Mechanism for nitrogen isotope fractionation during ammonium assimilation by *Escherichia coli* K12

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Organisms that use ammonium as the sole nitrogen source discriminate between [15N] and [14N] ammonium. This selectivity leaves an isotopic signature in their biomass that depends on the external concentration of ammonium. To dissect how differences in discrimination arise molecularly, we examined a wild-type (WT) strain of *Escherichia coli* K12 and mutant strains with lesions affecting ammonium-assimilatory proteins. We used isotope ratio mass spectrometry (MS) to assess the nitrogen isotopic composition of cell material when the strains were grown in batch culture at either high or low external concentrations of NH₃ (achieved by controlling total NH₄Cl and pH of the medium). At high NH₃ (≥0.89 μM), discrimination against the heavy isotope by the WT strain (−19.2‰) can be accounted for by the equilibrium isotope effect for dissociation of NH₃⁺ to NH₃ + H⁺. NH₃ equilibrates across the cytoplasmic membrane, and glutamine synthetase does not manifest an isotope effect in vivo. At low NH₃ (≤0.18 μM), discrimination reflects an isotope effect for the NH₄⁺ channel *AmtB* (−14.1‰). By making *E. coli* dependent on the low-affinity ammonium-assimilatory pathway, we determined that biosynthetic glutamate dehydrogenase has an inverse isotope effect in vivo (+8.8‰). Likewise, by making unmediated diffusion of NH₃ across the cytoplasmic membrane rate-limiting for cell growth in a mutant strain lacking *AmtB*, we could deduce an in vivo isotope effect for transport of NH₃ across the membrane (−10.9‰). The paper presents the raw data from which our conclusions were drawn and discusses the assumptions underlying them.

Fractionation of heavy and light isotopes of nitrogen ([15N] vs. [14N]), carbon ([13C] vs. [12C]), and hydrogen (D vs. H) can provide information about metabolic pathways and reaction mechanisms within living organisms (1–4). For example, fractionation between [15N] and [14N] during incorporation of ammonium N in a single-celled organism like *Escherichia coli* is determined by the rate-limiting step for assimilating it into glutamate, the precursor of 88% of cellular nitrogen-containing material (5). All nitrogen assimilated into this central metabolic intermediate goes on to be incorporated into cell material. Transfers from glutamate to other molecules are direct. Although transfers from glutamine, including transfers to glutamate, involve deamidation, the NH₃ released is carried directly to the assimilatory catalytic site through a tunnel and hence, cannot be protonated or diffused away (6). The overall ratio of [15N] to [14N] in biomass is, thus, controlled by steps at or before assimilation of ammonium N into glutamate.

In *E. coli*, the proteins participating in early and potentially rate-determining steps in the incorporation of NH₃⁺ into glutamate are (i) *AmtB*, its only membrane channel for NH₄⁺ (7), (ii) glutamine synthetase (GS), the first enzyme of the high-affinity ammonium-assimilatory pathway, (iii) glutamate synthase [glutamate(amide) 2-oxoglutarate amino transferase (GOGAT)], and (iv) glutamate dehydrogenase (GDH), the first enzyme of the low-affinity ammonium-assimilatory pathway (Fig. 1) (reviewed in ref. 8). To study effects of these proteins on the in vivo fractionation of ammonium N, we used WT and genetic mutant strains in which one or more was lacking or defective and studied these strains at high or low external concentrations of NH₃. To decrease the concentration of external NH₃ and still achieve significant cell yield, we lowered both the total concentration of NH₄Cl and the pH of the medium. Although *E. coli* lives in the human gut, which is nitrogen-rich, it also survives in fresh and brackish water, in which supplies of available ammonium can be limited (9, 10). Moreover, it acidifies its own environment by fermentation. Accordingly, the conditions that we have chosen to study the behavior of *E. coli* at low external NH₃ are pertinent to its normal life cycle.

Ammonium (NH₄⁺ + NH₃) is the optimal nitrogen source for *E. coli* (i.e., the source which yields most rapid growth). Ammonium (pKₐ = 9.25) enters cells in two forms (Fig. 1). NH₃, which is ∼2% of total ammonium at pH 7.4, crosses the cell membrane by unmediated diffusion, a process that cannot be altered genetically. When the pH is decreased to 5.5, NH₃ is only ∼0.02% of total ammonium. NH₄⁺, which is the bulk of total ammonium at both pH 7.4 and pH 5.5, can enter the cells if and only if the AmtB channel is expressed and functional. Its expression is controlled at the transcriptional level and regulated largely by the free-pool concentration of glutamine in the cell interior (8), whereas its activity is controlled by the regulatory protein GlnK, largely in response to the free-pool concentration of the precursor metabolite 2-oxoglutarate, which is an intermediate in the tricarboxylic acid cycle (ref. 11 and references cited therein). Expression of AmtB increases as the glutamine concentration declines, and the channel is activated as the concentration of 2-oxoglutarate rises.

