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 $^{15}$N and $^{14}$N ammonium. This 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 strain of *E. coli* K12 and mutant strains with lesions affecting ammonium-assimilatory proteins. We used isotope-ratio mass spectrometry 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 NH$_3$ (achieved by controlling total NH$_4$Cl and pH of the medium). At high NH$_3$ (≥ 0.89 µM), discrimination against the heavy isotope by the wild-type strain (-19.2‰) can be accounted for by the equilibrium isotope effect for dissociation of NH$_4^+$ to NH$_3$ + H$^+$. NH$_3$ equilibrates across the cytoplasmic membrane and glutamine synthetase does not manifest an isotope effect *in vivo*. At low NH$_3$ (≤ 0.18 µM), discrimination reflects an isotope effect for the NH$_4^+$ 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 NH$_3$ 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 NH$_3$ across the membrane (-10.9‰). The paper presents the raw data from which our conclusions were drawn and discusses the assumptions underlying them.
Introduction

Fractionation of heavy and light isotopes of nitrogen (\(^{15}\)N vs. \(^{14}\)N), carbon (\(^{13}\)C vs. \(^{12}\)C) and hydrogen (D vs. H) can provide information about metabolic pathways and reaction mechanisms within living organisms (1–4). For example, fractionation between \(^{15}\)N and \(^{14}\)N during incorporation of ammonium N in a single-celled organism like \(E. coli\) 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 that to glutamate, involve deamidation, the NH\(_3\) released is carried directly to the assimilatory catalytic site through a tunnel and hence cannot be protonated or diffuse away (6). The overall ratio of \(^{15}\)N to \(^{14}\)N in biomass is thus controlled by a step(s) at or prior to assimilation of ammonium N into glutamate.

In \(E. coli\), the proteins participating in early and potentially rate-determining steps in the incorporation of NH\(_4^+\) into glutamate are: 1) AmtB, its only membrane channel for NH\(_4^+\) (7); 2) glutamine synthetase (GS), the first enzyme of the high-affinity ammonium-assimilatory pathway; 3) glutamate synthase [glutamine(amide) 2-oxoglutarate amino transferase], GOGAT; and 4) glutamate dehydrogenase (GDH), the first enzyme of the low-affinity ammonium-assimilatory pathway (Fig. 1; reviewed in (8)). To study effects of these proteins on the \textit{in vivo} fractionation of ammonium N we used wild-type and genetic mutant strains in which one or more was lacking or defective and studied these strains at high or low external concentrations of NH\(_3\). To decrease the concentration of external NH\(_3\) and still achieve significant cell yield, we lowered both the total concentration of NH\(_4\)Cl and the pH of the medium. Though \(E. coli\) 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 we have chosen to study the behavior of *E. coli* at low external NH$_3$ are pertinent to its normal life cycle.

Ammonium (NH$_4^+$ + NH$_3$) is the optimal nitrogen source for *E. coli*, i.e., that which yields most rapid growth. Ammonium [pKa = 9.25] enters cells in two forms (Fig. 1). NH$_3$, 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, NH$_3$ is only ~ 0.02% of total ammonium. NH$_4^+$, 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 is 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 ([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 by 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 one mol of ATP per glutamate synthesized in the GS/GOGAT cycle drives assimilation of N even at extremely low concentrations of ammonium but is apparently detrimental when ammonium is abundant and energy is limiting (12). Both GS and GDH use NH$_3$ as their substrate because bond formation requires the lone pair of electrons on the N of NH$_3$ (6, 13). Hence, NH$_4^+$ must be dissociated to NH$_3$ + H$^+$ before either enzyme can use it. An equilibrium isotope effect associated with this spontaneous process leads to depletion of $^{15}$N in NH$_3$ relative to
NH₄⁺. Its magnitude is -19.2‰ (weighted mean, std. error = 0.4‰, n = 3; (14)). That is, at equilibrium, \(^{15}\text{N}/^{14}\text{N}\) in NH₃ is 19.2 parts per thousand lower than that in NH₄⁺.

