View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

FEBS Letters 585 (2011) 23–28

FEBS Letters
journal homepage: www.FEBSLetters.org

Hypothesis

AmtB-mediated NH_3 transport in prokaryotes must be active and as a consequence regulation of transport by GlnK is mandatory to limit futile cycling of NH_4^+/NH_3

Fred C. Boogerd [a,*], Hongwu Ma [b], Frank J. Bruggeman [a], Wally C. van Heeswijk [a], Rodolfo García-Contreras [a], Douwe Molenaar [a], Klaas Krab [a], Hans V. Westerhoff [a,c]

[a] Department of Molecular Cell Physiology, Faculty of Earth and Life Sciences, VU University Amsterdam, The Netherlands
[b] Computational Systems Biology, School of Informatics, University of Edinburgh, UK
[c] Manchester Centre for Integrative Systems Biology, The University of Manchester, UK

ARTICLE INFO

Article history:
Received 27 August 2010
Revised 29 November 2010
Accepted 29 November 2010
Available online 8 December 2010

Edited by Stuart Ferguson

Keywords:
AmtB
Active transport
Futile cycling
GlnK

ABSTRACT

The nature of the ammonium import into prokaryotes has been controversial. A systems biological approach makes us hypothesize that AmtB-mediated import must be active for intracellular NH_4^+ concentrations to sustain growth. Revisiting experimental evidence, we find the permeability assays reporting passive NH_3 import inconclusive. As an inevitable consequence of the proposed NH_4^+ transport, outward permeation of NH_3 constitutes a futile cycle. We hypothesize that the regulatory protein GlnK is required to fine-tune the active transport of ammonium in order to limit futile cycling whilst enabling an intracellular ammonium level sufficient for the cell's nitrogen requirements.
© 2010 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

1. Introduction

Until around 25 years ago, cell membranes were thought to be fairly permeable to small hydrophilic substances. Since then we have learned that any such permeability is not enough for the high metabolic fluxes that living organisms maintain. Even glycerol and water have been reported to use transport systems in certain tissues. Here we address the transport by the AmtB protein of ammonium,[1] which has often been thought to permeate as the NH_3 gas. For the bacterium *Escherichia coli* ammonium is the preferred nitrogen source [1].

The protonation and deprotonation of NH_3 is extremely fast and therefore NH_4^+ and NH_3 concentrations are in equilibrium in aqueous compartments. If the sum of NH_3 and NH_4^+, the pK (9.0 at 37 °C [2,3]) and the actual pH are known, the NH_3 and NH_4^+ concentrations can be calculated individually. At neutral to acidic pH, NH_4^+ dominates (>99%). We shall show that at acidic to neutral pHs the default route of ammonia-gas permeation would not suffice for cell growth and discuss the controversial alternative of NH_4^+ transport driven by the membrane potential and blocked by GlnK.

2. Mere passive diffusion of NH_3 may be insufficient for growth at low to neutral pH and low $NH_{(x)}$

Because some 14% (w/w) of cell dry weight is nitrogen and there is some 2 ml cytoplasm/g dry weight, a specific growth rate of 1.0 h^{-1} requires an N assimilation flux of

$$\frac{0.14\,\frac{\text{g N}}{\text{g DW}}\cdot 1.0\,\frac{1}{3600\text{ s}}}{14\,\frac{\text{g N}}{\text{mol N}}\cdot 2\,\frac{\text{ml}}{\text{g DW}}} \approx 1.4\text{ mM/s [4]}.$$

Using $[NH_3^{out}] - [NH_3^{in}] = \frac{J_{N\rightarrow biomass}\cdot V_{cell}}{P_{NH_3}\cdot A_{cell}}$,

$V_{cell}/A_{cell} = r_{cell}/3 \approx 1/6$ µm for a sphere of radius r (and somewhat more of a cigar-like *E. coli*) and P for NH_3 of 1000 µm/s,[2] we calculate that an NH_3 transmembrane concentration difference in excess

* Corresponding author. Address: Section of Molecular Cell Physiology, Netherlands Institute for Systems Biology, Faculty of Earth and Life Sciences, VU University Amsterdam, de Boelelaan 1085, NL-1081 HV Amsterdam, The Netherlands. Fax: +31 20 59 87229.
E-mail address: fred.boogerd@falw.vu.nl (F.C. Boogerd).

[1] In this paper, we will use the term '$NH_{(x)}$' when the nature of the molecular species is not relevant, but will write NH_4^+ and NH_3 when the protonation state is important. The term 'nitrogen' (N) will be used to refer to the element nitrogen.

[2] Experimentally a wide range of NH_3 permeabilities of liposomal membranes has been observed, ranging from 0.5 to 2000 µm/s; the variation may be partly due to differences in the experimental Ts from 10 °C to 'room temperature' [5–8].

0014-5793/$36.00 © 2010 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2010.11.055

of 0.2 μM is required to sustain growth. At pH 7 this corresponds to a 20 μM $NH_{(x)}$ concentration difference. This implies that growth of microorganisms at extracellular $NH_{(x)}$ concentrations below 0.02 mM at pH 7 and below 2 mM at pH 5 cannot really be supported by unfacilitated membrane permeation of NH_3. Growth of an *amtB*$^-$ mutant has been reported to be hampered at $NH_{(x)}$ concentrations below 1 mM at pH 5 [9] which is thereby just beyond the maximum capabilities of passive NH_3 diffusion.

