RESEARCH LETTER

Nitrogen source influences natural abundance ^{15}N of *Escherichia coli*

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Abstract

Escherichia coli cells were forced to mineralize or assimilate nitrogen *in vitro* by manipulating substrate carbon and nitrogen availability. When grown on an organic nitrogen source, *E. coli* cells released NH_4^+ and were enriched in ^{15}N relative to the nitrogen source (1.6–3.1‰). However, when cells were grown on an inorganic nitrogen source, the biomass was depleted (6.1–9.1‰) relative to the source. By measuring ^{15}N enrichment of microorganisms relative to nitrogen pools, ecosystem ecologists may be able to determine if microorganisms are assimilating or mineralizing nitrogen.

Introduction

Natural abundance stable isotopes are effective tools for elucidating biogeochemical processes in microbial ecology (Högberg, 1997; Robinson, 2001; Boschker & Middelburg, 2002; Staddon, 2004). Exploiting differences in isotope signatures between nutrient source and sink has made it possible to link microbial activity to ecosystem processes (Miyazaki *et al.*, 1980; Orphan *et al.*, 2001; Townsend *et al.*, 2003). Ecosystem ecologists are increasingly interested in measuring the natural abundance isotopic composition of the microbial biomass or DNA extracted from the environment in order to interpret dominant microbial processes (Dijkstra *et al.*, 2006a, b; Schwartz *et al.*, 2007). However, mixing of nitrogen (N) source pools and the great number of isotopically fractionating element transformations in the environment creates difficulty in interpreting environmental isotopic signatures (Högberg, 1997; Robinson, 2001). For this reason, researchers have called for controlled culture experiments to clarify mechanisms of cellular enrichment (Gannes *et al.*, 1997; Hobbie *et al.*, 2001). Past work shows that microbial cells can be either enriched or depleted in ^{15}N, with no clear consensus on the mechanism determining the outcome. For example, Macko & Estep (1984) showed that *Vibrio harveyi* cells cultured on different amino acids are either enriched or depleted in ^{15}N. Henn & Chapela (2004) showed that ammonia (NH_3) assimilation in fungi led to strong depletion of ^{15}N in the microbial biomass relative to the nitrogen source. Hobbie *et al.* (2001) have shown that ^{15}N natural abundance measurements can differentiate between organisms of different trophic strategies. To date there are no reports comparing ^{15}N enrichment of bacteria grown in media with inorganic vs. organic nitrogen sources.

In environments with low carbon (C) availability, organisms utilize organic nitrogen-substrates such as amino acids or proteins as a source of carbon. Following deamination of nitrogenous carbon compounds, NH_4^+ is released from the cell (Paul & Clark, 1996). Because transport of NH_3 is a fractionating process that strongly selects for the lighter nitrogen isotope, bacteria forced to mineralize nitrogen may become enriched in ^{15}N while cells that assimilate NH_3 will become depleted in ^{15}N (Henn & Chapela, 2004; Dijkstra *et al.*, 2006a, b). If true, this observation would be of great

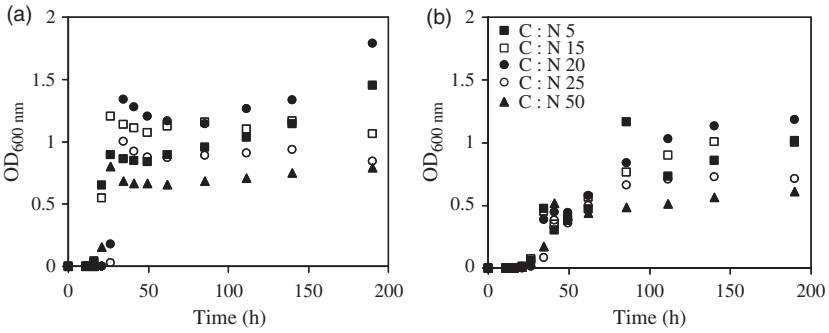

Fig. 1. Changes in OD of *Escherichia coli* cultures grown in (a) NH_4^+/glucose media or (b) glycine/glucose over time at different initial C : N ratios of the media.

use to ecosystem ecologists because microbial assimilation and mineralization of nitrogen strongly influence nitrogen availability to plants, and thus plant growth.

We cultured *E. coli* in defined media where the nitrogen source and concentration were controlled. Nutrient availability in the media was manipulated to force organisms to either mineralize or assimilate nitrogen. To test if cells become enriched in ^{15}N relative to the nitrogen source when releasing NH_4^+ and depleted in ^{15}N relative to source when assimilating NH_4^+, we measured the $\delta^{15}N$ of the *E. coli* cells among these treatments.