To control the site of rate limitation, we employed well-characterized mutant strains (Table 1). One such strain (\(\Delta\text{amtB}\)) lacked AmtB. A second strain (\(\text{AmtB}\Delta\text{C-term}\)) had a poorly active AmtB channel in which the normal membrane pores lacked the usual carboxy-terminal cytoplasmic extensions (15, 16). A third strain (\(\Delta\text{gdhA}::\text{kan}\)) lacked GDH, the low-affinity ammonium-assimilatory enzyme. The last two strains (\(\text{gltD}::\text{kan}\) and \(\text{gltD}::\text{kan}\Delta\text{amtB}\)) lacked GOGAT (Table 1; (8)). We did not employ 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 wild-type (18, 19), under both ammonium-excess and limiting conditions. We determined doubling time, cell yield, residual ammonium in the medium, and the isotopic fractionation associated with incorporation of ammonium into cell material (see 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 wild-type strain.** The doubling time was 50 min. at all values of \(c_0\), the initial external concentration of ammonia (\(n.\ b.,\ \text{NH}_3,\ \text{not}\ \text{NH}_3 + \text{NH}_4^+;\) Table 2, Fig. 2A). For \(0.89 \leq c_0 \leq 280\ \mu\text{M}\), measured isotopic fractionations (\(\varepsilon_b\)) ranged from -16.1 to -23.8‰ (mean and std. dev. -19.2 ± 2.6‰, \(n = 7\)), with negative values of \(\varepsilon\) indicating depletion of \(^{15}\text{N}\) 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 (Table 2, Fig. 2B). AmtB is highly expressed even when the concentration of external
NH$_3$ is 5 to 10 times higher, namely 1 μM (5 mM total ammonium at pH 5.5; (11)), but 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 μM (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 wild type under all conditions of nitrogen availability and its isotopic fractionations were very similar to those of the wild-type strain (Table 2). For 0.89 ≤ c$_0$ ≤ 280 μM, ε$_b$ ranged from -18.8 to -25.4‰ (mean and std. dev. -21.3 ± 2.6‰, n = 7). For c$_0$ = 89 nM, ε$_b$ decreased to -6.1‰. Because the ranges of ε$_b$ 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 (gltD::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 approximately 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 μM, the doubling time of the strain increased from 50 to 65 min. (Table 2, 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‰ (std. error = 0.3‰, n = 3) at both concentrations of NH$_3$ (Fig. S1D). This strain did not grow at all at c$_0$ ≤ 1 μM (23).

In agreement with its dependence on the low-affinity enzyme, GDH, for synthesis of glutamate, gltD::kan has an abnormally low internal-free-pool concentration of glutamate at low NH$_3$ (27).
The \textit{gltD::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 glutamine (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 (\textit{gltD::kan\Delta amtB}) to be certain that AmtB was completely absent. The doubling time of the \textit{gltD::kan\Delta amtB} strain was slightly longer than that of the \textit{gltD::kan} strain but the isotopic fractionation was unchanged at $-10.3\%$ (Table 2, Fig. S1D).

\textit{AmtB} and \textit{AmtB}-defective strains. Finally, the AmtB$^{-}$ strain (\textit{\Delta amtB}) lacks the NH$_4^+$ 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 \textit{\Delta amtB} strain and its isotopic fractionation were identical to those of the wild-type and of the \textit{\Delta gdhA} strain (Table 2). In fact, under these conditions, the wild type does not transcribe the \textit{glnKamtB} operon (11, 23, 31). When the external concentration of NH$_3$ was decreased to 1 $\mu$M, the \textit{\Delta 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 NH$_3$ concentration further (11, 23). Under these conditions, the weighted-mean isotopic fractionation was $-30.1\%$ (std. error = 0.6\%, $n = 2$), markedly larger than that observed in other strains.

For the strain in which the AmtB protein was modified at the carboxyl terminal (AmtB$\Delta$C-term; (15, 16)), both the doubling time and isotopic fractionation at $c_0 = 0.89$ $\mu$M did not differ from those of the wild-type, GDH-, and AmtB$^{-}$ strains (Table 2). However, when $c_0$ was decreased to 89
nM, AmtBΔC-term had a doubling time of 110 min., more than twice as long as that of the wild type and approximately half as long as that of ΔamtB. Under this condition, the activity of AmtB appeared to be rate-limiting for growth and the weighted-mean $\varepsilon_b$ was $-17.6\%$ (std. error $= 0.3\%$, $n = 2$).