3. Facilitated diffusion of NH_3 is not good enough either

Recent structural and activity assay data have been taken to suggest that NH_4^+ is bound at a high-affinity binding site in a periplasmic vestibule of the *E. coli* AmtB carrier protein, deprotonated before it migrates as NH_3 through a hydrophobic channel, and reprotonated and released as NH_4^+ in the cytoplasm [7,10]. This mode of transport constitutes catalyzed but passive permeation (Fig. 1, mechanism i). The experimental evidence (but see below) suggests that if there is any such carrier mediated transport it does not speed up the transport rate of NH_3 by more than a factor of 10. If P is taken to be 1000 μm/s, this would help to reach the required transport rate of NH_3. However, even if the transmembrane passive flux were fast enough, the facilitated ammonia diffusion mechanism would still not be able to explain growth at such low concentrations (10–50 μM) of ammonium, because the intracellular ammonium concentration would not become high enough. Because the sole driving force would be the NH_3 gradient, the only way for $NH_{(x)}$ to accumulate relative to its concentration in the external medium is by so-called acid trapping. Since *E. coli*'s pH^{in} is approximately 7.5, $NH_{(x)}$ can only be trapped in the cytosol if $pH^{out} > 7.5$. However, common growth media have a pH of 7.0–7.5 (see Table 1) and *E. coli* does grow at pHs much lower than 7.0.

At neutral pH^{out}, AmtB is present only at low external $NH_{(x)}$ concentrations, i.e. <10 μM [9,11]. At 10 μM $NH_{(x)}$ and a pH^{in} of 7.5, and assuming uptake of NH_3 via passive or facilitated NH_3 permeation, the intracellular NH_4^+ concentration will have been at most 3 μM. The K_M of glutamine synthetase (GS) for NH_4^+ is 100 μM [12–14], and the other enzyme capable of N assimilation, glutamate dehydrogenase (GDH), has an even higher K_M for NH_4^+ (2000 μM) [15,16]. Would metabolic trapping of $NH_{(x)}$ suffice?

The answer to this question comes from a systems biology model [4] of *E. coli*'s nitrogen assimilation network, based on kinetic characterization in vitro of its components, including the signal transduction cascades (ATase and UTase) and the regulatory protein GlnB. Implemented with protein and enzyme levels pertaining to cells growing under nitrogen-limitation this model showed that at least 45 μM of intracellular NH_4^+ is required to sustain the nitrogen assimilation flux needed for a specific growth rate of 0.35 h^{-1}. Thus the intracellular NH_4^+ concentration required for growth by the model was at least 15-fold higher than the maximum concentration of intracellular NH_4^+ (3 μM) achievable by passive or facilitated NH_3 permeation, independent of the rate at which this would occur.

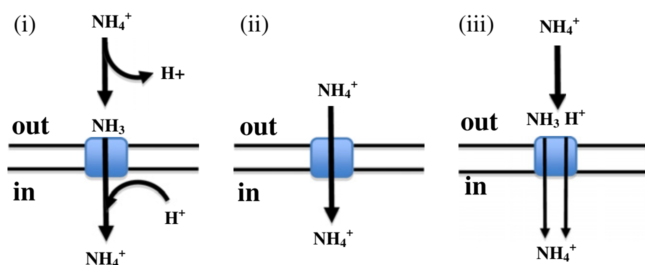

Fig. 1. Three AmtB-mediated mechanisms for $NH_{(x)}$ transport. The blue rounded rectangle represents the AmtB carrier. Mechanism (i): facilitated transport of NH_3; mechanism (ii): facilitated transport of NH_4^+, which corresponds to active transport of NH_3; mechanism (iii): active transport of NH_3 where NH_3 and the symported H^+ follow separate routes in space or time. Note that although at first glance it can appear that in mechanism (i) a proton leaves the cell for each NH_3 entering there is no movement of protons across the membrane and the mechanism is not an electrogenic antiport.

Table 1
Concentrations of NH_4^+ and NH_3 inside and outside cells as a function of the inside and outside pH.

pH^{out}	$NH_3^{out} + NH_4^{+,out}$ (mM)	$NH_4^{+,out}$ (mM)	$NH_3^{out} = NH_3^{in}$ (mM)	$NH_4^{+,in}$ (mM)	$NH_3^{in} + NH_4^{+,in}$ (mM)
4.0	10	10	0.0001	0.003	0.003
4.5	10	10	0.0003	0.01	0.01
5.0	10	10	0.001	0.03	0.03
5.5	10	10	0.003	0.10	0.10
6.0	10	10	0.01	0.32	0.33
6.5	10	10	0.03	1.0	1.0
7.0	10	9.9	0.1	3.1	3.2
7.5	10	9.7	0.3	9.7	10
8.0	10	9.1	0.9	29	30
8.5	10	7.6	2.4	76	78
9.0	10	5.0	5.0	158	163

The total $NH_{(x)}$ concentration outside cells was set at 10 mM. The p*K* of $NH_{(x)}$ inside and outside was taken to be 9.0 and the pH inside was set at 7.5. The hypothetical cells only allow permeation of NH_3 (AmtB is absent) and do not assimilate nitrogen. The table shows the inside and outside distribution of NH_4^+ and NH_3 at varying pH^{out}.