Within the cell, N is assimilated into glutamate, the organic precursor of most cellular nitrogen, by a high-affinity cycle and a low-affinity enzyme (Fig. 1). The high-affinity cycle is constituted by the exquisitely regulated GS and by GOGAT. The low-affinity enzyme is biosynthetic (NADPH-dependent) GDH (8). Use of 1 mol ATP per glutamate synthesized in the GS/GOGAT cycle drives assimilation of N even at extremely low concentrations of ammonium, but it is apparently detrimental when ammonium is abundant and energy is limiting (12). Both GS and GDH use NH₃ as their substrate, because bond formation requires the lone pair of electrons on the N of NH₃ (6, 13). Hence, NH₄⁺ must be dissociated to NH₃ + H⁺ before either enzyme can use it. An equilibrium isotope effect associated with this spontaneous process leads to depletion of [15N] in NH₃ relative to NH₄⁺. Its magnitude is −19.2‰ (weighted mean, SE = 0.4‰, n = 3) (14). That is, at equilibrium, [15N]/[14N] in NH₃ is 19.2 parts per thousand lower than that ratio in NH₄⁺.

To control the site of rate limitation, we used well-characterized mutant strains (Table 1). One such strain (*ΔamtB*) lacked AmtB. A second strain (*ΔamtBΔc-term*) had a poorly active AmtB channel, in which the normal membrane pores lacked the usual carboxyl-terminal cytoplasmic extensions (15, 16). A third strain

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Fig. 1. Schematic diagram of nitrogen assimilation from ammonium by E. coli K12.

(AgdhA::kan) lacked GDH, the low-affinity ammonium-assimilatory enzyme. The last two strains (gldD::kan and gldD::kanΔamtB) lacked GOGAT (Table 1) (8). We did not use a mutant lacking GS, because this strain is auxotrophic for glutamine and requires that glutamine be added to the medium in high concentrations, even in the presence of high concentrations of ammonium (17). We studied the mutant strains and their parental strain, which is a physiologically robust E. coli K12 WT (18, 19), under both ammonium-excess and -limiting conditions. We determined doubling time, cell yield, residual ammonium in the medium, and isotopic fractionation associated with incorporation of ammonium into cell material (Materials and Methods). When the absence or alteration of one protein increased the doubling time of the strain (i.e., decreased the growth rate), we could determine the rate-limiting step in transport or assimilation.

Results

Parental WT Strain. The doubling time was 50 min at all values of \( c_0 \), the initial external concentration of ammonia (note, NH\(_3\) not NH\(_3^+\) or NH\(_4^+\)) (Fig. 2A and Table 2). For 0.89 \( \leq c_0 \leq 280 \mu M \), measured isotopic fractionations (\( e_0 \)) ranged from −16.1‰ to −23.8‰ (mean and SD: −19.2 ± 2.6‰, \( n = 7 \)), with negative values of \( e \) indicating depletion of \(^1^N\) in the biomass relative to the dissolved inorganic N in the medium (Table 2). At lower concentrations (\( c_0 \leq 200 \text{ nM} \); i.e., at 1.0 or 0.5 mM total ammonium and pH 5.5), the isotopic fractionation decreased to −8.1‰ or −5.4‰, respectively (Fig. 2B and Table 2). AmtB is highly expressed, even when the concentration of external NH\(_3\) is 5–10 times higher, namely 1 \( \mu M \) (5 mM total ammonium at pH 5.5) (11); however, its activity is not needed for optimal growth, and it is inhibited by the regulatory protein GlnK (20–22). AmtB is not expressed when external NH\(_3\) is 10 \( \mu M \) (0.5 mM total ammonium at pH 7.4) or higher (11, 23).

GDH\(^−\) Strain. The GDH\(^−\) strain (ΔgdhA::kan) lacks the low-affinity pathway for ammonium assimilation and hence, is completely dependent on the high-affinity pathway for synthesis of glutamate and glutamine. Its doubling time was indistinguishable from that of WT under all conditions of nitrogen availability, and its isotopic fractionations were very similar to those of the WT strain (Table 2). For 0.89 \( \leq c_0 \leq 280 \mu M \), the isotopic fractionations were −18.8‰ to −25.4‰ (mean and SD: −21.3 ± 2.6‰, \( n = 7 \)). For \( c_0 = 89 \text{ nM} \), \( e_0 \) decreased to −6.1‰. Because the ranges of \( e_0 \) for cells having or lacking GDH overlap, these observations strongly support earlier reports that the GS/GOGAT cycle is the primary means for incorporating ammonium into biomass at all concentrations of NH\(_3\) (12, 24, 25).

GOGAT\(^−\) Strains. The GOGAT\(^−\) strain (gldD::kan) lacks the high-affinity ammonium-assimilatory cycle and depends on the linear, low-affinity pathway (biosynthetic NADPH-dependent GDH) for synthesis of glutamate. GS converts ~12% of the glutamate product of GDH to glutamine to meet biosynthetic needs. When initial external concentrations of NH\(_3\) were decreased from 70 to 7 \( \mu M \), the doubling time of the strain increased from 50 to 65 min (Table 2 and Fig. S1A), and hence, the activity of GDH [E. coli has only a biosynthetic GDH (26)] was apparently rate-limiting for cell growth under these conditions. The weighted-mean isotopic fractionation was −11.2‰ (SE = 0.3‰, \( n = 3 \)) at both concentrations of NH\(_3\) (Fig. S1D). This strain did not grow at all at ≤1 \( \mu M \) (23).

