molecular systems biology

Need-based activation of ammonium uptake in Escherichia coli

Minsu Kim, Zhongge Zhang, Hiroyuki Okano, Dalai Yan, Alexander Groisman and Terence Hwa

Corresponding author: Terence Hwa, University of California at San Diego

Review timeline:

Submission date:	01 June 2012
Editorial Decision:	04 July 2012
Revision received:	08 August 2012
Editorial Decision:	15 August 2012
Accepted:	15 August 2012

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 04 July 2012

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees who agreed to evaluate the study. As you will see, the referees find the topic of your study of potential interest and are largely supportive. They raise however a series of concerns and make suggestions for modifications, which we would ask you to carefully address in a revision of the present work. Addressing these concerns will likely require some additional analysis, in addition to clarifications and some textual revisions.

When preparing your revised work, please also address the following format and content issues:

1. Molecular Systems Biology generally encourages authors to provide numeric data underlying all key experimental results. To make these data more accessible, we provide a new functionality that allows readers to directly download the 'source data' associated with selected figure panels (e.g. <http://tinyurl.com/365zpej>). This sort of figure-associated data may be particularly appropriate for this work. Please see our Instructions of Authors for more details on preparation and formatting of figure source data (<http://www.nature.com/msb/authors/index.html#a3.4.3>).

2. Please provide three to four 'bullet points' highlighting the main findings of your study.

3. Please provide a 'standfirst text' summarizing the study in one or two sentences (approx. 250 characters).

4. Please provide a "thumbnail image" (width=211 x height=157 pixels, jpeg format), which can be used to highlight your paper on our homepage.

Please resubmit your revised manuscript online, with a covering letter listing amendments and responses to each point raised by the referees. Please resubmit the paper **within one month** and

ideally as soon as possible. If we do not receive the revised manuscript within this time period, the file might be closed and any subsequent resubmission would be treated as a new manuscript. Please use the Manuscript Number (above) in all correspondence.

Click on the link below to submit your revised paper.

<http://mts-msb.nature.com/cgi-bin/main.plex?el=A3BL6BsS4A3Dv3I7A9x8cyHTDlHfLcdCK0VYfpsQZ>

If you do choose to resubmit, please use the link below to access the Licence to Publish. Please complete and sign this on behalf of all authors, with their consent, and fax to +44 (0)1256 321670.

http://mts-msb.nature.com/letters/msb_copyright.pdf

Processing of your submission can proceed when we have received this form.

As a matter of course, please make sure that you have correctly followed the instructions for authors as given on the submission website.

Thank you for submitting this paper to Molecular Systems Biology.

Sincerely,
--

Editor - Molecular Systems Biology
msb@embo.org

REFEREE REPORTS

Reviewer #1

Summary of the review:
What is new here: The onset of transport varies under different growth conditions, but always occurring just when needed. The concept of homeostasis is not new of course, neither is the idea of the role of feedback in homeostasis. The new hypothesis raised from this work is that the level of α-ketoglutarate is the important signal that controls amtB activity.

Detailed Comments:

1. p5. The motivation for using glycerol as the carbon source should be noted. (For example, note that different carbon sources were used so as to vary the growth rate).

2. p6. "Km of GS is reported to be ~ 100 μM for NH4+". Actually, in Meeks and Villafranca, it is more complicated than as described here. A biphasic pattern was obtained as NH4+ was varied at fixed glutamate, with multiple Km values estimated (for the two branches of the biphasic pattern) at ~ 0.6 mM and 0.06 mM. This troubling result has never been further investigated. Notably, the double reciprocal plot 1/V vs 1/NH4+ was strongly non-linear when glutamate was at 5 mM, but became linear when glutamate was 50 mM. Thus, glutamate had a strong influence on the binding of ammonium to the enzyme. It may be helpful to consider these results in detail with regard to the conclusions from the current work. In Alibhai and Villafranca, the Km is indeed listed as 0.1 mM in both Mg2+ and Mn2+. In Alibhai and Villafranca, the non-varied substrates are stated in methods as being saturated (without stating the actual values used in the experiments), and the biphasic patterns were (apparently) not observed. This study was mainly focused on elucidating the roles of mutated side chains, and may be of limited usefulness in understanding the function of the wild-type enzyme. Notably, in the latter study the enzyme was not purified through any chromatography steps, only precipitation steps were used. In the former study, a number of cited enzyme purification methods were cited, but the paper does not state which studies were performed with which enzyme prep. Shorter version of comment: There are some issues with these prior data and conclusions. Luckily, I

believe you can safely sidestep many of these issues in your analysis, as you do.

3. Fig 1g. Higher expression of pamtB at low NH4+ when cell is amtB is providing some evidence of the feedback circuitry? Is the difference between wt and amtB significant? Same is seen in Fig 3B. It is a small effect, but seems to be reproducible.

