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

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Uptake of $^{14}$CH$_3$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 $^{14}$CH$_3$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 $^{14}$CH$_3$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 $^{14}$CH$_3$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 Gin and Ntr phenotypes were introduced into MX614 (F-, thi A(pro-lac) ilu680 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. $^{14}$CH$_3$NH$_3^+$ was obtained from New England Nuclear (50 mCi mmol$^{-1}$; 1.8 GBq mmol$^{-1}$); specific activity was lowered to 50 mCi mmol$^{-1}$ with nonradioactive CH$_3$NH$_3^+$. $^{14}$CH$_3$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 methyl-ammonium 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$_3$NH$_2$ and amino acids were from Sigma. NH$_3$ 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 $^{14}$CH$_3$NH$_3^+$ transport system**

Several characteristics of the $^{14}$CH$_3$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$_3^+$ ions but not by glutamate, and (d) variability in the levels of uptake (Table 2). In addition we found that glutamate at concentrations equivalent to those of NH$_3^+$ ions strongly inhibited $^{14}$CH$_3$NH$_3^+$ uptake (Table 1). Since we found that at equivalent concentrations neither CH$_3$NH$_3^+$ nor NH$_3^+$ ions inhibited L-[U-$^{14}$C]glutamate uptake (data not shown), the possibility that we were measuring $^{14}$CH$_3$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$_3^+$ 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 $^{14}$CH$_3$NH$_3^+$ transport**

MX614 cells grown under nitrogen limitation (0.5 mM-NH$_4^+$ or 6.8 mM-glutamine) developed a high capacity for $^{14}$CH$_3$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 $^{14}$CH$_3$NH$_3^+$. These results suggest that synthesis of the $^{14}$CH$_3$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 $^{14}$CH$_3$NH$_3^+$ transport was measured by transferring MX614 cells previously grown on 15 mM-NH$_4^+$ to nitrogen limiting conditions. The $^{14}$CH$_3$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$_3^+$ ions which could act as the effector. The de novo synthesis of the $^{14}$CH$_3$NH$_3^+$ carrier, and its polypeptide nature, were also suggested by the observation that chloramphenicol (34 µg ml$^{-1}$) completely inhibited its formation under otherwise derepressing conditions (Fig. 1a). A slow repression was observed when MX614 cells fully activated for $^{14}$CH$_3$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 $^{14}$CH$_3$NH$_3^+$ transport system**

$^{14}$CH$_3$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 $^{14}$CH$_3$NH$_3^+$ transport system of E. coli. (a) E. coli MX614 cells were grown aerobically on glucose/15 mM-NH$_4$Cl 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$_4$Cl (□), 6.8 mM-L-glutamine (■), or without nitrogen (○). Growth was also continued in the same 15 mM-NH$_4$Cl 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 $^{14}$CH$_3$NH$_3^+$ transport (○). (b) E. coli MX614 cells were grown aerobically on glucose/0.5 mM-NH$_4$Cl 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$_4$Cl medium (○) or in 15 mM-NH$_4$Cl medium (●). Samples were taken at appropriate intervals, washed, and tested for 6 µM-$^{14}$CH$_3$NH$_3^+$ uptake.

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

<table>
<thead>
<tr>
<th>Addition</th>
<th>pH 7.0</th>
<th>pH 9.0</th>
<th>Percentage inhibition at pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>13-8</td>
<td>19-7</td>
<td>-</td>
</tr>
<tr>
<td>1 mM-KCN</td>
<td>0-9</td>
<td>ND</td>
<td>93-5</td>
</tr>
<tr>
<td>50 µM-NH$_4$Cl</td>
<td>0-4</td>
<td>ND</td>
<td>97-1</td>
</tr>
<tr>
<td>50 µM-L-Glutamate</td>
<td>12-8</td>
<td>ND</td>
<td>7-2</td>
</tr>
<tr>
<td>50 µM-L-Glutamine</td>
<td>0-3</td>
<td>ND</td>
<td>97-8</td>
</tr>
</tbody>
</table>

Table 2. Effect of nitrogen availability on the capacity of E. coli to synthesize a CH$_3$NH$_3^+$ transport system

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

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>$^{14}$CH$_3$NH$_3^+$ uptake [nmol min$^{-1}$ (g cell dry mass)$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 mM-NH$_4$Cl</td>
<td>0-6 ± 0-1</td>
</tr>
<tr>
<td>0.5 mM-NH$_4$Cl</td>
<td>61-6 ± 6-3</td>
</tr>
<tr>
<td>6.8 mM-L-Glutamine</td>
<td>16-6 ± 3-9</td>
</tr>
<tr>
<td>15 mM-NH$_4$Cl + 6.8 mM-L-Glutamine</td>
<td>1-1 ± 0-3</td>
</tr>
</tbody>
</table>
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 $^{14}$CH$_3$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 adenilylated 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 $^{14}$CH$_3$NH$_3^+$ transport system whereas MX1019 cells did so (Table 3). Moreover, MX1019 cells built up a capacity to transport $^{14}$CH$_3$NH$_3^+$ under both nitrogen limitation or excess, i.e. they were constitutive for $^{14}$CH$_3$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 $^{14}$CH$_3$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 $^{14}$CH$_3$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