4. Active uptake of NH_3 would sustain the growth at low pH and low extracellular $NH_{(x)}$ concentrations

This discrepancy between the experimental and the modeling observation could be due to at least four factors: (a) The K_M of GS for NH_4^+ is much lower than the reported value of 100 μM. Considering the high intracellular glutamate concentration (75 mM) under N-limited growth conditions [11,17], the effective K_M should even be higher (±200 μM) [12]. In addition, the BRENDA database [18] reveals that most of the available K_M values for NH_4^+ in other prokaryotes are above 50 μM. (b) The experimentally determined maximum capacity of GS is underestimated. However, the measured expression levels (3% mass/mass of total protein) and maximal capacities of GS are already quite high (10 mM/s). (c) Channeling occurs between AmtB and GS. Although significant amounts of GS are membrane-bound, this membrane association occurred in an AmtB-independent fashion [19]. (d) Uptake of NH_3 does not occur via facilitated permeation, but through active transport. It is this possibility that we shall entertain further.

5. Our hypothesis

We hypothesize therefore that, at low external $NH_{(x)}$ concentrations, AmtB-mediated $NH_{(x)}$ transport *must* be active (i.e. active transport of NH_3 or facilitated diffusion of NH_4^+) to enable intracellular NH_4^+ concentrations to attain levels sufficient to sustain the biosynthetic nitrogen assimilation flux required for growth.

6. The existing evidence for permeation of $NH_{(x)}$ as NH_3, i.e. the evidence to the contrary of our hypothesis

Our hypothesis is inconsistent with the almost complete consensus that nitrogen moves as NH_3 through the AmtB channel [7,10], based on structural and activity data. On the basis of the X-ray structure of AmtB that was determined by two groups independently, both groups recognized a narrow hydrophobic channel in the protein monomer. The channel would not contain any water

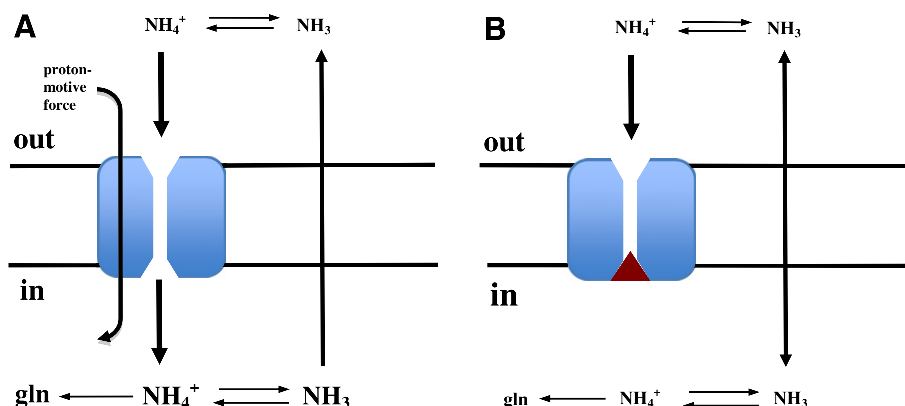

Fig. 2. Futile cycling of NH_4^+/NH_3 and interaction between AmtB and GlnK. The blue rounded rectangle represents the AmtB carrier. (A) Mechanisms (ii) and (iii) are combined in one scheme featuring active transport of ammonium. Intracellular accumulation of NH_4^+ and NH_3 is indicated by an increased font size. Passive outward permeation of NH_3 is denoted by the longest arrow. After protonation of NH_3, NH_4^+ will again be actively taken up via AmtB. This futile cycling goes on until NH_4^+ gets incorporated into glutamine. (B) The brown triangle represents GlnK. It physically blocks the exit of $NH_{(x)}$ from the cytoplasmic side of AmtB into the cytoplasm.

molecules, but might contain a single file of NH_3 molecules [7]. However, a recent X-ray crystallographic study suggests that it could also be filled with water molecules [20,21]. Also, X-ray structures reveal molecules such as water and NH_3 that are bound statically, but may not do so when they are bound dynamically. Solute transfer in transporter proteins is known to be a dynamic process. The conditions of crystallization lack the extremely high electric field corresponding to a 150 mV membrane potential. Membrane potentials of this magnitude have been observed to change the spectral dynamics of bacteriorhodopsin, probably reflecting structural changes [22]. Any NH_4^+ channel should operate only at high membrane potentials. Indeed, activity data are needed for more definitive conclusions: Neuhäuser et al. [23] demonstrated that of two paralogous ammonium transporters from Arabidopsis, with expected structural similarity, one transported NH_4^+ and one NH_3.