4. p15, last sen of results. In the field of study of nitrogen assimilation, it has long been thought/known/hypothesized that feedback in the system is used to maintain homeostasis of the levels of internal metabolites. For example, as the gln level becomes high in cells, GS becomes adenylylated so as to slow down the glutamine synthesis rate. Conversely, if the level of gln falls in the cell, GS can be activated by deadenylylation, increasing the rate of glutamine synthesis. Such feedback by the adenylylation system and other signaling systems maintains the homeostasis of the gln concentration. Therefore, it is not really surprising that the AmtB expression and activity should be tightly controlled such that AmtB activity is only present as necessary. This is exactly the expected result. Therefore, the italics on p15 are not appropriate, as the result that was obtained was the expected one.

5. Supplementary Information, p16, section titled "Influence of akG on amtB activity. In this section, it is argued that as aKG goes from 0.3 mM to 1.5 mM, transcription of amtB should be hardly changed, since the percentage of NtrC that was phosphorylated in an in vitro study only went from 1.5% to 2.5%. Missing from this discussion is the significance of the rise in the NtrC~P--- was such a rise significant? Furthermore, it is hard to see where the authors come up with their rise of NtrC~P from 1.5% to 2.5%. In fig 5 of the paper that was cited, we can see that aKG had various effects on the fractional phosphorylation of NtrC (called NRI), depending on the gln concentration. When gln was very low (as in N-limited cells), change of aKG from 0.3 mM to 1.5 mM caused NtrC~P to go from ~9% to ~12% phosphorylated when gln was 0.08 mM, and to go from ~ 4% to about 7% when gln was 0.2 mM. These may not be inconsequential changes, hand-waving in this section notwithstanding. There is no basis for assuming that the reconstituted in vitro system perfectly re-iterates the cellular regulation; what if there is an additional component in cells that was not know and therefore not present in the in vitro system? Furthermore, the discussion/analysis assumes that all aKG effects are the known ones, this is unlikely as new mechanisms for regulation by aKG are continuously being discovered, such as regulation of pts flux by aKG. Note also that aKG might affect the synthesis/decay of AmtB at multiple steps. All-in-all, I think this small section of the supplementary materials could use some additional work and its assumptions need to be re-evaluated.

6. A prior study concluded that enteric bacteria percieve nitrogen limitation as internal glutamine limitation. However, here you hypothesize that an important role is played by the internal ketoglutarate concentration. This disagreement with the conclusion of the earlier paper should be noted.

7. The title of the manuscript is somewhat off the mark. Robustness is a frequently mis-used term; robustness always pertains to a parameter that can be changed without effect. What parameter does the term "Robust" pertain to in this case? Surely, it cannot pertain to the external ammonium concentration, as it was shown in the paper that transport was activated differently, depending on growth rate. I do agree that the control is "sensitive" in both the colloquial and the technical sense. But, I note that this term is dangerous, precisely because "sensitivity" has both colloquial and technical meanings and in the two meanings can be almost opposite in certain circumstances. Finally, what was studied here (directly) was growth rate and ammonium transporter expression and GS expression, not nitrogen sequestration. There was no control of nitrogen sequestration, just control of its uptake and assimilation. Therefore, I suggest changing the word sequestration. This is especially important since some beautiful systems biology work has recently appeared on the roles of sequestration in shaping signaling system outputs; it would not be appropriate to use the word here and have this article pop up in searches for the other topic. Shorter version of comment: try to make a better title.

typos, trivia:

8. page 3, line 2: high--> sufficient

9. For consistency, Fig 2B needs labeling of the X-axis.

10. page 3, final sen of 1st paragraph: "unusual" problem? Why is it unusual? You are pointing out that several molecules fall into this category. Suggest deleting "unusual"

11. page 4, paragraph 1: .... "but is always set slightly above the point where".... This phrase is troublesome, because of the anthropomorphic shadings of the word "set" and because the "point" (actually, a concentration) is judged by what would happen if there was no transport (i.e what does not happen). Please try to re-word this sentence, focusing on what does happen, at which concentrations.

Reviewer #2

This is an outstanding paper. The authors have found very clever means of indirectly measuring the level of NH4+ inside living cells of E. coli. Moreover they have used this information and additional insightful analysis to reveal the workings of the nitrogen assimilation system, and have discovered an elegant regulation system based on integral feedback. This is really a paradigm of creative and clearheaded systems biology.

I have only a few technical questions/suggestions:

1. The authors should provide evidence or at least an estimate to show that the cells in their microfluidics device don't appreciably deplete the NH4+ concentration in their own vicinity.

2. One could imagine product-inhibition of GS by glutamine at high ammonium levels. Again an estimate and some discussion would be helpful here.

3. The authors implicitly assume that GS levels follow those of the GS-promoter fusion. It would be reassuring to have this assumption checked via a translational fusion, as there could in principle be post-transcriptional regulation of GS levels.

4. There is a central assumption that the only relevant input to amtB promoter activity comes from the internal NH4+ level. Do the experiments on different carbon sources provide a means of verifying that assumption?