Process-related summary of isotopic observations. Table 2 includes 11 different observations of $\varepsilon_b$ for cells equilibrating NH$_3$ by diffusion and having both the low- and high-affinity pathways for its assimilation (Fig. 1). These include seven wild-type cultures with $0.89 \leq c_0 \leq 280$ µM, three ΔamtB cultures with $0.89 \leq c_0 \leq 70$ µM and one AmtBΔC-term strain with $c_0 = 0.89$ µM. Values of $\varepsilon_b$ range from -16.1 to -23.8‰. The weighted mean is $-19.6\%$ (std. error = 0.7‰). There were seven different observations for cells equilibrating NH$_3$ by diffusion and lacking the low-affinity GDH pathway for its assimilation. For these ΔgdhA cultures, as for wild-type, $0.89 \leq c_0 \leq 280$ µM. Values of $\varepsilon_b$ range from -18.8 to -25.4‰. The weighted mean is $-22.2\%$ (std. error = 0.9‰). In total, there are 18 different observations of $\varepsilon_b$ for cells equilibrating NH$_3$ by diffusion and using the high-affinity GS-GOGAT cycle to incorporate ammonium N into organic molecules. Values of $\varepsilon_b$ range from -16.1 to -25.4‰. The weighted mean is $-21.1\%$ (std. error = 0.6). Variations of $\varepsilon_b$ are not correlated with $c_0$ ($r^2 = 0.13$). Three values of $\varepsilon_b$ were obtained for cells relying on the AmtB channel for transport of NH$_4^+$ and assimilating ammonium N via GS + GOGAT. In those cases, two wild-type cultures with $c_0 = 0.089$ and 0.18 µM and one ΔgdhA culture with $c_0 = 0.089$ µM, $\varepsilon_b$ ranged from -5.4 to -8.1‰. In four cases, cells incorporated N using GS + GOGAT but either had no AmtB channel or an AmtB channel that was impaired by deletion of the C-terminal extensions. When AmtB was entirely absent (two cases, $c_0 = 0.18$ and 0.089 µM), weighted-mean $\varepsilon_b$ was $-30.1\%$. When AmtB was only modified ($c_0 = 0.089$ µM, two cases), the weighted-mean $\varepsilon_b$ was $-17.6\%$. Finally, when cells lacked GOGAT (three cultures of ΔgltD and one of ΔgltDamtB::kan), values of $\varepsilon_b$ varied from -10.3 to -11.5‰ with a weighted mean of $-10.5\%$ (std. error = 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. Though 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 (see Process-related summary), which reduces the outputs to two. The isotopic budget is summarized in Fig. 3A. Balancing the input against the outputs, we can write

\[
\delta_{ae} + \varepsilon_{at} = g(\delta_{ai} + \varepsilon_{GS}) + (1 - g)(\delta_{ai} + \varepsilon_{at})
\]

where the \(\delta\) values are defined in Fig. 3A, \(\varepsilon_{at}\) is the isotope effect associated with transport of NH₃ across the membrane, and \(g\) is the fraction of the input that is incorporated into biomass. When equilibration of NH₃ across the membrane is rapid in comparison to the rate of assimilation, \(g \to 0\) (n. b., \(g\) is not the amount of NH₃ that is assimilated but instead the fraction which is assimilated) and \(\delta_{ae} = \delta_{ai}\). Because \(\delta_{ii} = \delta_{ai} + \varepsilon_{GS}\), the isotopic composition of the external NH₃ is related to that of the cells by \(\delta_{ae} = \delta_{i} - \varepsilon_{GS}\). Since essentially all external N is in the form of NH₄⁺, \(\delta_{ae} = \delta_{b} + \varepsilon_{h}\), where \(\delta_{b}\) is the measured isotopic composition of the N supplied to the medium and \(\varepsilon_{h}\) is the equilibrium isotope effect relating NH₄⁺ and NH₃. Finally, recalling that measured values of \(\varepsilon_{b}\) are equal to \(\delta_{b} - \delta_{h}\), we obtain \(\varepsilon_{GS} = \varepsilon_{b} - \varepsilon_{h}\). As noted above and summarized in Table 3, 18 experiments yielded \(\varepsilon_{h} \approx -21.1\%\). The 95% confidence interval of that value overlaps with the 95% confidence interval for \(\varepsilon_{h}\). Accordingly, there is no evidence for fractionation by GS.