Khademi et al. [7] incorporated AmtB into liposomes loaded with a pH indicator and 20 mM HEPES (pH 6.8) and then mixed the liposomes with 5 mM NH_4Cl. They reported an increase in the intraliposomal pH, relaxing with a rate constant of 116 s^{-1}, i.e. 10 times faster than in the corresponding experiment with liposomes without protein reconstituted into them.[3] They took this alkalinization to reflect NH_3 entry and subsequent protonation to NH_4^+. However, this experiment may not discriminate between NH_3 and NH_4^+ transport. When measuring ion transport across a liposomal membrane by measuring the chemical activity of the ion, one must ensure that there is sufficient permeability for other ions to dissipate the electric transmembrane potential that will arise (cf. [26]). Khademi et al. [7] had no readily permeant counterions in their medium at significant concentrations. Consequently, movement of NH_4^+ through AmtB would have been hindered by the rising membrane potential, which would cause outward movement of protons leading to the same internal alkalinization as they found indicative of NH_3 transport. Had the authors added ammonium thiocyanate then the alkalinization might not have been observed. In addition, the experiments lacked controls such as with inactive AmtB mutant protein, or demonstrating of saturability: The K_M for NH_4^+ that can be calculated from the data given in [7] amounts to 400 μM, which is 50-fold higher than the concentration (8 μM) that gives complete inhibition of methylamine uptake in vivo [19], again suggesting that Khademi et al. [7] may not have been looking at the physiologically relevant transport mode. Indeed, in a systematic effort to reproduce the experiments of Khademi et al. [7], Javelle et al. [8] failed to observe a significant difference in the internal alkalinization rate between the proteoliposomes and the protein-free liposomes.

7. Evidence in favour of active transport of NH_3, i.e. in favour of our hypothesis

At least for some homologous, plant Amts, electrogenic transport of NH_3 was shown convincingly by current measurements [27,28]. Studies with AmtB variants, including plant's, were not thought to be compatible with NH_4^+ as the permeant species [23], but net electrogenic transport was not always ruled out [29]. Methylammonium uptake studies with *E. coli* [30] and *Corynebacterium glutamicum* [31] provided evidence for Amt-mediated electrogenic transport. Although active transport in the form of an NH_4^+ uniport system (Fig. 1, mechanism ii) seemed not very likely considering the available evidence, an NH_3/H^+ symport mechanism might constitute a plausible electrogenic mode of transport in bacterial AmtB (Fig. 1, mechanism iii). After dissociation of NH_4^+, the released proton might move separated from NH_3 in space or time through the AmtB channel [21]. Provided that the two transports would be coupled [the mechanism for which is unclear at present], such an NH_3/H^+ co-symport mechanism would correspond to active transport driven by the NH_3 gradient plus the proton-motive force. Fong et al. [30] who showed that a single amino acid substitution at the periplasmic interface of AmtB (W148L) greatly increased the active uptake of methylamine in a protonophore sensitive manner, also demonstrated active transport of methylamine by the wild type AmtB, supporting our hypothesis.

8. A problem generated by NH_4^+ transport: futile cycling of NH_4^+/NH_3

Active transport of NH_3 comes at a price: combined with passive outward NH_3 permeation it constitutes a futile cycle (Fig. 2A) that wastes Gibbs energy. Since the membrane potential is around 150 mV [32], in principle the concentration of $NH_{(x)}$ inside the cells could reach around 300-times the external concentration, assuming an infinite uptake rate. In a medium with a pH of 7.5 and a constant $NH_{(x)}$ concentration of 10 μM, the cytosolic

[3] We are afraid that based on the primary data shown in this paper, we cannot reproduce this factor of 10, i.e. 116/13. We calculate a factor of 2, i.e. 28/13, only, if we fit the data for the proteoliposomes with a mono-exponential. Although the authors did not specifically mention how the fitting was done, this is what a single rate constant implies. However, in our hands, the fit was not very good; in contrast, a close fit was obtained with a double-exponential, featuring an initial fast k of 60 s^{-1} and a second slow k of 13 s^{-1}. Two papers on the homologous rhesus factor RhCG, the first of which is by the same group, conclude to a rate constant of 23 s^{-1} and not the >100 s^{-1} claimed here [24,25]. The k = 23-curve in Fig. 4a of Gruswitz et al. [24] virtually coincides with the k = 116-curve of Fig. 5 in Khademi et al. [7].

$NH_{(x)}$ concentration would then be some 3 mM. An outward NH_3 gradient of around 90 μM would exist. Under this theoretical condition and at $P = 200$ μm/s, futile cycling would occur on a huge scale: the cycle number we calculate is 77 molecules flowing outward for each 78 flowing inward if μ is maintained at 0.1 h^{-1}.

How would the cell manage to live this dangerously? We propose that GlnK is the answer.

9. The second part of our hypothesis

The second part of our hypothesis postulates that the regulatory protein GlnK fine-tunes the active AmtB-mediated uptake of NH_3 from full activity to no activity at all so as to balance the trade-off between [negative effects of] futile cycling and [positive effects of a high] intracellular $NH_{(x)}$ level.