Materials and methods

Stock *E. coli* strain DH5α-T1 (Invitrogen Corp., Carlsbad, CA) was inoculated (*c.* 10^6 cells) into flasks with 250 mL of minimal medium made with either $(NH_4)_2SO_4$ or glycine as the sole nitrogen source. Cultures were incubated at 37 °C with shaking. Carbon and nitrogen in media were added to yield C : N molar ratio of 5, 15, 20, 25, and 50 for each nitrogen source. All media contained 7 g L^{-1} Na_2HPO_4, 2 g L^{-1} KH_2PO_4, 0.1 g L^{-1} $MgSO_4 \cdot 7H_20$, 0.02 g L^{-1} $CaCl_2 \cdot 2H_20$, 10 mL L^{-1} ATCC Trace Element Supplement, and 10 mL L^{-1} ATCC Vitamin Supplement, and were adjusted to pH 7.5. The media were filter sterilized using a 0.2 μm Nalgene vacuum filter. Ammonium/glucose medium with C : N ratios of 5, 15, 20, 25 and 50 contained 1 g L^{-1} $(NH_4)_2SO_4$ and 2.5 g L^{-1} D(+)-glucose, 0.5 g L^{-1} $(NH_4)_2SO_4$ and 3.75 g L^{-1} D(+)-glucose, 0.5 g L^{-1} $(NH_4)_2SO_4$ and 5.0 g L^{-1} D(+)-glucose, 0.25 g L^{-1} $(NH_4)_2SO_4$ and 3.12 g L^{-1} D(+)-glucose, and 0.20 g L^{-1} $(NH_4)_2SO_4$ and 5.0 g L^{-1} D(+)-glucose, respectively. Glycine/glucose medium with C : N ratios of 5, 15, 20, 25, and 50 contained 1.14 g L^{-1} glycine and 1.5 g L^{-1} D(+)-glucose, 0.5 g L^{-1} glycine and 2.86 g L^{-1} D(+)-glucose, 0.5 g L^{-1} glycine and 3.96 g L^{-1} D(+)-glucose, 0.25 g L^{-1} glycine and 2.52 g L^{-1} D(+)-glucose, and 0.20 g L^{-1} glycine and 4.22 g L^{-1} D(+)-glucose, respectively.

Each treatment had a total of three replicates. At five points along the growth curve, subsamples were removed from each flask. *Escherichia coli* growth was determined through absorbance measurements at 600 nm with a spectrophotometer (Fig. 1) (Eppendorf North America Inc., Westbury, NY). At all harvest times the concentration of cells was much higher than the 4000 cells mL^{-1} added to the medium at the beginning of the incubation so that the isotopic signature of the inoculant did not impact the isotopic composition of the cells at harvest. Cells were separated from media by centrifugation and were washed twice with 0.085% (w/v) NaCl. Media and cells were maintained at −20 °C before analysis. Cells were dried under laminar flow for *c.* 24 h, or until completely dry and ground into a fine powder by hand with a mortar and pestle and packed into 3.5 mm × 9 mm tin capsules (Costech Analytical Technologies Inc., Valencia, CA). Ammonium concentration in media was determined by flow injection analysis on a Lachat QuickChem 8000 Automated Ion Analyzer (Lachat Instruments, Loveland, CO) (Prokopy, 2003).

To measure $\delta^{15}N$ of NH_4^+, acid–base diffusion was used to prepare NH_4^+ samples for $\delta^{15}N$ analysis (Stark & Hart, 1996). The volume of sample required per diffusion was targeted for 40 μg of nitrogen. National Institute of Standards and Technology (NIST) standard reference materials NBS-1 and NBS-2, and $(NH_4)_2SO_4$ were also diffused using this procedure. For each sample, a 7-mm diameter Whatman GF/F filter disc was acidified with 20 μL of 2.5 M $KHSO_4$ and sandwiched between two layers of Teflon tape (Stark & Hart, 1996). This acid trap package was added to media and 0.5 M NaCl solution. The solution was saturated with MgO and sealed, forcing the NH_4^+ in the solution to NH_3, which volatilized and collected on the acid trap as NH_4^+. Samples were incubated at 30 °C for 7 days to maximize recovery of NH_4^+ and NH_3 (Sigman *et al.*, 1997; Holmes *et al.*, 1998). Acid traps and filters were dried in a desiccator equipped with an acid trap to avoid contamination by atmospheric NH_3. Once dry, filters were removed from the Teflon tape and packed into 5 mm × 9 mm tin capsules (Costech). Samples and diffused standards were analyzed on the mass spectrometer within 48 h, or were stored on desiccant to avoid disintegration of the tin from $KHSO_4$.