An alternative interpretation, with \(g\) appreciably greater than zero, is not tenable. Specifically, the 18 experiments yielding \(\varepsilon_{b} \approx \varepsilon_{h}\) could then be explained only if \(i) g, \varepsilon_{GS}, and \varepsilon_{at}\) happened in all
cases to have values satisfying the relationship \( g = \varepsilon_{GS}/(\varepsilon_{GS} - \varepsilon_{at}) \) 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 \(-0.7 \pm 0.6\%\) (mean and standard deviation, \( n = 7 \), (35)).

It is unlikely that GS limits growth because it is synthesized in excess when supplies of NH\(_3\) are plentiful and its catalytic activity is regulated downwards 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 (\( \Delta \)amtB) depend on uncatalyzed transport of NH\(_3\) across the membrane. At \( c_0 \leq 0.2 \) µM, growth of the \( \Delta \)amtB strain is extremely slow and the rate decreases as the external concentration of NH\(_3\) declines (Table 2, Fig. 2A). The mass balance described by Eq. 1 applies but the conditions differ from those just discussed. Instead, \( g \neq 0 \) and, because GS imposed no fractionation even when supplies of NH\(_3\) were abundant, we know that \( \delta_{ai} = \delta_{a} \) and \( \varepsilon_{GS} = 0 \). Making these substitutions and simplifying, we obtain \( \delta_{ae} = \delta_{a} - g\varepsilon_{at} \). Substituting \( \delta_{ae} = \delta_{e} + \varepsilon_{h} \) and \( \varepsilon_{b} = \delta_{b} - \delta_{e} \) leads to \( \varepsilon_{b} = g\varepsilon_{at} + \varepsilon_{h} \). At the limit in which growth is limited by transport of NH\(_3\) and \( g \rightarrow 1 \), \( \varepsilon_{at} = \varepsilon_{b} - \varepsilon_{h} \). If the slowest growing cultures (experiments 27 and 1, Table 2) represent that case, \( \varepsilon_{at} = -30.1 + 19.2 = -10.9 \pm 0.7\% \) (std. error 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., 20\%\(_{oo}\), almost twice that estimated here).
For cells with a normal AmtB channel (wild-type or ΔgdhA strain) and with \( c_0 = 0.089 \, \mu\text{M} \), \( \varepsilon_b \) was observed as low as -5.4 ± 0.3‰, much lower than that of a strain lacking the channel (ΔamtB). The corresponding mass balance is shown schematically in Fig. 3B and expressed mathematically in Eq. 2.

\[
\delta_e + \varepsilon_{ht} = g \delta_b + (1 - g)(\delta_b + \varepsilon_{at})
\]

Here, \( \varepsilon_{ht} \) is the isotope effect associated with transport of \( \text{NH}_4^+ \) by the AmtB channel and substitutions introduced above have been adopted where appropriate (\( \delta_{he} = \delta_e \), \( \delta_{ai} = \delta_b \)). Simplifying gives \( \varepsilon_b = \varepsilon_{ht} - (1 - g)\varepsilon_{at} \). A recent quantitative study of AmtB function (11) indicates that \( g \) is ~ 0.2. For \( \varepsilon_b = -5.4 \, \text{‰} \), adopting \( \varepsilon_{at} \approx -10.9 \, \text{‰} \) (see above), we find \( \varepsilon_{ht} = -14.1 \, \text{‰} \). If an uncertainty of 0.05 is assigned to \( g \), the estimated std. error of \( \varepsilon_{ht} \) is 0.7‰. Notably, the reduced fractionation at low values of \( c_0 \), a condition that may be encountered in nature, derives not only from fractionations associated with the AmtB channel but from an interplay between fractionations associated with \( \varepsilon_{ht} \) and \( \varepsilon_{at} \).

