similarity between these compounds that would allow their competition for binding at the carrier molecule. On the other hand, if we invoke the formation of a metabolic product of glutamine as responsible for the inhibition, an immediate mol per mol conversion of glutamine into NH_4^+ ions (and glutamate) should take place in order for cells to attain the 0.05 mM external concentration required to inhibit $^{14}\text{CH}_3\text{NH}_3^+$ transport by 90% (Table 1). The fact that glutamine acts as a nitrogen limiting substrate for activation of expression of $^{14}\text{CH}_3\text{NH}_3^+$ transport further suggests that a high external concentration of NH_4^+ ions is not built up by *E. coli* cells when they are grown on 6.8 mM-glutamine (1 mg ml⁻¹) as the only nitrogen source. If such were the case, they would be unable to activate their $^{14}\text{CH}_3\text{NH}_3^+$ transport system under this condition. More experimentation is required to explain the nature of the inhibition of $^{14}\text{CH}_3\text{NH}_3^+$ uptake by glutamine.

Results presented in Tables 2 and 3 show that in wild-type *E. coli* cells synthesis of both glutamine synthetase and $^{14}\text{CH}_3\text{NH}_3^+$ transport are simultaneously activated by nitrogen limitation and repressed by conditions of nitrogen excess. By use of mutants affected in Ntr regulation we demonstrated that both *glnG* and *glnF* products are required to activate synthesis of the $^{14}\text{CH}_3\text{NH}_3^+$ transport system. The results also show that the *glnL* product is required for its repression. Mutation *glnL83* or the combination *glnL82::Tn5-glnG85* leads to constitutivity of both glutamine synthetase and $^{14}\text{CH}_3\text{NH}_3^+$ transport. Even though strains MX1019 (*glnL83*) and MX1029 (*glnL82::Tn5-glnG85*) were constitutive, they were unable to attain full derepression for both $^{14}\text{CH}_3\text{NH}_3^+$ uptake and glutamine synthetase, particularly when grown on 15 mM- NH_4^+ medium (Table 3). An explanation for this is not available. It could be related to the fact that the control region of the *glnALG* operon has three potential promoters (Covarrubias & Bastarrachea, 1983), the functionality of which have not been ascertained. We still do not know how they respond to different physiological conditions or to qualitative and quantitative changes of the *glnG* and *glnL* regulatory proteins.

Finally, we found that MX988 cells carrying the *gltB31* mutation are also impaired in their ability to activate expression of $^{14}\text{CH}_3\text{NH}_3^+$ transport and to derepress glutamine synthetase fully (Table 3). These results were expected, since *gltB* mutants are known to be affected in their ability to synthesize glutamine synthetase, to be phenotypically Ntr⁻ (Pahel *et al.*, 1978) and unable to increase their levels of glutamine synthetase in response to nitrogen limitation (Magasanik, 1982). The inability of *gltB31* cells to activate synthesis of their $^{14}\text{CH}_3\text{NH}_3^+$

transport system strongly suggests this, rather than the high substrate K_m values of glutamate dehydrogenase (Tyler, 1978), as the most likely explanation for their inability to grow on low NH_4^+ ion concentrations. The reasons for the failure of cells carrying *gltB* mutations to activate other Ntr regulated systems, however, is still unknown.

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