In agreement with its dependence on the low-affinity enzyme GDH for synthesis of glutamate, gldD::kan has an abnormally low internal free-pool concentration of glutamate at low NH\(_3\) (27). The gldD::kan strain also has an unusually high free-pool concentration of glutamine (27–29), the primary metabolic indicator of nitrogen sufficiency and hence, the primary metabolic regulator of the transcriptional response to nitrogen availability (8, 27). This strain fails to express a number of proteins under the control of nitrogen regulatory protein C (NtrC), which is active at low internal concentrations of glutamate (8, 30).

Although we presumed that AmtB, which is one of the proteins controlled by NtrC, was poorly expressed (23, 31), we constructed a double-mutant strain (gldD::kanΔamtB) to be certain that AmtB was completely absent. The doubling time of the gldD::kanΔamtB strain was slightly longer than the doubling time of the gldD::kan strain, but N and C utilization rates (Fig. S1B and C) and the isotopic fractionation was unchanged at −10.3‰ (Table 2 and Fig. S1D).

AmtB\(^−\) and AmtB-Defective Strains. Finally, the AmtB\(^−\) strain (ΔamtB) lacks the NH\(_3^+\) channel. For acquisition of ammonium, it must depend on unmediated diffusion of NH\(_3\) across the cytoplasmic membrane. At high external concentrations of NH\(_3\), both the doubling time of the ΔamtB strain and its isotopic fractionation were identical to those of the WT and the ΔgdhA strain (Table 2). In fact, under these conditions, the WT does not

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**Table 1. Bacterial strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Phenotype</th>
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<tr>
<td>NCM3722: E. coli K12 WT</td>
<td>WT</td>
<td></td>
</tr>
<tr>
<td>NCM4199: AmtB, tesB::kan</td>
<td>AmtB &amp; C-term*</td>
<td></td>
</tr>
<tr>
<td>NCM4453: gldD::kan</td>
<td>GOGAT(^−)</td>
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</tr>
<tr>
<td>NCM4590: ΔgldB</td>
<td>GDH(^−)</td>
<td></td>
</tr>
<tr>
<td>NCM4701: gldD::kanΔamtB</td>
<td>GOGAT(^−), AmtB(^−)</td>
<td></td>
</tr>
</tbody>
</table>

*Provided by Dalai Yan, Indiana University School of Medicine, Indianapolis.

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**Fig. 2.** (A) Growth and (B) determination of \( c_0 \) for assimilation of external ammonium \( (c_0 = 0.089 \text{ mM NH}_3, 0.5 \text{ mM total NH}_4\text{Cl}, 0.1% \text{ glucose, pH 5.5}) \) into biomass in WT (○) and AmtB\(^−\) strains (△). Cultures in B were different from cultures in A, which were sampled more frequently.
transcribe the glnKamtB operon (11, 23, 31). When the external concentration of NH₃ was decreased to 1 μM, the AmtB strain grew very slowly; its doubling time was initially 200 min, and growth slowed even more as the strain consumed ammonium and thereby decreased the external NH₃ concentration further (11, 23). The AmtB strain also failed to consume all of the NH₃ supplied in the media and it displayed a high C/N ratio in its biomass (see Supporting Information and Figs. S2 and S3). Under these conditions, the weighted-mean isotopic fractionation was −30.1‰ (SE = 0.6‰, n = 2), markedly larger than the value observed in other strains.

For the strain in which the AmtB protein was modified at the carboxy terminal (AmtBΔC-term) (15, 16), both the doubling time and isotopic fractionation at c₀ = 0.89 μM did not differ from those of the WT, GDH⁺, and AmtB strains (Table 2). However, when c₀ was decreased to 89 nM, AmtBΔC-term had a doubling time of 110 min, more than two times as long as that of the WT and approximately one-half as long as that of ΔamtB. Under this condition, the activity of AmtB seemed to be rate-limiting for growth, and the weighted-mean εᵇ was −17.6‰ (SE = 0.3‰, n = 2).