When the wild-type strain was grown with a slightly higher \( c_0 = 0.18 \, \mu\text{M} \), the observed fractionation increased to -8.1‰. The AmtB channel also functions at this \( \text{NH}_3 \) concentration because an AmtB- strain continues to grow suboptimally. Hence eq’n 2 applies. Using \( \varepsilon_{ht} = -14.1 \pm 0.7 \, \text{‰} \) 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 \( \text{NH}_3 \) at higher external \( \text{NH}_3 \) concentrations (11).

For cells with an altered AmtB channel lacking the cytoplasmic C-terminal extensions (16), \( \varepsilon_b = -17.6 \pm 0.3 \, \text{‰} \) (wt’d. mean and std. error) at \( c_0 = 0.089 \, \mu\text{M} \). The fraction of N assimilated by GS is 0.5 (11) and solving as above yields \( \varepsilon'_{ht} = -23.0 \pm 0.7 \, \text{‰} \) (where a prime is used to denote the
altered channel). If the uncertainty assigned to $g$ is doubled (to 0.1), the standard error increases only to $\pm 1.2\%$. Hence the isotope effect for the mutant AmtB channel is significantly larger than that for the wild-type channel. The mutant channel is known to lack coordination between the function of its individual monomers and to have other unusual properties (40).

To make *E. coli* dependent on the low-affinity pathway for assimilation of ammonium we inactivated GOGAT. This eliminates the GOGAT cycle and makes the organism dependent on biosynthetic GDH. At an external NH$_3$ concentration of 10 $\mu$M, GDH activity already limits the growth rate of the GOGAT$^-$ strain, and the strain does not grow at all at $c_0 = 1$ $\mu$M. The fractionation observed for the GOGAT$^-$ strains with $c_0 \geq 7$ $\mu$M is $-10.5\%$. Assuming that NH$_3$ inside and outside the cell was in equilibrium, as for the wild-type, $\Delta$gdhA, and $\Delta$amtB 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 $\varepsilon_b = -10.5\%$ requires inverse fractionation of $^{15}$N by GDH (*i.e.*, enrichment of the product relative to the reactant) with $\varepsilon_{GDH} = 8.7 \pm 0.4\%$. 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 effect for transport of NH$_3$ is the first for a biological membrane. A previous measurement was made *in vitro* with a membrane filter (14).

That $\varepsilon_b$ for the $\Delta$gdhA strain is the same as that for the wild-type strain at all external concentrations of NH$_3$ confirms the finding of Yuan et al. (24, 25) that the GOGAT cycle is the major means for assimilation of NH$_3$ by *E. coli* K12 not only at low but also at high concentrations. Although the GOGAT cycle is widespread in bacteria and archaea (8, 42, 43), whereas the occurrence of biosynthetic GDH appears to be more restricted (44), several important examples of organisms naturally lacking GOGAT have recently come to light (45). Determining $\varepsilon_b$ for the
abundant ocean archaean *Nitrosopumilis maritima* will be particularly interesting not only because it lacks GOGAT and depends on GDH for ammonium assimilation but also because it oxidizes ammonium extracellularly as its primary energy source.

We hope that the present results will help future workers to correlate environmental genomic data with isotopic variations observed in nature.

*Comparison to earlier work.* Studying the γ-proteobacterium *Vibrio harveyi*, a close evolutionary relative of *E. coli* K12, Hoch *et al.* (46) also found that isotopic fractionation between external ammonium and cell material varied with external ammonium availability. Now, by using AmtB- strains, we have determined that the decrease in $\varepsilon_b$ from $\sim -20\%$ to $\sim 4\%$ that they observed as external ammonium was dropped from an initial concentration of $\sim 500$ to $\sim 25$ μM (pH 7.4) did, as they proposed, depend on the activity of an active ammonium channel. Specifically, $\varepsilon_b \sim -5\%$ results from (i) acquisition of NH$_4^+$ rather than NH$_3$ by the growing cells, (ii) an isotope effect associated with transport of NH$_4^+$ by the AmtB channel ($\varepsilon_{ht} \sim -14\%$), (iii) equilibration of NH$_4^+$ and NH$_3$ inside the cell, (iv) an absence of isotopic fractionation during assimilation of NH$_3$ by GS, and (v) leakage of $^{15}$N-depleted NH$_3$ from the cells.