10. Evidence for our second hypothesis

In most eubacteria and archaea, the *glnK* and the *amtB* gene form an operon [33,34]. During growth with excess $NH_{(x)}$ as the N-source, the operon is not expressed [35]. When $NH_{(x)}$ was reduced to low levels, AmtB and GlnK expression became apparent [9,11,36–39]. The operon is also transcribed when *E. coli* (*Salmonella typhimurium*) grows in a medium with glutamine or arginine as the single N-source [11,35,36,40]. The evolutionary conservation of the *glnK–amtB* gene pair could be understood if $NH_{(x)}$ transport is regulated by GlnK [39,41–43]. Trimeric GlnK forms a stable 1:1 complex with trimeric AmtB [43], reversibly interacting via the T-loop of the former. The tip of the T-loop inserts deeply into the cytoplasmic pore exit, thereby presumably blocking conduction [44] (Fig. 2B). The inactivation may occur within seconds and in response to micromolar changes (10–200 μM) in the extracellular $NH_{(x)}$ concentration [19,39,45]. In response to low intracellular glutamine levels, which is indicative of severe N-limitation, GlnK is uridylylated at a tyrosine residue (Y51) in the T-loop [39]. Because uridylylated GlnK cannot bind to AmtB, $NH_{(x)}$ uptake is then not hampered. In vitro and in vivo, ADP favours complex formation between un-uridylylated GlnK and AmtB, whereas MgATP and α-ketoglutarate can replace ADP on GlnK leading to dissociation of the GlnK–AmtB complex [43,45,46]. Under N-limiting conditions α-ketoglutarate accumulates [45], up to 10 mM [17]. Upon a sudden $NH_{(x)}$ pulse, α-ketoglutarate decreases to submillimolar levels within a few minutes [17,45] and full inhibition of AmtB by GlnK should take place.

In view of the above conditional and partial inhibition of AmtB by GlnK, we propose that GlnK's cellular function is to prevent the *unnecessary* use of the proton-motive force for NH_3 uptake. Depending on the conditions, two cases can be distinguished here: (1) minimisation and (2) complete prevention of the use of the proton-motive force. At a constant and low level (say 10 μM) of $NH_{(x)}$, active transport via AmtB should be constrained by GlnK such that the cost of futile cycling is adapted to the benefit of a higher cytosolic NH_4^+ concentration in order not to waste Gibbs energy. If NH_3 is taken up in symport with one proton (0.33 mol ATP-equivalent) and if GlnK action constrains AmtB activity such that it results in a lower cytosolic NH_4^+ concentration (but high enough to maintain growth) and brings the cycle number down to say 3, the rate of ATP-equivalent consumption for futile cycling would go down to some 10% of the total ATP-equivalent consumption rate $\left(100\,\frac{\text{mmol ATP}}{\text{g DW}}\cdot 0.5\,\frac{\text{g DW}}{\text{ml}}\cdot 1000\cdot 1.0\,\frac{1}{3600\text{ s}} = 14\text{ mM/s}\right)$ at $\mu = 1.0\ h^{-1}$ ($J_{N\rightarrow \text{biomass}} = 1.4$ mM/s). This seems an investment of free energy in the fine-tuning of the AmtB-mediated active transport that might pay off. The second condition refers to the case when N-limited cells are confronted with a large and sudden $NH_{(x)}$ upshift (say 10 mM). Immediately after the upshift, proton-motive force driven transport of NH_3 can and should be completely stopped, because passive permeation of NH_3 then suffices.

An alternative would be that GlnK shifts the specificity of the AmtB channel from NH_4^+ to NH_3. The latter option seemed unrealistic until Fong et al. [30] suggested that a single amino acid substitution in AmtB greatly affected this specificity.

Recently, the AmtB-mediated uptake of $NH_{(x)}$ in N-limited *E. coli* was modeled [47]. Passive permeation of NH_3 was also included, as well as ammonium assimilation and a simplified form of AmtB-regulation by GlnK. Uptake of $NH_{(x)}$ was implicitly modeled to be active. The model showed that, in steady state at low external $NH_{(x)}$ concentrations (5 μM, pH 7), a small intracellular accumulation of NH_3 (1.3-fold) occurred, whereas AmtB activity was partially blocked by GlnK. Also, a small outward passive permeation flux of NH_3 existed. Thus, this model behaved in a manner qualitatively consistent with our hypothesis.

11. Conclusion

With this paper we hope to have indicated that there is insufficient experimental evidence to conclude whether AmtB uptake of $NH_{(x)}$ into *E. coli* involves transport of NH_3 or NH_4^+. We have demonstrated how in a field where the molecular data seem to indicate one type of mechanisms, a systems biology analysis suggests strongly that an alternative mechanism is operative. The analysis led us to experimental design: Activity assays should be done in the presence of agents dissipating the membrane potential, should measure the generation of that membrane potential, and if performed with intact cells and their high membrane potential, should be done at the lower concentrations of ammonia where the AmtB protein is actually expressed. Structural evidence should extend to mutant proteins that have been shown to catalyze NH_4^+ uptake. The possibility should be entertained that the specificity of transporters such as AmtB for NH_3 can shift to NH_4^+ due to a single amino acid change, binding of a second protein, or covalent modification of the protein.

12. Other observations supporting the hypothesis

Below we put forward a disparate collection of seven observations that are either consistent with our general line of reasoning (1, 2, 3) or may be considered in support of (4, 5, 6, 7) our hypothesis if the results – except for those in [48] – are interpreted differently from the original publications:

(1) The observation that the enterobacter *Klebsiella pneumoniae* exhibited nitrogen-limited behavior while growing in a chemostat ($D = 0.4\ h^{-1}$) at pH 4.25 in the presence of excess (80 mM) external $NH_{(x)}$ [49] is understandable, because the NH_3 gradient should have been insufficient to sustain the internal NH_4^+ concentration needed to attain the required saturation level of GS to allow for a growth rate of 0.4 h^{-1}; the active transport we propose would explain the growth.