**Process-Related Summary of Isotopic Observations.** Table 2 includes 11 different observations of εᵇ for cells equilibrating NH₃ by diffusion and having both the low- and high-affinity pathways for its assimilation (Fig. 1). These observations include seven WT cultures with 0.89 ≤ c₀ ≤ 280 μM, three ΔamtB cultures with 0.89 ≤ c₀ ≤ 70 μM, and one AmtBΔC-term strain with c₀ = 0.89 μM. Values of εᵇ range from −16.1‰ to −23.8‰. The weighted mean is −19.6‰ (SE = 0.7‰). There were seven different observations for cells equilibrating NH₃ by diffusion and lacking the low-affinity GDH pathway for its assimilation. For these ΔgdhA cultures, as for WT, 0.89 ≤ c₀ ≤ 280 μM. Values of εᵇ range from −18.8‰ to −25.4‰. The weighted mean is −22.2‰ (SE = 0.9‰). In total, there are 18 different observations of εᵇ for cells equilibrating NH₃ by diffusion and using the high-affinity GS/GOGAT cycle to incorporate ammonium N into organic molecules. Values of εᵇ range from −16.1‰ to −25.4‰. The weighted mean is −21.1‰ (SE = 0.6‰). Variations of εᵇ are not correlated with c₀ (r² = 0.13). Three values of εᵇ were obtained for cells relying on the AmtB channel for transport of NH₄⁺ and assimilating ammonium N by GS + GOGAT. In those cases (two WT cultures with c₀ = 0.089 μM and c₀ = 0.18 μM and one ΔgdhA culture with c₀ = 0.089 μM), εᵇ ranged from −5.4‰ to −8.1‰. In four cases, cells incorporated N using GS + GOGAT but had either no AmtB channel or an AmtB channel that was impaired by deletion of the C-terminal extensions. When AmtB was entirely absent (two cases, c₀ = 0.18 μM and c₀ = 0.089 μM), weighted-mean εᵇ was −30.1‰. When AmtB was only modified (c₀ = 0.089 μM; two cases), the weighted-mean εᵇ was −17.6‰. Finally, when cells lacked GOGAT (three cultures of ΔgltD and one culture of ΔgltD::kan), values of εᵇ varied from −10.3‰ to −11.5‰ with a weighted mean of −10.5‰ (SE = 0.2‰).

**Discussion**

When external concentrations of NH₃ exceed 0.18 μM, NH₃ can rapidly equilibrate across the cytoplasmic membrane of many bacteria by unmediated diffusion, and NH₄⁺ channels are not expressed (7, 11, 23, 32–34). The interior pool of NH₃ (in equilibrium with intracellular NH₄⁺) has only one input: diffusion of NH₃ from the external medium. Although it has three potential outputs—assimilation of NH₃ by GS, assimilation of NH₃ by GDH, and leakage from the cell—our results are not significantly affected by the absence of GDH (Process-Related Summary of Isotopic Observations), which reduces the outputs to

**Table 2. Summary of cultures and measured isotopic fractionations**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Strain*</th>
<th>c₀ (NH₃)</th>
<th>DT (min)</th>
<th>n</th>
<th>εᵇ (%)</th>
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<td>50</td>
<td>8</td>
<td>−20.4 ± 2.1</td>
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<td>50</td>
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<td>50</td>
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<td>0.089</td>
<td>110</td>
<td>4</td>
<td>−17.8 ± 0.4</td>
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</table>

*Strain number (phenotypes listed in Table 1).

**Concentrations of NH₃ determined from total concentrations of NH₄Cl, which were 71-fold higher than the concentrations of NH₃ at pH 7.4 and 5,620-fold higher at pH 5.5. Cultures with c₀ ≤ 0.89 were grown at pH 5.5.

**Doubling time.

**Number of points used in the determination of εᵇ.

**Reported uncertainty is the SE of the slope derived from the regression described in Calculations.
two. The isotopic budget is summarized in Fig. 3A. Balancing the input against the outputs, we can write

$$\delta_{ae} + e_{at} = g(\delta_{ai} + e_{GS}) + (1 - g)(\delta_{ai} + e_{at}),$$

[1]

where the δ-values are defined in Fig. 3A, $e_{at}$ is the isotope effect associated with transport of NH$_3$ across the membrane, and $g$ is the fraction of the input that is incorporated into biomass. When equilibration of NH$_3$ across the membrane is rapid in comparison to the rate of assimilation, $g \to 0$ (n.b.; $g$ is not the amount of NH$_3$ that is assimilated but instead the fraction that is assimilated) and $\delta_{ai} = \delta_{ai}$. Because $\delta = \delta_{ai} + e_{GS}$, the isotopic composition of the external NH$_3$ is related to the isotopic composition of the NH$_3$ within the cells by $\delta_{ai} = \delta_{ai} - e_{GS}$. Because essentially all external N is in the form of NH$_4$ and carbon isotope effects associated with glutamine synthetase are small, we obtain $e_{GS} = e_{at} = e_{ai}$. As noted above and summarized in Table 3, 18 experiments yielded $e_{ai} \sim -21.1\%$. The 95% confidence interval of that value overlaps with the 95% confidence interval for $e_{ai}$. Accordingly, there is no evidence for fractionation by GS.

An alternative interpretation, with $g$ appreciably greater than zero, is not tenable. Specifically, 18 experiments yielding $e_{ai} \equiv e_{ai}$ could then be explained only if (i) $g$, $e_{GS}$ and $e_{at}$ happened in all cases to have values satisfying the relationship $g = e_{GS}/(e_{GS} - e_{at})$ and (ii) $g$ was independent of the external concentration of NH$_3$. Additionally, a well-documented experimental study of nitrogen and carbon isotope effects associated with glutamine synthetase from E. coli found a near-zero nitrogen isotope effect of $-0.7 \pm 0.6\%e$ (mean and SD, $n = 7$) (35).