Given the very large $\varepsilon_b$ characteristic of *E. coli* AmtB- strains at low external NH$_3$ ($\sim 30\%$), which appears to result from rate-limiting diffusion of NH$_3$ across the cytoplasmic membrane, it is tempting to speculate that the fractionation observed in *V. harveyi* as the external concentration of ammonium decreased from 182 to 107 μM in a single experiment [$\varepsilon_b = -26.5\%$; (46)] may indicate the precise range of external ammonium concentrations at which unmediated diffusion of NH$_3$ becomes limiting in wild-type *V. harveyi*, just prior to activation of its AmtB channel. In *E. coli* expression of AmtB occurs in response to a decrease in the internal-free-pool concentration of glutamine, whereas activation requires, in addition, an increase in the pool concentration of its
precursor metabolite 2-oxoglutarate (11). The latter occurs at lower external NH$_3$ concentrations than the former. Activation requires release of the inhibitory gating protein GlnK (20–22).

Finally, we think that the decrease in $\varepsilon_b$ from -21 to -14‰, which Hoch et al. observed above 5 mM external ammonium, is an artifact of growth inhibition (doubling time increased from the optimal of 84 min. to 138 min. at high ammonium). Whatever the explanation, the $\varepsilon_b$ of -14‰ cannot be characteristic of GDH, as they proposed, because the NADH-dependent GDH they characterized is a catabolic enzyme. The genome sequence of *V. harveyi* is now known and it apparently lacks a biosynthetic, NADPH-dependent GDH. Moreover, we find that the biosynthetic, NADPH-dependent GDH of *E. coli* contributes very little to ammonium assimilation even at 20 mM ammonium, their highest concentration and ours.

**Materials and Methods**

**Bacterial Strains and Cultures.** NCM3722 (18) was the parental strain for all genetic mutant strains used 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 of 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 optical density 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 were removed from the supernatant by high-speed centrifugation. The cell-free supernatant was frozen at -80°C for later measurement of residual ammonium, glucose, and final pH. The cells were washed twice 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 pre-weighed tin capsules (part number 240-053-00, Costech Analytical Technologies, Inc.). Capsules containing only the reactant glucose and ammonium chloride used in the media were also prepared. All samples were analyzed at the UC Berkeley Center for Stable Isotope Biogeochemistry. δ⁵N and %N were determined by using a PDZ Europa system consisting of an ANCA-NT carbon/nitrogen analyzer in combination with a 2020 mass spectrometer (48). The isotopic-abundance parameters are defined as follows:

\[ \delta^{15}N = 10^{3}\left(\frac{^{15}R_{\text{sample}}}{^{15}R_{\text{standard}}} - 1\right) \]

Where \( ^{15}R \equiv ^{15}N/^{14}N \) and the isotopic standard for nitrogen is N₂ in air, for which \( ^{15}R = 0.0036765 \) (49). Values of \( \delta^{5}N \) express the relative difference between the isotope ratio in the sample and in the standard, expressed in parts per thousand (‰). A value of \( \delta^{5}N = -12.2\%_0 \), for example, indicates that \( ^{15}R_{\text{sample}} \) is 0.0036316. The precision of the analyses, expressed as a standard deviation of a single observation and based on five pairs of duplicates and four sets of triplicates collected during the analyses (thus 13 degrees of freedom) is 0.06‰.

Calculations. The objective of the isotopic analyses is to determine \( \epsilon_b \), the overall isotope effect associated with the assimilation of N. This 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 following inoculation. In mathematical terms, the isotopic difference is expressed as a ratio of isotope ratios. Since the isotope ratios are very similar, a notation is used that expresses the
difference in terms of parts per thousand:

\[ [4] \varepsilon_b = 10^3\left([\frac{^{15}R_{e0}}{^{15}R_{b0}}] - 1\right) \]

where \(^{15}R_{e0}\) is the ratio of \(^{15}\)N to \(^{14}\)N in the initial medium and \(^{15}R_{b0}\) 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 \(\varepsilon_b\) must take this into account.