(2) It has been shown for some bacteria, including *E. coli*, that growth in the exponential phase at neutral pH in batch culture was not affected by $NH_{(x)}$ concentrations up to 500 mM [50], which by inference implies a similarly high cytoplasmic concentration. So, it seems that for the usual media $NH_{(x)}$ as such is not toxic for bacteria, in contrast to plants and animals. Although not addressed in [50], an interesting question is what happens immediately after inoculation of the batch culture, when cells are suddenly confronted with 500 mM $NH_{(x)}$. We surmise that passive influx of NH_3 occurs on a massive scale and that active uptake via AmtB, if pres-

ent, will be of negligible importance. As a consequence, cells in the lag phase will have to deal with a substantial (but probably temporary) increase of the internal pH due to reprotonation of NH_3.

(3) Growth on organic N-compounds (and nitrate or N_2) will lead to $NH_{(x)}$ production inside cells and as a consequence NH_3 will leak out of the cell. Active transport then makes it possible to scavenge lost NH_3, but this would also induce futile cycling [51].

(4) AmtB (over)expression alone (by induction from different expression vectors) impaired or abolished growth of *E. coli* [37,52,53]. The adverse effects on growth could be conventionally explained as the deleterious effect of (over)expressing an integral membrane protein [54], but, alternatively, in this case, it could have been (partly) caused by excessive futile cycling due to the drastic disturbance of the balance between $NH_{(x)}$ transport and assimilation in the absence of GlnK.

(5) In the presence of high $NH_{(x)}$ concentrations, NH_4^+ can be taken up via the high affinity active K^+-transporter Kdp under K^+-limiting conditions. Coupled to permeation of NH_3 out of the cell, a Gibbs energy-wasting futile cycle emerged, as the authors concluded [48]. A Kdp-deficient mutant strain did not show this behavior. Other limitations, i.e. conditions where Kdp was not expressed, also did not show this negative effect on growth[4] [48].

(6) *Mutatis mutandis*, a similar story can be told for methyl-$NH_{(x)}$ (MA) and AmtB. MA has been used to determine the AmtB activity in vivo (e.g. [19,30]). If the hypothesis also holds for MA, it would imply that the AmtB activity as measured in whole cells using MA is being underestimated to the extent that futile cycling occurs during the uptake assay. Moreover, Javelle et al. calculated a ratio of 3.9 for internal/external MA concentrations for a cell lacking GS, which is indeed much smaller than a ratio of 200, expected if the membrane potential is 140 mV and MA transport is active transport [19]. Similarly, Fong et al. observed a 5- to 10-fold accumulation of MA in cells that lack GS [30]. Nevertheless both ratios are higher than 1.0. And since GlnK-dependent regulation of AmtB did not occur with MA [19], our interpretation is that accumulation was limited because of unmitigated futile cycling of MA.

(7) Growth of a *glnK*$^-$*amtB*$^+$ strain was in comparison to a *glnK*$^-$*amtB*$^-$ strain and the *glnK*$^+$*amtB*$^+$ (wild type) negatively affected in liquid cultures when after a period of N-starvation excess $NH_{(x)}$ was added: the lag phase was longer and growth occurred at a slower specific growth rate. Growth of the former strain on plates was similarly affected compared to the latter two strains. The same phenomena were observed when glutamine was the sole N-source [37,55]. We interpret these finding as to indicate that in the absence of GlnK and presence of AmtB, futile cycling will not be prevented by GlnK and growth is slower because of extra Gibbs energy dissipation. In the wild type, GlnK will stop AmtB activity and in the double mutant, no futile cycling can occur whatsoever.

Acknowledgements

This research was supported in part by the FP6-NEST-STREP project 043235 (EC-MOAN), by the SysMO project MOSES, by the BBSRC (BB/C008219/1; BB/F003528/1; BB/I00470X/1), and by the FP7 project UNICELLSYS.

[4] Since under all tested growth conditions, $NH_{(x)}$ was present in excess, with hindsight it can be concluded that AmtB was not present in these cells and consequently that AmtB-mediated futile cycling was absent.

References

[1] Reitzer, L. (2003) Nitrogen assimilation and global regulation in *Escherichia coli*. Annu. Rev. Microbiol. 57, 155–176.

[2] Martinelle, K. and Häggström, L. (1997) On the dissociation constant of ammonium: effects of using an incorrect pK_a in calculations of the ammonia concentration in animal cell cultures. Biotechnol. Tech. 8, 549–551.

[3] Lang, W., Blöck, T.M. and Zander, R. (1998) Solubility of NH_3 and apparent pK of NH_4^+ in human plasma, isotonic salt solutions and water at 37 °C. Clin. Chim. Acta 273, 43–58.

[4] Bruggeman, F.J., Boogerd, F.C. and Westerhoff, H.V. (2005) The multifarious short-term regulation of ammonium assimilation of *Escherichia coli*: dissection using an in silico replica. FEBS J. 272, 1965–1985.