It is unlikely that GS limits growth, because it is synthesized in excess when supplies of NH$_3$ are plentiful; also, its catalytic activity is regulated downward by covalent modification (36–38). It is more likely that the flux of N into glutamate, the most plentiful intermediate in central nitrogen metabolism, is limited by the capacity of GOGAT. No isotopic fractionation is associated with GOGAT, because practically all of the amide N in gln is transferred to 2-oxoglutarate to produce glu.

When external NH$_3$ concentrations are below 0.89 μM, E. coli K12 depends on the ammonium channel AmtB to maintain an optimal growth rate. Cells lacking this channel (ΔamtB) depend on uncatalyzed transport of NH$_3$ across the membrane. At $c_0 \leq 0.2$ μM, growth of the ΔamtB strain is extremely slow, and the rate decreases as the external concentration of NH$_3$ declines (Fig. 2A and Table 2). The mass balance described by Eq. 1 applies, but the conditions differ from the conditions just discussed. Instead, $g \neq 0$, and because GS imposed no fractionation (even when supplies of NH$_3$ were abundant), we know that $\delta_{ai} = \delta_{ai}$ and $e_{GS} = 0$. Making these substitutions and simplifying, we obtain $\delta_{ai} = \delta_{ai} - g e_{at}$. Substituting $\delta_{ai} = \delta_{ai} + e_{at}$, we get $e_{at} = e_{GS} = e_{at} + e_{at}$. At the limit in which growth is limited by transport of NH$_3$ and $g \to 1$, $e_{ai} = e_{ai} - e_{ai}$. If the slowest growing cultures (Table 2, experiments 1 and 27) represent that case, $e_{ai} = -30.1 + 19.2 = -10.9 \pm 0.7\%e$ (SE from combining 0.5 and 0.4 in quadrature). This relatively large value suggests that transport of NH$_3$ across the membrane is limited by some process other than simple diffusion. Polar interactions within the membrane may play a role. Rishavy and Cleland (39) commented that the isotope effect in a related case “could easily be 2%” (i.e., $\sim 20\%e$; almost two times the value estimated here) (39).

For cells with a normal AmtB channel (WT or Δgdh4 strain) and $c_0 = 0.089$ μM, $e_{ai}$ was observed as low as $-5.4 \pm 0.3\%e$, much lower than the value of a strain lacking the channel (ΔamtB). The corresponding mass balance is shown schematically in Fig. 3B and expressed mathematically in Eq. 2:

$$\delta_{ai} + e_{at} = g \delta_{ai} + (1 - g)(\delta_{ai} + e_{at}),$$

[2]

Here, $e_{at}$ is the isotope effect associated with transport of NH$_4^+$ by the AmtB channel, and substitutions introduced above have been adopted where appropriate ($\delta_{ai} = \delta_{ai}$, $\delta_{ai} = \delta_{ai}$). Simplifying gives $e_{ai} = e_{ai} = e_{ai} + e_{at}$. A recent quantitative study of AmtB function (11) indicates that $g \sim 0.2$. For $e_{ai} = -5.4\%e$, adopting $e_{at} = -10.9\%e$ (see above), we find $e_{at} = -14.1\%e$. If an uncertainty of 0.05 is assigned to $g$, the estimated SE of $e_{ai}$ is 0.7%e. Notably, the reduced fractionation at low values of $c_0$, a condition that may be encountered in nature, derives from not only fractionations associated with the AmtB channel but also, an interplay between fractionations associated with $e_{at}$ and $e_{ai}$.

When the WT strain was grown with a slightly higher $c_0 = 0.18$ μM, the observed fractionation increased to $-8.1\%e$. The AmtB channel also functions at this NH$_3$ concentration, because an AmtB$^+$ strain continues to grow suboptimally. Hence, Eq. 2 applies. Using $e_{at} = -14.1 \pm 0.7\%e$ and solving for $g$, we find $g = 0.45 \pm 0.1$. When AmtB functions, the internal ammonium concentration is held constant, and hence, there is less leakage of NH$_3$ at higher external NH$_3$ concentrations (11).

For cells with an altered AmtB channel lacking the cytoplasmic C-terminal extensions (16), $e_{ai} = -17.6 \pm 0.3\%e$ (weighted mean and SE) at $c_0 = 0.089$ μM. The fraction of N assimilated by GS is 0.5 (11), and solving as above yields $e_{at} = -23.0 \pm 0.7\%e$.