Here, we employ the regression of \(\delta\) on \([f/(1 - f)] \cdot \ln f\) (1), thus fitting the observations to a linear equation of the form:

\[ [5] \delta_b = \delta_0 - \left[\frac{f}{(1 - f)}\right] \varepsilon_b \cdot \ln f \]

where \(\delta_b\) is the measured \(\delta^{15}\)N of the biomass, \(\delta_0\) is the measured \(\delta^{15}\)N of the medium at \(t = 0\), and \(f\) is the fraction of ammonium unutilized. If, for example, \(\varepsilon_b = -18.8\%\), it indicates that \(^{15}\)N is assimilated and used to produce biomass 18.8 parts per thousand more slowly than \(^{14}\)N.

Values of \(\delta_0\) 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 standard deviation, \(n = 8\)); 6-15, 1.43 ± 0.06\% (\(n = 3\)); 16-17, 1.12 ± 0.19\% (\(n = 2\)); 18-19, 0.96 ± 0.19\% (\(n = 2\)); 20-21, 1.15 ± 0.21\% (\(n = 2\)); 22-24, 1.36 ± 0.09\% (\(n = 3\)); and 25-29, 0.91 ± 0.08\% (\(n = 5\)).

Uncertainties in \(\varepsilon_b\), calculated from the variance about the regression and expressed as standard errors 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 standard errors 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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former colleague, Wally van Heeswijk. Deceased at 49, Wally discovered GlnK, which is the most
widespread regulatory protein known in the living world.
References


Figure legends

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

**Fig. 2.** Growth (A) and (B) determination of $\varepsilon_b$ for assimilation of external ammonium ($c_0 = 0.089 \mu M \text{NH}_3$, 0.5 mM total NH$_4$Cl, 0.1% glucose, pH 5.5) into biomass in wild-type (squares) and AmtB strains (triangles). Cultures in panel B were different from those in panel A, which were sampled more frequently.

**Fig. 3.** Flows of N across the cell membrane and within cells. Isotopic compositions are denoted by $\delta$ terms and isotope effects are denoted by $\varepsilon$ terms. In subscripts, a designates NH$_3$; b, biomass; e, external; GS, glutamate synthetase; h, NH$_4^+$ or protonation; i, internal; and t, transport. Isotopic compositions of the N fluxes are then given by expressions such as $\delta_{he} + \varepsilon_{ht}$, which indicates the isotopic composition of the ammonium being transported by the AmtB channel. (A) Cells not utilizing the AmtB channel to actively transport NH$_4^+$. (B) Cells in which the AmtB channel is used.
A)  

B)
### Table 1. Bacterial strains*

<table>
<thead>
<tr>
<th>Strain: Genotype</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCM3722: <em>E. coli</em> K12 wild-type</td>
<td>wild-type</td>
</tr>
<tr>
<td>NCM4199: <em>amtB</em>, <em>tesB</em>::kan</td>
<td>AmtBΔC-term†</td>
</tr>
<tr>
<td>NCM4453: <em>gltD</em>::kan</td>
<td>GOGAT‡</td>
</tr>
<tr>
<td>NCM4454: Δ<em>gdhA</em>::kan</td>
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</tr>
<tr>
<td>NCM4590: Δ<em>amtB</em></td>
<td>AmtB</td>
</tr>
<tr>
<td>NCM4701: <em>gltD</em>::kanΔ<em>amtB</em></td>
<td>GOGAT, AmtB</td>
</tr>
</tbody>
</table>

*All strains were constructed in the background of a physiologically robust *E. coli* K12 wild-type strain, NCM3722 ([18, 19]; see Materials and Methods).
† Residues from position 382 onward were deleted (15, 16).
‡ Dalai Yan, Indiana University School of Medicine.
<table>
<thead>
<tr>
<th>Exp.</th>
<th>Strain*</th>
<th>(c_0) (NH₃) † µM</th>
<th>D.T.‡ min.</th>
<th>(n^§)</th>
<th>(\varepsilon_b) ¶ ‰</th>
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<td>110</td>
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<td>-17.8 ± 0.4</td>
</tr>
</tbody>
</table>