[5] Lande, M.B., Donovan, J.M. and Zeidel, M.L. (1995) The relationship between membrane fluidity and permeabilities to water, solutes, ammonia, and protons. J. Gen. Physiol. 106, 67–84.

[6] Mathai, J.C., Sprott, D. and Zeidel, M.L. (2001) Molecular mechanisms of water and solute transport across Archaebacterial lipid membranes. J. Biol. Chem. 276, 27266–27271.

[7] Khademi, S., O'Connell III, J., Remis, J., Robles-Colmenares, Y., Miercke, L.J.W. and Stroud, R.M. (2004) Mechanism of ammonia transport by Amt/MEP/Rh: Structure of AmtB at 1.35 Å. Science 305, 1587–1594.

[8] Javelle, A., Lupo, D., Li, X.-D., Merrick, M., Chami, M., Ripoche, P. and Winkler, F.K. (2007) Structural and mechanistic aspects of Amt/Rh proteins. J. Struct. Biol. 158, 472–481.

[9] Soupene, E., He, L., Yan, D. and Kustu, S. (1998) Ammonia acquisition in enteric bacteria: physiological role of the ammonium/methylammonium transport B (AmtB) protein. Proc. Natl. Acad. Sci. USA 95, 7030–7034.

[10] Zheng, L., Kostrewa, D., Bernèche, S., Winkler, F.K. and Li, X.-D. (2004) The mechanism of ammonia transport based on the crystal structure of AmtB of *Escherichia coli*. Proc. Natl. Acad. Sci. USA 101, 17090–17095.

[11] Soupene, E., Lee, H. and Kustu, S. (2002) Ammonium/methylammonium transport (Amt) proteins facilitate diffusion of NH_3 bidirectionally. Proc. Natl. Acad. Sci. USA 99, 3926–3931.

[12] Meek, T.D. and Villafranca, J.J. (1980) Kinetic mechanism of *Escherichia coli* glutamine synthetase. Biochemistry 19, 5513–5519.

[13] Colanduoni, J., Nissan, R. and Villafranca, J.J. (1987) Studies of the mechanism of glutamine synthetase utilizing pH-dependent behavior in catalysis and binding. J. Biol. Chem. 262, 3037–3043.

[14] Alibhai, M. and Villafranca, J.J. (1994) Kinetic and mutagenic studies of the role of the active site residues Asp-50 and Glu-327 of *Escherichia coli* glutamine synthetase. Biochemistry 33, 682–686.

[15] Sakamoto, N., Kotre, A.M. and Savageau, M.S. (1975) Glutamate dehydrogenase from *Escherichia coli*: purification and properties. J. Bacteriol. 124, 775–783.

[16] Sharkey, M.A. and Engel, P.C. (2008) Apparent negative co-operativity and substrate inhibition in overexpressed glutamate dehydrogenase from *Escherichia coli*. FEMS Microbiol. Lett. 281, 132–139.

[17] Yuan, J., Doucette, C.D., Fowler, W.U., Feng, X., Piazza, M., Rabitz, H.A., Wingreen, N.S. and Rabinowitz, J.D. (2009) Metabolomics-driven quantitative analysis of ammonia assimilation in *E. coli*. Mol. Syst. Biol. 5, 302–318.

[18] Chang, A., Scheer, M., Grote, A., Schomburg, I. and Schomburg, D. (2009) BRENDA, AMENDA and FRENDA the enzyme information system: new content and tools in 2009. Nucleic Acids Res. 37, D588–D592.

[19] Javelle, A., Thomas, G., Marini, A., Krämer, R. and Merrick, M. (2005) In vivo functional characterization of the *Escherichia coli* ammonium channel AmtB: evidence for metabolic coupling of AmtB to glutamine synthetase. Biochem. J. 390, 215–222.

[20] Lamoureux, G., Klein, M.L. and Bernèche, S. (2007) A stable water chain in the hydrophobic pore of the AmtB ammonium transporter. Biophys. J. 92, L82–L84.

[21] Lamoureux, G., Javelle, A., Baday, S., Wang, S. and Bernèche, S. (2010) Transport mechanisms in the ammonium transporter family. Transfus. Clin. Biol. 17, 168–175.

[22] Hellingwerf, K.J., Schuurmans, J.J. and Westerhoff, H.V. (1978) Demonstration of coupling between the protonmotive force across bacteriorhodopsin and the flow through its photochemical cycle. FEBS Lett. 92, 181–186.

[23] Neuhäuser, B., Dynowski, M. and Ludewig, U. (2009) Channel-like NH_3 flux by ammonium transporter AtAMT2. FEBS Lett. 583, 2833–2838.

[24] Gruswitz, F., Chaudhary, S., Ho, J.D., Schlessinger, A., Pezeshki, B., Ho, C.-M., Sali, A., Westhoff, C.M. and Stroud, R.M. (2010) Function of human Rh based on structure of RhCG at 2.1 Å. Proc. Natl. Acad. Sci. USA 107, 9638–9643.

[25] Mouro-Chanteloup, I., Cochet, S., Chami, M., Genetet, S., Zidi-Yahiaoui, N., Engel, A., Colin, Y., Bertrand, O. and Ripoche, P. (2010) Functional reconstitution into liposomes of purified human RhCG ammonia channel. Plos One 5 (1), e8921. doi:10.1371/journal.pone.0008921.