The natural abundances of ^{15}N of cells and of glass filters were measured using a Carlo Erba NC 2100 Elemental Analyzer (CE Instruments, Milan, Italy) interfaced with a Thermo-Finnigan Delta Plus XL isotope ratio mass spectrometer (Thermo-Electron Corp., Bremen, Germany). The ^{15}N compositions were expressed in standard delta notation ($\delta^{15}N$) in parts per thousand (‰) relative to atmospheric N_2, where $\delta = 1000 \times [(R_{sample}/R_{standard}) - 1]$ and R is the molar ratio $^{15}N/^{14}N$ (Lajtha & Michener, 1994). The external precision on repeated measurements of an internal standard (NIST 1547 – peach leaves) was < 0.20‰. The SD of standards prepared by diffusion was 0.8‰ or better.

Repeated measures multivariate ANOVA (MANOVA) test was used to determine if isotopic composition of microbial cells were significantly different between treatments. A separate MANOVA was run for each nitrogen source. The response variables, time, C : N ratio, and the time by C : N interaction, were determined in cultures of *E. coli*. All statistical analyses were performed using JMP 4 statistical package (SAS Institute, Cary, NC). Alpha values <0.05 were considered statistically significant in these analyses.

Results and discussion

The aim of this study was to test if the nitrogen isotopic composition of *E. coli* was impacted by growth on an inorganic vs. organic nitrogen source and to determine whether the relative availability of carbon and nitrogen influenced the degree of ^{15}N enrichment.

Escherichia coli cells grown in NH_4^+/glucose media, regardless of C : N, were initially strongly depleted in ^{15}N (5.9–8.4‰) relative to the nitrogen source (Fig. 2a). Henn & Chapela (2004) also found that fungal biomass was depleted relative to the nitrogen source when grown on NH_4^+. Hobbie *et al.* (2001) showed that when host plants assimilated nitrogen from ectomycorrhizal fungi they became depleted in ^{15}N relative to the nitrogen source. Two factors may influence the depletion in biomass when assimilating NH_4^+. The activation energy of deprotonation of $^{14}NH_4^+$ is smaller than $^{15}NH_4^+$. Therefore, NH_3 tends to be depleted in ^{15}N relative to NH_4^+ (Bigeleisen, 1965). Ammonia, not NH_4^+, is likely the form taken up by cells because at high concentrations NH_3 can passively diffuse through cell membranes or ammonia transporters (Burkovski, 2003). In addition, fractionation by glutamate dehydrogenase in the NH_4^+ assimilation pathway may have resulted in depletion in microbial biomass (Hoch *et al.*, 1994; Henn & Chapela, 2004). C : N ratio did affect the ^{15}N signature of *E. coli* over time. After 50 h of growth cells in the NH_4^+/glucose medium with a C : N ratio of 5 became less depleted relative to the initial isotopic signature of the NH_4^+ on which the cells were grown (Fig. 2a). Cells in this treatment were carbon limited and were unable to utilize all available nitrogen (Fig. 3a). Different amounts of nitrogen-subtrates were added to the C : N ratio treatments, which impacted how much biomass accumulated in the cultures and the composition of the remaining media. The degree of fractionation depends on how much of the nitrogen source is consumed and

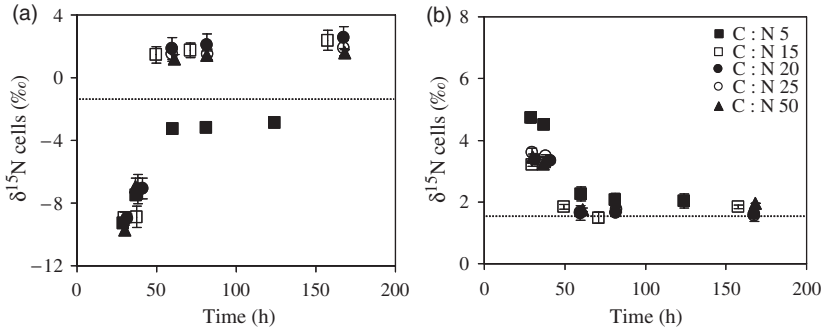

Fig. 2. Changes in $\delta^{15}N$ of *Escherichia coli* cells grown in (a) NH_4^+/glucose media or (b) glycine/glucose over time at different initial C : N ratios of the media. Symbols represent mean values and error bars represent ± 1 SE. For samples without error bars, the SE was smaller than the symbol. Dotted line represents $\delta^{15}N$ of (a) NH_4^+ or (b) glycine used to make media.