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Glutamine synthetase activity [nmol min$^{-1}$ (mg protein)$^{-1}$]</th>
<th>$^{14}$CH$_3$NH$_3^+$ uptake [nmol min$^{-1}$ (g cell dry mass)$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>15 mM-NH$_4$Cl 6·8 mM L-Glutamine</td>
<td>15 mM-NH$_4$Cl 6·8 mM L-Glutamine</td>
</tr>
<tr>
<td>MX614</td>
<td>Wild-type</td>
<td>150</td>
<td>0·6</td>
</tr>
<tr>
<td>MX705</td>
<td>glnA70</td>
<td>NG&lt;10</td>
<td>NG0·2</td>
</tr>
<tr>
<td>MX727</td>
<td>glnA71::Tn5</td>
<td>NG&lt;10</td>
<td>NG0·4</td>
</tr>
<tr>
<td>MX852</td>
<td>$\Delta$(glnA)</td>
<td>NG&lt;10</td>
<td>NG0·6</td>
</tr>
<tr>
<td>MX856</td>
<td>glnD84</td>
<td>NG50</td>
<td>NG0·5</td>
</tr>
<tr>
<td>MX902</td>
<td>glnG74::Tn5</td>
<td>50</td>
<td>ND0·6</td>
</tr>
<tr>
<td>MX960</td>
<td>glnL82::Tn5</td>
<td>80</td>
<td>0·5</td>
</tr>
<tr>
<td>MX1019</td>
<td>glnL83</td>
<td>1190</td>
<td>3·7</td>
</tr>
<tr>
<td>MX1029</td>
<td>glnL82::Tn5 glnG85</td>
<td>1170</td>
<td>5·0</td>
</tr>
<tr>
<td>MX848</td>
<td>glnF73::Tn5</td>
<td>NG&lt;10</td>
<td>ND0·5</td>
</tr>
<tr>
<td>MX988</td>
<td>glnB31</td>
<td>110</td>
<td>ND0·5</td>
</tr>
</tbody>
</table>

ND, Not done; NG, no growth.
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 (Castano & Bastarrachea, 1984).

All the $^{14}$CH$_3$NH$_3^+$ uptake assays described in Table 3 were carried out at pH 7.0. The fact that strains MX902 (glnG74::Tn5) and MX988 (gltB3I) failed to take up $^{14}$CH$_3$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 $^{14}$CH$_3$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 $^{14}$CH$_3$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 $^{14}$CH$_3$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 $^{14}$CH$_3$NH$_3^+$ carrier showed a strict dependence for de novo polypeptide synthesis, as demonstrated by its inhibition by chloramphenicol. Once activated, $^{14}$CH$_3$NH$_3^+$ uptake could be inhibited by NH$_3^+$ ions but not by glutamate. Surprisingly, $^{14}$CH$_3$NH$_3^+$ uptake was also inhibited by glutamine. Further evidence suggested that $^{14}$CH$_3$NH$_3^+$ does not enter the cells via the high affinity glutamine transport system. Glutamine inhibition of $^{14}$CH$_3$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$_3^+$ ions (and glutamate) should take place in order for cells to attain the 0.05 mM external concentration required to inhibit $^{14}$CH$_3$NH$_3^+$ transport by 90% (Table 1). The fact that glutamine acts as a nitrogen limiting substrate for activation of expression of $^{14}$CH$_3$NH$_3^+$ transport further suggests that a high external concentration of NH$_3^+$ 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 $^{14}$CH$_3$NH$_3^+$ transport system under this condition. More experimentation is required to explain the nature of the inhibition of $^{14}$CH$_3$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 $^{14}$CH$_3$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 $^{14}$CH$_3$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 $^{14}$CH$_3$NH$_3^+$ transport. Even though strains MX1019 (glnL83) and MX1029 (glnL82::Tn5-glnG85) were constitutive, they were unable to attain full derepression for both $^{14}$CH$_3$NH$_3^+$ uptake and glutamine synthetase, particularly when grown on 15 mM-NH$_3^+$ 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 gltB3I mutation are also impaired in their ability to activate expression of $^{14}$CH$_3$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 glutamate synthase, 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 gltB3I cells to activate synthesis of their $^{14}$CH$_3$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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with mutations in a nitrogen regulatory gene, \textit{ntrC}, that is near \textit{glnA}. Molecular and General Genetics 183, 392–399.