Reviewer #3

Report ms.no. 12-380: "Robust and sensitive control of nitrogen sequestration in Escherichia coli", by Kim et al.

Reviewing manuscripts is often a burden, but sometimes it is a real joy. The latter is the case here.

Firstly, the results of Kim et al. are very interesting. The authors study the nitrogen uptake system of E. coli. They show that when the ambient nitrogen concentration is high, passive diffusion of ammonium across the membrane suffices to drive the nitrogen uptake. Yet, when the ambient concentration drops below a certain critical level, ammonium needs to be imported actively, to counteract passive diffusion (leakage) out of the cell. The authors show that the uptake machinery is activated strongly at a very well defined ammonium-concentration threshold. Moreover, they demonstrate that this threshold is just above the level needed to sustain cell growth; the costly uptake machinery is thus activated only when it is really needed. Furthermore, they provide strong evidence that the regulation of the activation of the uptake machinery follows an integral-feedback control mechanism. All these results are very interesting. People in the field often talk about how their work "elucidates design principles", but I think here

these words are truly appropriate: the work of Kim et al. reveals the design rationale of the nitrogen uptake system.

Secondly, the results are very well presented. The data is beautiful; the experiments have been very carefully chosen. But the data is also clearly presented. The figures and the captions are self-explanatory; one can get the main points of the story without having to read the main text (even though the text is also well written).

It is indeed a beautiful piece of work, and I can recommend this manuscript most highly for publication in MSB. I only have a few points.

- I understand the integral-feedback mechanism presented in the main text, using Eqs. 6-8. Also the math of the more detailed description of AmtB regulation in the SI, Eqs. S21-S35, is clear; it elucidates why AmtB activation is strong and sudden. Yet, I would appreciate a more intuitive explanation; in addition, the relationship between the aKG concentration and the internal ammonium concentration can be elucidated further. Specifically, when the ammonium concentration is high, the internal ammonium concentration is high, the aKG concentration is low, and AmtB activation is low. When the ammonium concentration decreases, V_GS expression/activity increases, counterbalancing the lower internal ammonium concentration and sustaining the flux J_GS. Yet, at some point V_GS expression/activity does not increase further, which means that the flux J_GS \propto V_GS x [NH4+]_int can only be maintained by upholding [NH4+]_int. This requires active uptake, and hence activation of AmtB. The point is now that when [NH4+]_int would drop, the aKG concentration would rise, which would then activate AmtB. What is still not clear to me is how the aKG concentration increases and how this is coupled to the internal ammonium concentration. In the main text the authors argue that when the internal ammonium level drops, aKG drainage slows down, leading to a rise of aKG; this argument is clear and convincing. But in the mathematical model in the SI, this argument is no longer used. Here, in Eqs. S21-S35, V_GS is taken to be a function of both [NH4+]_int and [aKG]. Do the authors now mean that the activity and/or the expression of the enzyme GS itself depends on [aKG], or should we imagine that the flux J_GS depends on [aKG]? Is J_GS \propto V_GS ([NH4+]_int) x [NH4+]_int x [aKG]? In other words, we recognize that the flux depends on the "substrate concentration" [aKG], but the activity/expression of GS itself is independent of aKG? I presume this gives the same result for AmtB as a function of [NH4+]_int, but it would be good to clarify this.

- If V_GS([NH4+]_int) could continue to rise further with decreasing [NH4+]_int, then (I expect) AmtB would be activated later, i.e. for lower ammonium concentrations. Could nature have chosen to not ever invoke active transport? I guess then the expression of GS would have to be increased to very high levels (becoming infinite when the internal ammonium concentration drops to zero). Is it indeed more efficient to actively import ammonium, rather than increase V_GS further?

- Caption Fig.2: "specific GS activity"; to make also this point in the caption self-explanatory, it would be useful to briefly explain what this means (and how it is determined), and/or refer to the caption of Fig.4A.

1st Revision - authors' response 08 August 2012

Reviewer #1

Summary of the review:
What is new here: The onset of transport varies under different growth conditions, but always occurring just when needed. The concept of homeostasis is not new of course, neither is the idea of the role of feedback in homeostasis. The new hypothesis raised from this work is that the level of α-ketoglutarate is the important signal that controls amtB activity.

We are grateful to the reviewer's constructive and educational comments, most of which we have incorporated into the revised text as described below.

Detailed Comments:

1. p5. The motivation for using glycerol as the carbon source should be noted. (For example, note that different carbon sources were used so as to vary the growth rate).

In numerous batch culture experiments in the past (e.g. Ikeda *et al*, 1996; Okano *et al*, 2010), nitrogen-limited conditions were realized by using organic nitrogen sources such as proline and arginine. For such experiments, glucose – a common choice of carbon source – could not be used as catabolite repression impairs the utilization of these organic nitrogen sources. Thus, glycerol was often used as the carbon source in those studies. In our study, we described results on glycerol first because we wanted to directly compare our data with previous batch culture data, and also use those data for our analysis (e.g. Supplementary Figure. 2). We do not feel this explanation is necessary in the text. However, we take the reviewer's point that the motivation for using different carbon sources should be explained better. This is done by first adding a clause on p.5 (where glycerol is first mentioned) that the effect of different carbon sources would be described later. Then where different carbon sources were first mentioned (bottom of p.14 to the top of p.15), we explained that the reason for using different carbon sources was to vary the growth rate in ammonium-replete conditions, in order to generate different demand for ammonium.