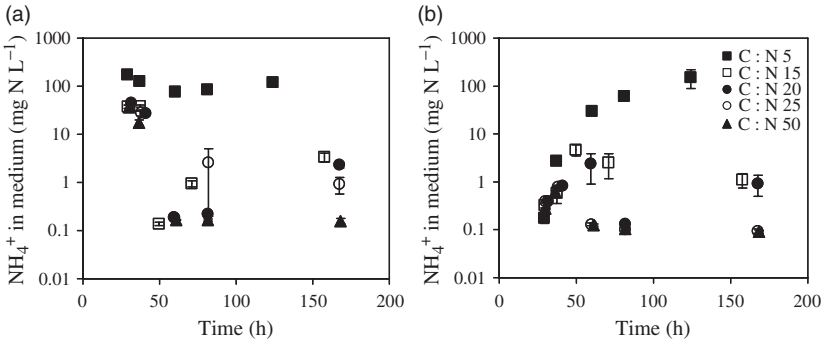

Fig. 3. Changes in NH_4^+ concentrations in (a) NH_4^+/glucose or (b) glycine/glucose media with different initial C : N. Symbols represent mean values and error bars represent ± 1 SE. For samples without error bars, the SE was smaller than the symbol.

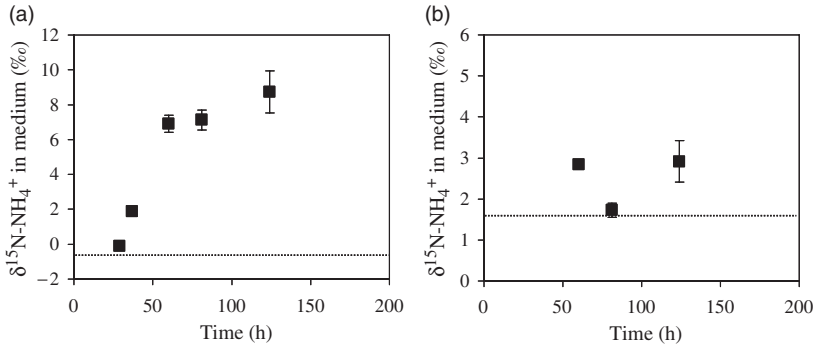

Fig. 4. Temporal dynamics of $\delta^{15}N$ of NH_4^+ in (a) NH_4^+/glucose or (b) glycine/glucose media with an initial C : N ratio of 5. Symbols represent mean values and error bars represent ± 1 SE. For samples without error bars, the SE was smaller than the symbol. Dotted line represents $\delta^{15}N$ of (a) NH_4^+ or (b) glycine used to make media. The $\delta^{15}N$ of NH_4^+ in media with C : N ratios of 15, 20, 25, and 50 could not be measured due to low NH_4^+ concentrations in these treatments.

diminishes as more of the source pool of nitrogen is utilized (Robinson, 2001). In treatments with C : N ratios of 15, 20, 25, and 50, the $\delta^{15}N$ of microbial biomass increased over time and became enriched (1.9–3.2‰) relative to the source nitrogen. Carbon starvation may have forced these cells to metabolize their own proteins and other biomolecules as a source of carbon and energy, resulting in nitrogen mineralization. Time, medium C : N ratio, and the interaction between these factors significantly affected the $\delta^{15}N$ content of microbial biomass when grown in inorganic nitrogen ($F = 8.06$, $P < 0.01$; $F = 994.87$, $P < 0.01$; $F = 16.24$, $P < 0.01$, respectively).

In the NH_4^+/glucose media, NH_4^+ concentrations declined over time, showing cells were taking up and assimilating NH_4^+ (Fig. 3a). When NH_4^+ was offered as a nitrogen source, cells assimilated nitrogen regardless of the substrate C : N ratio, as demonstrated by the rapid decrease in NH_4^+ concentrations in the media. NH_4^+ was eventually exhausted in media of all C : N treatments except for the medium with a C : N ratio of 5, where NH_4^+ remained in high concentrations.