### Table 3. Process-related isotope effects

<table>
<thead>
<tr>
<th>Process</th>
<th>Related experiments</th>
<th>Result*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Assimilation of NH$_3$ by GS</td>
<td>WT, $c_0 \geq 0.89$ μM, $n = 7$</td>
<td>$\delta_{ai} \sim 95%$ confidence intervals: $\delta_{ai} = -21.1 \pm 1.3%e$</td>
</tr>
<tr>
<td>For $g = 0$</td>
<td>AmtB$^+$, $c_0 \geq 0.89$ μM, $n = 3$</td>
<td>$\delta_{ai} = -19.2 \pm 1.7%e$</td>
</tr>
<tr>
<td>$e_{GS} = e_{ai} - e_{ai}$</td>
<td>AmtB$^+$C-term, $c_0 = 0.89$ μM, $n = 1$</td>
<td>$e_{GS} \sim 0$</td>
</tr>
<tr>
<td>2. Transmembrane transport of NH$_3$</td>
<td>AmtB$^+$, $c_0 &lt; 0.2$ μM, $n = 2$</td>
<td>$\delta_{ai} = -30.1 \pm 0.5%e$</td>
</tr>
<tr>
<td>For $g = 1$</td>
<td>$e_{at} = e_{ai} - e_{ai}$</td>
<td>$e_{at} = -10.9 \pm 0.7%e$</td>
</tr>
<tr>
<td>3. Transport of NH$_4^+$ by AmtB</td>
<td>WT, $c_0 = 0.089$ μM, $n = 1$</td>
<td>$\delta_{ai} = -5.5 \pm 0.3%e$</td>
</tr>
<tr>
<td>$e_{ai} = e_{ai} + (1 - g)e_{at}$</td>
<td>GDH$^+$, $c_0 = 0.089$ μM, $n = 1$</td>
<td>For $g = 0.2 \pm 0.05$, $e_{at} = -14.1 \pm 0.8%e$</td>
</tr>
<tr>
<td>4. Transport of NH$_4^+$ by altered AmtB</td>
<td>AmtB$^+$C-term, $c_0 = 0.089$ μM, $n = 2$</td>
<td>$\delta_{ai} = -17.6 \pm 0.3%e$</td>
</tr>
<tr>
<td>$\tau_{ai} = e_{ai} + (1 - g)e_{at}$</td>
<td>For $g = 0.5 \pm 0.1$, $\tau_{ai} = -23.1 \pm 1.2%e$</td>
<td></td>
</tr>
<tr>
<td>5. Assimilation of NH$_3$ by GDH</td>
<td>GOGAT$^+$, AmtB$^+$, $c_0 = 7$ μM, $n = 1$</td>
<td>$\delta_{ai} = -10.5 \pm 0.2%e$</td>
</tr>
<tr>
<td>$e_{GDH} = e_{ai} - e_{ai}$</td>
<td>GOGAT$^+$, $c_0 \geq 7$ μM, $n = 3$</td>
<td>$e_{GDH} = 8.8 \pm 0.4%e$</td>
</tr>
</tbody>
</table>

*Denotes weighted mean. Indicated uncertainties are SEs except for process 1, where 95% confidence intervals are specified.
(where a prime is used to denote the altered channel). If the uncertainty assigned to $g$ is doubled (to 0.1), the SE increases only to $0.15\%$. Hence, the isotope effect for the mutant AmtB channel is significantly larger than that for the WT channel. The mutant channel is known to lack coordination between the function of its individual monomers and have other unusual properties (40).

To make E. coli dependent on the low-affinity pathway for assimilation of ammonium, we inactivated GOGAT. This inactivation eliminates the GOGAT cycle and makes the organism dependent on biosynthetic GDH. At an external NH$_3$ concentration of 10 kM, GDH activity already limits the growth rate of the GOGAT$-$ strain, and the strain does not grow at all at $c_0 = 1$ kM. The fractionation observed for the GOGAT$-$ strains with $c_0 \geq 7$ kM is $10.5\%$. Assuming that NH$_3$ inside and outside the cell was in equilibrium, such as for the WT, $\Delta$gdhA, and $\Delta$amnB strains at the same concentrations, it follows that internal NH$_3$ was depleted in $^{15}$N by 19.2% relative to external dissolved inorganic N and therefore, that the observation of $e_{\text{GDH}} = -10.5\%$ requires inverse fractionation of $^{15}$N by GDH (i.e., enrichment of the product relative to the reactant) with $e_{\text{GDH}} = 8.7 \pm 0.4\%$. An inverse isotope effect has also been reported for bovine liver GDH (41).

Conclusion. Our studies of E. coli K12 have yielded in vivo isotope effects as summarized in Table 3. To our knowledge, the isotope effects relative to the reactant) with $e_{\text{GDH}} = 8.7 \pm 0.4\%$. An inverse isotope effect has also been reported for bovine liver GDH (41).

Comparison with Earlier Work. Studying the $\gamma$-proteobacterium Vibrio harveyi, a close evolutionary relative of E. coli K12, Hoch et al. (46) also found that isotope fractionation between external ammonium and cell material varied with external ammonium availability. Now, by using AmtB$^+$ strains, we have determined that the decrease in $e_{\text{GDH}}$ from $-20\%$ to $-4\%$ as $c_0$ increased from 6 kM in this work (Table 1). Additional details of strain construction are in SI Materials and Methods. For growth experiments, bacterial cultures were grown on the minimal medium by Neidhardt et al. (47) in MOPS buffered medium, pH 7.4, with 0.1% glucose as sole carbon source and NH$_4$Cl as sole nitrogen source. For experiments at pH 5.5, cultures were additionally adapted to low pH in minimal medium buffered with MES at pH 5.5. Growth and doubling time were determined by measuring OD at 420 nm.