2. p6. "Km of GS is reported to be ~ 100 μM for NH4+". Actually, in Meeks and Villafranca, it is more complicated than as described here. A biphasic pattern was obtained as NH4+ was varied at fixed glutamate, with multiple Km values estimated (for the two branches of the biphasic pattern) at ~ 0.6 mM and 0.06 mM. This troubling result has never been further investigated. Notably, the double reciprocal plot 1/V vs 1/NH4+ was strongly non-linear when glutamate was at 5 mM, but became linear when glutamate was 50 mM. Thus, glutamate had a strong influence on the binding of ammonium to the enzyme. It may be helpful to consider these results in detail with regard to the conclusions from the current work. In Alibhai and Villafranca, the Km is indeed listed as 0.1 mM in both Mg2+ and Mn2+. In Alibhai and Villafranca, the non-varied substrates are stated in methods as being saturated (without stating the actual values used in the experiments), and the biphasic patterns were (apparently) not observed. This study was mainly focused on elucidating the roles of mutated side chains, and may be of limited usefulness in understanding the function of the wild-type enzyme. Notably, in the latter study the enzyme was not purified through any chromatography steps, only precipitation steps were used. In the former study, a number of cited enzyme purification methods were cited, but the paper does not state which studies were performed with which enzyme prep. Shorter version of comment: There are some issues with these prior data and conclusions. Luckily, I believe you can safely sidestep many of these issues in your analysis, as you do.

We thank the reviewer for pointing out the subtleties of these *in vitro* studies. Although the study by Meek & Villafranca (1980) showed a biphasic pattern in the double reciprocal plot 1/V vs $1/NH_4^+$ of GS at low glutamate levels (below 5 mM), at the physiological glutamate levels (~50 mM, see Okano *et al*, 2010; Yuan *et al*, 2009), the plot became linear for the entire range of NH_4^+ concentration, producing one K_m for GS, ~200 μM as reported in that study. This is consistent with the value of 100 μM reported in Alibhai & Villafranca (1994), conducted supposedly in saturating conditions for the nonvaried substrates including glutamate. We added a paragraph (Supplementary

Note 2, p19) mentioning this subtlety; a pointer to this Note is inserted in the main text where K_m is first mentioned (p.6).

Moreover, on p6 where the K_m of GS is first mentioned, the only point we made was that the ammonium concentration where growth slows down, ~20 μM, was well below the reported range of K_m. Although we did use the actual value of the K_m of GS later in our analysis (Eq. (3)) to estimate the absolute value of V_{GS}, the V_{max} of GS, the only bearing V_{GS} has on our main finding – the ordered, seamless integration of the two lines of defense by GS and AmtB against nitrogen limitation – is the *relative* form of the dependence of V_{GS} on the ammonium concentration. Thus, the accurate value of K_m of GS is not critical for our study. We added this discussion in Supplementary Note 2 on p. 19 of the Supplementary Information.

3. Fig 1g. Higher expression of pamtB at low NH4+ when cell is amtB is providing some evidence of the feedback circuitry? Is the difference between wt and amtB significant? Same is seen in Fig 3B. It is a small effect, but seems to be reproducible.

Yes. At low ambient ammonium levels, the P_{AmtB}–GFP level of Δ*amtB* strain is 3-5x higher than that of wild type cells (open and filled symbols respectively in the grey region of Figures 1G and 3B), and it is reproducible. The differences in promoter activities between the two strains indicate different internal nitrogen status of the two strains, i.e., in the Δ*amtB* strain where P_{AmtB}–GFP level is higher, the internal nitrogen level is lower. It does not necessarily indicate the existence of feedback circuitry. However, the steady maintenance of P_{AmtB}–GFP level in the wild type cells at low ambient ammonium levels (solid green symbols in the grey region of Figures 1G and 3B) suggests feedback mechanism to keep the internal nitrogen status steady.

4. p15, last sen of results. In the field of study of nitrogen assimilation, it has long been thought/known/hypothesized that feedback in the system is used to maintain homeostasis of the levels of internal metabolites. For example, as the gln level becomes high in cells, GS becomes adenylylated so as to slow down the glutamine synthesis rate. Conversely, if the level of gln falls in the cell, GS can be activated by deadenylylation, increasing the rate of glutamine synthesis. Such feedback by the adenylylation system and other signaling systems maintains the homeostasis of the gln concentration. Therefore, it is not really surprising that the AmtB expression and activity should be tightly controlled such that AmtB activity is only present as necessary. This is exactly the expected result. Therefore, the italics on p15 are not appropriate, as the result that was obtained was the expected one.