When *E. coli* was cultured in NH_4^+/glucose medium with a C : N ratio of 5, the $\delta^{15}N$ of NH_4^+ increased over time from 1.9‰ to 6.9‰ (Fig. 4a) showing that *E. coli* preferentially assimilated the light isotope of nitrogen, leaving heavier NH_4^+ behind. This pattern is representative of closed, unidirectional systems in which isotopic fractionation follows Raleigh kinetics (Evans, 2001; Robinson, 2001). We observed a consistent isotope separation between cells and ammonium of 10.1‰ (± 0.3). In a study of ammonium assimilation by marine bacterial assemblages, Hoch *et al.* (1994) estimated isotopic fractionation factors to range between 5‰ and 20‰. The pH remained below 8 in all experiments, well below the pH at which the equilibrium between NH_3 and NH_4^+ favors volatilization of isotopically depleted NH_3 (Bigeleisen, 1965). Therefore, it is unlikely that pH influenced the isotopic composition of microorganisms or nitrogen pools. The $\delta^{15}N$ of NH_4^+ in media with C : N ratios of 15, 20, 25, and 50 could not be measured due to low NH_4^+ concentrations in these treatments.

Escherichia coli cells grown in glycine/glucose media were initially enriched in ^{15}N (1.6–3.1‰) relative to the nitrogen source (Fig. 2b). In the first 50 h of growth, cells in media with a C : N of 5 had higher $\delta^{15}N$ enrichment values than C : N treatments 15, 20, 25, and 50. Exudation of $^{14}NH_3$ has been suggested as a mechanism of microbial ^{15}N enrichment when cells utilize organic nitrogen-substrates as a carbon source (Dijkstra *et al.*, 2006b). The cells in the C : N 5 treatment metabolized all the glucose in the media and were forced to utilize nitrogenous substrates as a source of carbon before the other treatments, as indicated by higher ammonium concentrations in the medium (Fig. 3b). This probably led to higher microbial enrichment in ^{15}N. However, over time *E. coli* cells in all C : N treatments had $\delta^{15}N$ values that were similar to the initial signature of glycine. Like cultivation in NH_4^+/glucose media, time, medium C : N, and the interaction of these factors significantly affected the $\delta^{15}N$ of microbial cells across all C : N treatments when grown on organic nitrogen ($F = 16.32$, $P < 0.01$; $F = 51.49$, $P < 0.01$; $F = 2.21$, $P = 0.05$, respectively).

In the glycine/glucose media, the NH_4^+ concentration increased over time in all C : N treatments, indicating cells were secreting NH_4^+ (Fig. 3b). Over time, media with C : N ratios higher than 5 had very little remaining NH_4^+, indicating the released NH_4^+ was assimilated by *E. coli*. However, mineralization of glycine remained high in the C : N 5 treatment, demonstrating cells remained carbon limited.

Isotopic composition of NH_4^+ in glycine/glucose medium with a C : N ratio of 5 was similar to the $\delta^{15}N$ of glycine after 75 h of growth (Fig. 4b). Before this, the NH_4^+ concentration in the medium was too low to measure $\delta^{15}N$ of NH_4^+. At all time points in C : N treatments 15, 20, 25, and 50, the $\delta^{15}N$ of NH_4^+ could not be measured because NH_4^+ concentrations were too low to obtain sufficient nitrogen for analysis.

Both NH_4^+ assimilation and nitrogen mineralization require NH_3 to be transported across the plasma membrane of microorganisms. Our results suggest that this process fractionates nitrogen isotopes, favoring ^{14}N uptake into the cell cytoplasm over ^{15}N. Consequently, microorganisms become enriched in ^{15}N when secreting NH_4^+ and depleted

in ^{15}N when importing NH_4^+. Alternatively, enzymatic reactions inside the cell such as deamination or transamination of specific amino acids (Hoch *et al.*, 1994) may explain the differences in isotopic fractionation when cells are assimilating vs. mineralizing nitrogen. These results can be used to interpret ^{15}N isotopic patterns of microbial communities in the environment. We predict that microorganisms will be enriched in ^{15}N relative to the source nitrogen in carbon limited environments where nitrogen-substrates are important sources of carbon and nitrogen mineralization rates are high. Conversely, in environments where large amounts of carbon and NH_4^+ are available, microorganisms will assimilate nitrogen and consequently be depleted in ^{15}N relative to their nitrogen source. The degree to which microorganisms will be depleted in ^{15}N relative to their nitrogen source in environmental samples could be very small if microbial uptake causes the concentrations of NH_4^+ to be so low that fractionation barely occurs.

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