*Strain number (for phenotype see Table 1).
†Concentrations of NH₃ determined from total concentrations of NH₄Cl, which were 71-fold higher than that of NH₃ at pH 7.4 and 5620-fold higher at pH 5.5. Cultures with \(c_0 \leq 0.89\) were grown at pH 5.5.
‡Doubling time.
§Number of points used in the determination of \(\varepsilon_b\).
¶Reported uncertainty is the standard error of the slope derived from the regression described in the section on calculations.
Table 3. Process-related isotope effects

<table>
<thead>
<tr>
<th>Process Related Experiments</th>
<th>Related Experiments</th>
<th>Result*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Assimilation of NH$_3$ by GS</td>
<td>Wild type, $c_0 \geq 0.89$ µM, $n = 7$</td>
<td>95% Confidence intervals:</td>
</tr>
<tr>
<td>For $g \to 0$</td>
<td>AmtB, $c_0 \geq 0.89$ µM, $n = 3$</td>
<td>$\bar{\delta}_b = -21.1 \pm 1.3%$</td>
</tr>
<tr>
<td>$\delta_{GS} = \delta_b - \delta_h$</td>
<td>AmtBΔC-term, $c_0 = 0.89$ µM, $n = 1$</td>
<td>$\bar{\delta}_h = -19.2 \pm 1.7%$</td>
</tr>
<tr>
<td></td>
<td>GDH, $c_0 \geq 0.89$ µM, $n = 7$</td>
<td>$\delta_{GS} \sim 0$</td>
</tr>
<tr>
<td>2. Transmembrane transport of NH$_3$</td>
<td>AmtB, $c_0 &lt; 0.2$ µM, $n = 2$</td>
<td>$\bar{\delta}_b = -30.1 \pm 0.5%$</td>
</tr>
<tr>
<td>For $g \to 1$</td>
<td>GDH, $c_0 = 0.089$ µM, $n = 1$</td>
<td>$\bar{\delta}_{at} = -10.9 \pm 0.7%$</td>
</tr>
<tr>
<td>$\delta_{at} = \delta_h - \delta_h$</td>
<td>Wild type, $c_0 = 0.089$ µM, $n = 1$</td>
<td>$\bar{\delta}_{ht} = -5.5 \pm 0.3%$</td>
</tr>
<tr>
<td>3. Transport of NH$_4^+$ by AmtB</td>
<td>GDH, $c_0 = 0.089$ µM, $n = 1$</td>
<td>for $g = 0.2 \pm 0.05$,</td>
</tr>
<tr>
<td>$\bar{\delta}<em>{ht} = \delta_h + (1 - g)\delta</em>{at}$</td>
<td>$\bar{\delta}_{ht} = -14.1 \pm 0.8%$</td>
<td></td>
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<tr>
<td>4. Transport of NH$_4^+$ by altered AmtB</td>
<td>AmtBΔC-term, $c_0 = 0.089$ µM, $n = 2$</td>
<td>$\bar{\delta}_b = -17.6 \pm 0.3%$</td>
</tr>
<tr>
<td>$\bar{\delta}<em>{ht} = \delta_h + (1 - g)\delta</em>{at}$</td>
<td>for $g = 0.5 \pm 0.1$,</td>
<td>for $g = 0.5 \pm 0.1$,</td>
</tr>
<tr>
<td>$\bar{\delta}_{ht} = -23.1 \pm 1.2%$</td>
<td>$\bar{\delta}_{ht} = -23.1 \pm 1.2%$</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>$\bar{\delta}_b = -10.5 \pm 0.2%$</td>
</tr>
<tr>
<td>$\delta_{GDH} = \delta_h - \delta_h$</td>
<td>GOGAT, $c_0 \geq 7$ µM, $n = 3$</td>
<td>$\delta_{GDH} = 8.8 \pm 0.4%$</td>
</tr>
</tbody>
</table>

* $\bar{\delta}$ denotes weighted mean. Indicated uncertainties are standard errors except for process 1, where 95% confidence intervals are specified.