We removed the italics on the phrase (the last sentence of the first paragraph in p16 of the main text).

5. Supplementary Information, p16, section titled "Influence of akG on amtB activity. In this section, it is argued that as aKG goes from 0.3 mM to 1.5 mM, transcription of amtB should be hardly changed, since the percentage of NtrC that was phosphorylated in an in vitro study only went from 1.5% to 2.5%. Missing from this discussion is the significance of the rise in the NtrC~P--- was such a rise significant? Furthermore, it is hard to see where the authors come up with their rise of NtrC~P from 1.5% to 2.5%. In fig 5 of the paper that was cited, we can see that aKG had various effects on the fractional phosphorylation of NtrC (called NRI), depending on the gln concentration. When gln was very low (as in N-limited cells), change of aKG from 0.3 mM to 1.5 mM caused NtrC~P to go from ~9% to ~12% phosphorylated when gln was 0.08 mM, and to go from ~ 4% to about 7% when gln was 0.2 mM. These may not be inconsequential changes, hand-waving in this section notwithstanding. There is no
basis for assuming that the reconstituted in vitro system perfectly re-iterates the cellular regulation; what if there is an additional component in cells that was not know and therefore not present in the in vitro system? Furthermore, the discussion/analysis assumes that all aKG effects are the known ones, this is unlikely as new mechanisms for regulation by aKG are continuously being discovered, such as regulation of pts flux by aKG. Note also that aKG might affect the synthesis/decay of AmtB at multiple steps. All-in-all, I think this small section of the supplementary materials could use some additional work and its assumptions need to be re-evaluated.

This section was written to argue that as the concentration of aKG increases to turn on AmtB activity, it does not significantly affect the P_{AmtB}-GFP level. This might be a concern because it is

known from *in vitro* studies that aKG level could affect the phosphorylation state of NtrC, and NtrC~P is the key regulator of P_{AmtB}. In the original text, we made our argument based on the estimated concentration ranges of various metabolites. The reviewer pointed out that since the *in vitro* system may not accurately reflect *in vivo* cellular regulation, the parameter ranges obtained from the *in vitro* studies may not be applicable to our argument. The reviewer was right of course.

In fact we can make a much more direct argument based on our *in vivo* data. Note that in the region of low ambient NH_4^+ concentrations where AmtB activity of wild type cells is turned on, the P_{AmtB}-GFP level remains at an approximately constant level (solid green circles in the grey region of Figure 3B). Since P_{AmtB} activity has not saturated in this range (see the higher P_{AmtB} activity of the Δ*amtB* strain, open green circles), these data directly indicate that the expected increases in the aKG concentration in that region (cyan line in the grey region of Figure 5C) do not have a significant effect on P_{AmtB}-GFP level.

We completely re-wrote the paragraph as Supplementary Note 3 (p.19-20).

6. A prior study concluded that enteric bacteria percieve nitrogen limitation as internal glutamine limitation. However, here you hypothesize that an important role is played by the internal ketoglutarate concentration. This disagreement with the conclusion of the earlier paper should be noted.

Glutamine has long been established as a signal of the internal nitrogen status, serving as a major effector of GS expression and activity. Our study suggests that when the expression and activity of GS, the first line of defense against nitrogen limitation, becomes saturated, aKG plays an important role by turning on AmtB activity. Thus, our study does not exclude glutamine as a signal for internal nitrogen status. The key conclusion from our study is that two independent signals, glutamine and aKG, are employed to sense the internal nitrogen status in two different regions (above and below N^*_{ext}), and coordinate the two lines of defense seamlessly. We clarified this in the main text (the last paragraph of Analysis and Discussion, p.22).

7. The title of the manuscript is somewhat off the mark. Robustness is a frequently mis-used term; robustness always pertains to a parameter that can be changed without effect. What parameter does the term "Robust" pertain to in this case? Surely, it cannot pertain to the external ammonium concentration, as it was shown in the paper that transport was activated differently, depending on growth rate. I do agree that the control is "sensitive" in both the colloquial and the technical sense. But, I note that this term is dangerous, precisely because "sensitivity" has both colloquial and technical meanings and in the two meanings can be almost opposite in certain circumstances. Finally, what was studied here (directly) was growth rate and ammonium transporter expression and GS expression, not nitrogen sequestration. There was no control of nitrogen sequestration, just control of its uptake and assimilation. Therefore, I suggest changing the word sequestration. This is especially important since some beautiful systems biology work has recently appeared on the roles of sequestration in shaping signaling system outputs; it would not be appropriate to use the word here and have this article pop up in searches for the other topic. Shorter version of comment: try to make a better title.