Ammonia Assay. Residual ammonium in cell-free supernatants was assayed in a GDH catalyzed reaction (AA0100 kIt; Sigma). In the assay, 2-oxoglutarate is reduced to l-glutamate by GDH using ammonium as substrate and NADPH as the cofactor providing reducing equivalents. Oxidation of NADPH is measured by a change in absorbance at 340 nm.

Sample Preparation and Isotopic Analyses. Bacterial cell samples were taken at various points during growth and removed from the supernatant by high-speed centrifugation. The cell-free supernatant was frozen at $-80^\circ$C for later measurement of residual ammonium, glucose, and final pH. The cells were washed two times in medium without additional glucose or ammonium and dried in air overnight. Uniform amounts of 2 mg dry weight, yielding 0.8 mg carbon and 0.2 mg nitrogen, were transferred into preweighed tin capsules (part number 240-053-00; Costech Analytical Technologies, Inc.). Capsules containing only the reactant glutamine and ammonium chloride used in the media were also prepared. All samples were analyzed at the University of California at Berkeley Center for Stable Isotope Biogeochemistry. $\delta^{15}$N and $\%N$ were determined by using an Europa 2020 mass spectrometer coupled to an automatic elemental analyzer (40). The isotopic abundance parameters are defined as follows:

$$\delta^{15}N = [\frac{^{15}N_{\text{sample}}}{^{15}N_{\text{standard}}} - 1] \times 1000$$

where $^{15}N_{\text{sample}}$ and $^{15}N_{\text{standard}}$ are the isotopic composition of nitrogen in the sample and the standard, expressed in parts per thousand ($\‰$). A value of $\delta^{15}N = -12.2\‰$ for example, indicates that $^{15}N_{\text{sample}}$ is 12.2$‰$ lower than the standard.

Calculations. The objective of the isotopic analyses is to determine $e_{\text{GDH}}$, the overall isotope effect associated with the assimilation of N. The isotope effect is most simply expressed by the isotopic difference between the starting pool of inorganic N in the medium and the first increment of biomass formed after inoculation. In mathematical terms, the isotopic difference is expressed as a ratio of isotope ratios. Because the isotope ratios are very similar, a notation is used that expresses the difference in terms of parts per thousand:

$$e_{\text{GDH}} = \frac{\delta^{15}N_{\text{sample}} - \delta^{15}N_{\text{initial medium}}}{1000}$$

where $\delta^{15}N_{\text{sample}}$ is the ratio of $^{15}$N to $^{14}$N in the initial medium and $\delta^{15}N_{\text{sample}}$ is the same ratio in the first increment of biomass. As growth proceeds, the measured isotopic compositions of both the medium and the biomass
change as a result of preferential transfer of either $^{15}$N or $^{14}$N (depending on the sign of the isotope effect) from the medium to the biomass. Measurements of $\varepsilon_0$ must take this change into account.

Here, we use the regression of $\varepsilon_0$ on $f_l(l−f_l)$ ln f, thus fitting the observations to a linear equation of the form:

$$\varepsilon_0 = \varepsilon_f - \frac{f_l}{1−f_l} \ln f$$

where $\varepsilon_0$ is the measured $\delta^{15}$N of the biomass, $\varepsilon_f$ is the measured $\delta^{15}$N of the medium at $t = 0$, and $f$ is the fraction of ammonia unused. If, for example, $\varepsilon_f = -18.8\%$, it indicates that $^{15}$N is assimilated and used to produce biomass 18.8 parts per thousand more slowly than $^{14}$N.

Values of $\varepsilon_0$ vary between experiments depending on the batch of NH$_4$Cl that was used. Specific values are, for experiments 1–5, 3.25 ± 0.19% (mean and SD, n = 8); for experiments 6–15, 1.43 ± 0.06% (n = 3); for experiments 16 and 17, 1.12 ± 0.19% (n = 2); for experiments 18 and 20, 21, 1.15 ± 0.21% (n = 2); for experiments 22–24, 1.36 ± 0.09% (n = 3); and for experiments 25–29, 0.91 ± 0.08% (n = 5).

Uncertainties in $\varepsilon_0$, calculated from the variance about the regression and expressed as SEs of the slope, are reported in Table 2 and range from 0.1% to 2.9%. Where weighted means are reported, the weighting factor is the inverse variance. The reported SEs of weighted means are conventional or dispersion-corrected, whichever is greater. Uncertainties reported for calculated isotope effects are derived by conventional propagation of errors.