We changed the title to **"**Need-based activation of ammonium uptake in *Escherichia coli"*. We think this reflects most directly the reviewer's comment on what this paper is about.

typos, trivia:

8. page 3, line 2: high--> sufficient

Changed

9. For consistency, Fig 2B needs labeling of the X-axis.

Changed

10. page 3, final sen of 1st paragraph: "unusual" problem? Why is it unusual? You are pointing out

that several molecules fall into this category. Suggest deleting "unusual"

Deleted

11. page 4, paragraph 1: .... "but is always set slightly above the point where".... This phrase is troublesome, because of the anthropomorphic shadings of the word "set" and because the "point" (actually, a concentration) is judged by what would happen if there was no transport (i.e what does not happen). Please try to re-word this sentence, focusing on what does happen, at which concentrations.

Changed to "but it always occurs just at the concentration where an isogenic strain unable to transport ammonium begins to show a growth defect".

Reviewer #2

This is an outstanding paper. The authors have found very clever means of indirectly measuring the level of NH4+ inside living cells of E. coli. Moreover they have used this information and additional insightful analysis to reveal the workings of the nitrogen assimilation system, and have discovered an elegant regulation system based on integral feedback. This is really a paradigm of creative and clearheaded systems biology.

We are grateful to the reviewer's positive comments.

I have only a few technical questions/suggestions:

1. The authors should provide evidence or at least an estimate to show that the cells in their microfluidics device don't appreciably deplete the NH4+ concentration in their own vicinity.

In a microfluidic chamber, we monitored a single cell growing exponentially to form a monolayer of a cell cluster (see Methods and materials). To estimate the depletion of ammonium by the growing cluster, we calculated the profile of ammonium concentration when the cluster was located in the middle of the chamber in Supplementary Note 1 (p.17-19). It showed that up to 3 doublings (within which duration the growth rate was determined; see Supplementary Figure 1C), the depletion of ammonium due to cell growth is less than 1 μM. In our study, two key conditions from which we defined the mode of AmtB activation, the onset of AmtB activation in wild type cells (~30 μM for glycerol, green arrow in Figure 1G) and the onset of growth defect in Δ*amtB* strain (~ 20 μM for glycerol, black arrow in Figure 1E), are hardly affected by a 1 μM depletion. Thus, the effect of ammonium depletion by a growing cluster in our experiment is negligible. We added this discussion in Supplementary Note 1 (p. 18) and a pointer to this Note in the main text when a microfluidic chamber was introduced (p.5).

2. One could imagine product-inhibition of GS by glutamine at high ammonium levels. Again an estimate and some discussion would be helpful here.

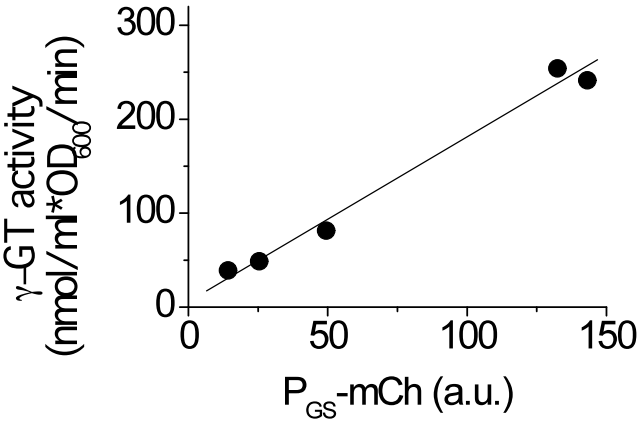
GS activity was inhibited allosterically by several products of glutamine metabolism, but not by glutamine itself (Woolfolk & Stadtman, 1964; Woolfolk & Stadtman, 1967) . Glutamine instead inhibits GS by affecting the ability of another enzyme (GlnE) which modifies the activity of GS by adenylylating/deadenylylating it (Kingdon *et al* 1967; Wulff *et al* 1967; Okano *et al*, 2010).

In our study, we deduced from our data a 10-fold change of total GS activity between growth medium with high- and low- ammonium (V_{GS}, brown symbols in Figure 2D). Together with the 5-fold change in the GS promoter activity, we deduced a 2-fold change in the specific activity of GS (k_{GS}, cyan symbols in Figure 2D). This 2-fold change in k_{GS} would include all the effect of product inhibition that the reviewer asked about, as well as the effect of GS adenylylation by GlnE. A prior study showed that GS adenylylation state changed by ~ 2-fold between nitrogen-poor and nitrogen-rich conditions (Okano *et al*, 2010). Assuming that accounts for the bulk of the change in the deduced activity, our results suggest that the product-inhibition does not play a significant role under the conditions we used.

We added one sentence to clarify the known molecular mechanisms in the main text (last paragraph of p.9) and another phrase to indicate that most of the deduced change in k_{GS} appears to be derived from the effect of adenylylation (towards the bottom of p.10). We note that for our main finding about the ordered, seamless integration of the two lines of defense by GS and AmtB against nitrogen limitation, we only need the dependence of GS activity (V_{GS}) on the ammonium concentration, and do not need to distinguish whether the changes are derived from gene expression or specific activity.

3. The authors implicitly assume that GS levels follow those of the GS-promoter fusion. It would be reassuring to have this assumption checked via a translational fusion, as there could in principle be post-transcriptional regulation of GS levels.

As in the answer to the previous question, we only need the total GS activity, V_{GS}, which includes all effects due to transcriptional, post-transcriptional, and post-translational regulations, as well as allosteric inhibition. We have actually checked that P_{GS}-mCh level reflects the GS amount by comparing the former with γ-glutamyltransferase activity of GS (Stadtman *et al*. *Anal. Biochem.* 95, 275) for cells growing on different organic nitrogen sources in the batch culture; see figure below. The γ-GT activity is measured at the condition in which GS in adenylylated and unadenylylated states is equally active and represents the total GS amount.



We do not think it is necessary to publish the above data in this study, as post-transcriptional regulation of GS has never been established after 50 years of careful molecular studies and the issue is anyway not relevant to the conclusions.

4. There is a central assumption that the only relevant input to amtB promoter activity comes from the internal NH4+ level. Do the experiments on different carbon sources provide a means of verifying that assumption?

Cells growing on the different carbon sources have different growth rate (Figure 3A). Since the gene expression changes globally when the growth rate changes (Scott *et al*. *Science*, 330, 1099), AmtB promoter activity for cells growing on different carbon sources cannot be directly compared. (Throughout this work, we never compared gene expression for cells growing at different rates; see the last paragraph of Supplementary Note 3 on p. 20).

The assumption we actually made is that AmtB promoter activity is primarily determined by the internal nitrogen status for a fixed carbon source. The validity of this assumption is justified in detail in Supplementary Note 3 (p. 20).

Reviewer #3

Report ms.no. 12-380: "Robust and sensitive control of nitrogen sequestration in Escherichia coli", by Kim et al.

Reviewing manuscripts is often a burden, but sometimes it is a real joy. The latter is the case here.

Firstly, the results of Kim et al. are very interesting. The authors study the nitrogen uptake system of E. coli. They show that when the ambient nitrogen concentration is high, passive diffusion of ammonium
across the membrane suffices to drive the nitrogen uptake. Yet, when the ambient concentration drops below a certain critical level, ammonium needs to be imported actively, to counteract passive diffusion
(leakage) out of the cell. The authors show that the uptake machinery is activated strongly at a very well defined ammonium-concentration threshold. Moreover, they demonstrate that this threshold is just above
the level needed to sustain cell growth; the costly uptake machinery is thus activated only when it is really needed. Furthermore, they provide strong evidence that the regulation of the activation of the uptake machinery follows an integral-feedback control mechanism. All these results are very interesting. People in the field often talk about how their work "elucidates design principles", but I think here these words are truly appropriate: the work of Kim et al. reveals the design rationale of the nitrogen uptake system.

Secondly, the results are very well presented. The data is beautiful; the experiments have been very carefully chosen. But the data is also clearly presented. The figures and the captions are self-explanatory; one can get the main points of the story without having to read the main text (even though the text is also well written).

It is indeed a beautiful piece of work, and I can recommend this manuscript most highly for publication in MSB. I only have a few points.

We are grateful to the reviewer's compliments and the concise summary/perspective of our work.

- I understand the integral-feedback mechanism presented in the main text, using Eqs. 6-8. Also the math of the more detailed description of AmtB regulation in the SI, Eqs. S21-S35, is clear; it elucidates
why AmtB activation is strong and sudden. Yet, I would appreciate a more intuitive explanation; in addition, the relationship between the aKG concentration and the internal ammonium concentration can be elucidated further. Specifically, when the ammonium concentration is high, the internal ammonium concentration is high, the aKG concentration is low, and AmtB activation is low. When the ammonium
concentration decreases, V_GS expression/activity increases,counterbalancing the lower internal ammonium concentration and sustaining the flux J_GS. Yet, at some point V_GS expression/activity does not increase further, which means that the flux J_GS \propto V_GS x [NH4+]_int can only be maintained by upholding [NH4+]_int. This requires active uptake, and hence activation of AmtB. The point is now that when [NH4+]_int would drop, the aKG concentration would rise, which would then activate AmtB.

The reviewer summarized the system's behavior perfectly.

What is still not clear to me is how the aKG concentration increases and how this is coupled to the internal ammonium concentration. In the main text the authors argue that when the
internal ammonium level drops, aKG drainage slows down, leading to a rise of aKG; this argument is clear and convincing. But in the mathematical model in the SI, this argument is no longer used. Here,
in Eqs. S21-S35, V_GS is taken to be a function of both [NH4+]_int and [aKG]. Do the authors now mean that the activity and/or the expression of the enzyme GS itself depends on [aKG], or should we
imagine that the flux J_GS depends on [aKG]? Is J_GS \propto V_GS ([NH4+]_int) x [NH4+]_int x [aKG]? In other words, we recognize that the flux depends on the "substrate concentration" [aKG], but the activity/expression of GS itself is independent of aKG? I presume this gives the same result for AmtB as a function of [NH4+]_int, but it would be good to clarify this.