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Supporting Information

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SI Results

TheAmtB–strain failed to consume all of the ammonium in the medium under NH₃-limiting conditions (0.5 mM total ammonium at pH 5.5) (Fig. S2A). Whereas residual ammonium left behind by the parental strain fell below our detection limit of 25 μM, the residual ammonium in the AmtB– strains remained ≥50 μM. We infer that, at ≤2 μM internal ammonium [assuming an internal pH 7.0 at an external pH 5.5 (1)], glutamine synthetase ceases to function in synthesis of glutamine (2). The AmtB– strains showed unusually high light scattering at the end of growth (~10% above the parental strain), and ~10% of the residual ammonium was unused. Moreover, at c₀ = 180 mM and c₀ = 89 mM (experiments 1 and 27), the molar C/N ratio in the biomass of the AmtB– strain was significantly elevated, rising from 4.3 to 4.9 and from 4.4 to 5.2, respectively, as ammonia in the growth medium was depleted. These values are sharply higher than those values observed in all other experiments (C/N ~ 4.0) (Fig. S3) and indicate a significant difference in cellular composition. We have not pursued the basis for increased light scattering by AmtB– strains (e.g., whether it is because of a difference in cell size or shape), and we have not explored the basis for changed cellular composition. There is precedent for changes in composition of the cell envelope (apart from proteins) in nutrient-limited WT bacteria of various sorts (3–5).

Because the growth of AmtB– strains ceases but detectable ammonium is left behind in the medium, the cells begin to consume glucose rapidly, whereas this rapid consumption of glucose occurs in the WT strain only after stationary phase has been reached (Fig. S1B). The phenomenon is very striking. We do not know whether the reasons for it are nonspecific (i.e., somehow because of slow growth generally) or specific. A particularly intriguing possibility is that the rapid consumption of glucose is caused by spontaneous cyclization of γ-glutamyl-phosphate, the high-energy intermediate in the glutamine synthetase reaction, to γ-glutamyl pyridolinedione (2-pyridolinedione 5-carboxylate) (5). If this compound is a dead end, its formation would require 1 mol glucose/mol.


SI Materials and Methods

Strain Construction. NCM3722 (6) was the parental strain for all genetic mutant strains used in this work (Table 1). NCM4587 (∆amtB::kan) was constructed by transducing NCM3722 to kanamycin resistance with P1 vir bacteriophage grown on Keio strain JWKO441-1 (7, 8). Analogously, strains carrying ∆gdhA::kan (NCM4454) and gldD::kan (NCM4453) were obtained by transducing NCM3722 to kanamycin resistance with phage grown on JWKO1750-6 or a gldD::kan insertion strain from the collection of Kang et al. (9), respectively. The kanamycin insert in NCM4587 was removed by site-directed recombination (7) to yield NCM4590 (∆amtB). NCM4701 (gdhD::kan ∆amtB) was constructed by transducing NCM44590 (∆amtB) to kanamycin resistance with phage grown on strain NCM4453 (gdhD::kan). NCM4199, which lacks the C terminus of AmtB, was constructed as described (10, 11).

amtB and gldD Gene Arrangement. The amtB gene is the second and last gene in the glnK amtB operon, and the gldA gene is in an operon by itself. The gldD gene is the second gene in the gldBDF operon. The lesion in gldD may be polar on gldF, but gldF has no known function under our growth conditions (12, 13).

Justification for gldD Mutant. The gldD gene codes for the small subunit of glutamine(amide)-2-oxoglutarate amino transferase (GOGAT), which has an electron transfer function (14). Although the small subunit was thought to have the capacity for NH₃-dependent (but not glutamine-dependent) synthesis of glutamate (15), which is why we originally inactivated it rather than gldB. Although the gldB product (large subunit of GOGAT) may, in fact, have the capacity for NH₃-dependent synthesis of glutamate, this capacity is low in vitro (16, 17); we do not think it influenced our experiments, because the gldD strain grew poorly at pH 7.4 on 0.5 mM NH₄Cl and did not grow at all at pH 5.5, even at 5 mM NH₄Cl (Results).


Fig. S1. (A) Growth (B) ammonium consumption, (C) glucose consumption, and (D) determination of $f_0$, for assimilation of external ammonium ($c_0 = 7 \mu M NH_4$, 0.5 mM NH$_4$Cl, 0.1% glucose, pH 7.4) by GOGAT$^-$ strains: $gltD$::kan from experiment 12 (open circles), experiment 29 (filled circles), and $gltD$::kan$\Delta$amtB from experiment 28 (open diamonds).

Fig. S2. (A) Ammonium consumption and (B) glucose consumption for WT (open squares) and AmtB$^-$ strains (open triangles, $c_0 = 0.089 \mu M NH_4$, 0.5 mM total NH$_4$Cl, 0.1% glucose, pH 5.5).
Fig. S3. C/N molar ratio of biomass. Experiments 2–30, excluding experiment 27, were combined. AmtB− strains (experiments 1 and 27, \( c_0 = 0.089 \) μM NH₃, 0.5 mM total NH₄Cl, 0.1% glucose, pH 5.5) are shown individually.