We apologize for the confusion due to the lack of motivation provided at the beginning of the more detailed model described in the Supplementary Discussion. In the main text, we presented a simplified model (equations (6)-(8) and Figure 5A) to explain qualitatively the abrupt onset of AmtB activity mediated by the rise of aKG concentration below N^*_{ext}. This simplified model ignores the known weak dependence of V_{GS} on aKG, and predicts an infinitely sharp rise of AmtB activity at N^*_{int} (Figure 5D). However, the actual AmtB activity deduced from our data is not infinitely sharp, but occurs over the narrow range of the internal ammonium concentration (green symbols in Figure 3D). This finite slope is accounted for upon including the known (weak) dependence of V_{GS} on aKG in the more elaborate model (Supplementary Equations (S24)-(S39)). Intuitively, when the aKG pool rises upon internal NH_4^+ depletion, not only is AmtB activated but GS expression and activity are also *further* enhanced. This slightly reduces the cell's reliance on AmtB, making the transition less abrupt. Quantitatively, the lengthy analysis in the supplement shows that the slope is determined by the ratio between the relative response of V_{GS} to changes in aKG concentration,

$$\kappa_{GS,\alpha} = \left(\frac{1}{V_{GS}} \frac{\partial V_{GS}}{\partial [aKG]} \right)^{-1}$$

and the response of AmtB activity to changes in aKG concentration (determined by the inhibition constant K_{AmtB}); see Supplementary Equation (S34).

We clarified this in the main text (second half of p.21 through the top of p.22), and in the Supplementary Discussion before presenting the more elaborate model (second paragraph on p.11).

- If V_GS([NH4+]_int) could continue to rise further with decreasing [NH4+]_int, then (I expect) AmtB would be activated later, i.e. for lower ammonium concentrations. Could nature have chosen to not ever
invoke active transport? I guess then the expression of GS would have to be increased to very high levels (becoming infinite when the internal ammonium concentration drops to zero). Is it indeed more efficient to actively import ammonium, rather than increase V_GS further?

The reviewer raised a very interesting issue – the rationale by which the maximum GS level is set. It is known that even under nitrogen-replete conditions, the amount of GS in the cell is already very high (1~2% of the total proteome, Okano *et al*, 2010). Upon ammonium limitation, the GS expression level is found to increase 5x (red symbols in Figure 2D), making it possibly one of the most abundant proteins. To maintain cell growth at even lower ammonium concentrations, the cell cannot increase GS expression indefinitely as it would eventually limit the fraction of proteome devoted to translation, which has an obligatory dependence on the growth rate (Scott *et al*. *Science*, 330, 1099). We do not believe this is what sets the ceiling in GS expression though. A more likely scenario is the tradeoff between the cost of further increasing the GS expression vs the cost of dealing with the futile cycle introduced by the use of AmtB. This would be a very interesting issue to pursue in a future study.

- Caption Fig.2: "specific GS activity"; to make also this point in the caption self-explanatory, it would be useful to briefly explain what this means (and how it is determined), and/or refer to the caption
of Fig.4A.

We included the definition of the specific activity of GS (k_{GS}) in the caption for Figure 2D (p.29) as well as in the main text where k_{GS} is first introduced (middle of p.10). At the bottom of p.9, we further mentioned the known molecular mechanisms by which the specific activity may be modified. It is thus not necessary to refer to the caption of Figure 4A which would be out of order.

2nd Editorial Decision 15 August 2012

Thank you again for sending us your revised manuscript. The reviewers agreed that the revisions made had satisfied their concerns, and they are now strongly supportive of publication. As such, I am pleased to inform you that your paper has been accepted, in principle, for publication, pending

correction of the very minor textual issues raised by Reviewer #1 (see below).

A revised manuscript document correcting these issues can be send as an attachment to a reply email, ideally within the next week.

Thank you very much for submitting your work to Molecular Systems Biology.

Sincerely,
--

Editor - Molecular Systems Biology
msb@embo.org

REFEREE REPORTS

Reviewer #1

I believe the revised version of the manuscript is improved over the already-strong initial submission. In particular, I note that the authors have responded appropriately to all of my comments, and made appropriate changes to the manuscript.

The most important of these changes was the title. I believe the new title is much stronger. I also appreciate the changes to supplementary notes 2 and 3; I believe these improve things. I also think the response to my point 6 was appropriate and helpful.

I have only a few exceptionally minor comments on the revised version, three of which deal with typographical errors and one of which requests the change of a single word:

p14, line 12: slows-->slow
p15, last line: I suggest dropping the word "genetically". The regulatory system is genetically encoded too, of course. The critical distinction is between pre-set and dynamically determined.
p24, missing spaces between values and "um" (just to show you that I really did read it very carefully)
p27, advices-->advice.

Reviewer #2

The authors have satisfactorily addressed all my concerns.