[26] Arents, J.C., van Dekken, H., Hellingwerf, K.J. and Westerhoff, H.V. (1981) Linear relations between proton current and pH gradient in bacteriorhodopsin liposomes. Biochemistry 20, 5114–5123.

[27] Ludewig, U., von Wirén, N. and Frommer, W.B. (2002) Uniport of NH_4^+ by the root hair plasma membrane ammonium transporter LeAMT1;1. J. Biol. Chem. 277, 13548–13555.

[28] Ludewig, U., Neuhäuser, B. and Dynowski, M. (2007) Molecular mechanisms of ammonium transport and accumulation in plants. FEBS Lett. 581, 2301–2308.
[29] Javelle, A., Lupo, D., Ripoche, P., Fulford, T., Merrick, M. and Winkler, F.K. (2008) Substrate binding, deprotonation, and selectivity at the periplasmic entrance of the *Escherichia coli* ammonia channel AmtB. Proc. Natl. Acad. Sci. USA 105, 5040–5045.
[30] Fong, R.N., Kim, K., Yoshihara, C., Inwood, W.B. and Kustu, S. (2007) The W148L substitution in the *Escherichia coli* ammonium channel AmtB increases flux and indicates that the substrate is an ion. Proc. Natl. Acad. Sci. USA 104, 18706–18711.
[31] Walter, B., Küspert, M., Ansorge, D., Krämer, R. and Burkovski, A. (2008) Dissection of ammonium uptake systems in *Corynebacterium glutamicum*: mechanism of action and energetics of AmtA and AmtB. J. Bacteriol. 190, 2611–2614.
[32] Jensen, P.R. and Michelsen, O. (1992) Carbon and energy metabolism of *at*p mutants of *Escherichia coli*. J. Bacteriol. 174, 7635–7641.
[33] Thomas, G.H., Mullins, J.G.L. and Merrick, M. (2000) Membrane topology of the Mep/Amt family of ammonium transporters. Mol. Microbiol. 37, 331–344.
[34] Sant'Anna, F.H., Trentini, D.B., de Souto Weber, S., Cecagno, R., Ceroni da Silva, S. and Schrank, I.S. (2009) The PII superfamily revised: a novel group and evolutionary insights. J. Mol. Evol. 68, 322–336.
[35] van Heeswijk, W.C., Hoving, S., Stegeman, B., Kahn, D. and Westerhoff, H.V. (1996) An alternative PII protein in the regulation of glutamine synthetase in *Escherichia coli*. Mol. Microbiol. 21, 133–146.
[36] Atkinson, M.R., Blauwkamp, T.A., Bondarenko, V., Studitsky, V. and Ninfa, A.J. (2002) Activation of the *glnA*, *glnK*, and *nac* promoters as *Escherichia coli* undergoes the transition from nitrogen excess growth to nitrogen starvation. J. Bacteriol. 184, 5358–5363.
[37] Blauwkamp, T.A. and Ninfa, A.J. (2003) Antagonism of PII signalling by the AmtB protein of *Escherichia coli*. Mol. Microbiol. 48, 1017–1028.
[38] Maheswaran, M. and Forchhammer, K. (2003) Carbon-source-dependent nitrogen regulation in *Escherichia coli* is mediated through glutamine-dependent GlnB signalling. Microbiology 149, 2163–2172.
[39] Javelle, A., Severi, E., Thornton, J. and Merrick, M. (2004) Ammonium sensing in *Escherichia coli*. Role of the ammonium transporter AmtB and AmtB–GlnK complex formation. J. Biol. Chem. 10, 8530–8538.
[40] van Heeswijk, W.C., Wen, D., Clancy, P., Jaggi, R., Ollis, D.L., Westerhoff, H.V. and Vasudevan, S.G. (2000) The *Escherichia coli* signal transducers PII (GlnB) and GlnK form heterotrimers in vivo: fine tuning the nitrogen signal cascade. Proc. Natl. Acad. Sci. USA 97, 3942–3947.
[41] Javelle, A. and Merrick, M. (2005) Complex formation between AmtB and GlnK: an ancestral role in prokaryotic nitrogen control. Biochem. Soc. Trans. 33, 170–172.
[42] Coutts, G., Thomas, G., Blakey, D. and Merrick, M. (2002) Membrane sequestration of the signal transduction protein GlnK by the ammonium transporter AmtB. EMBO J. 21, 536–545.
[43] Durand, A. and Merrick, M. (2006) In vitro analysis of the *Escherichia coli* AmtB–GlnK complex reveals a stoichiometric interaction and sensitivity to ATP and 2-oxoglutarate. J. Biol. Chem. 281, 29558–29567.
[44] Conroy, M.J., Durand, A., Lupo, D., Li, X., Bullough, P.A., Winkler, F.K. and Merrick, M. (2007) The crystal structure of the *Escherichia coli* AmtB–GlnK complex reveals how GlnK regulates the ammonia channel. Proc. Natl. Acad. Sci. USA 104, 1213–1218.
[45] Radchenko, M.V., Thornton, J. and Merrick, M. (2010) Control of AmtB–GlnK complex formation by intracellular levels of ATP, ADP and 2-oxoglutarate. J. Biol. Chem. 285, 31037–31045.
[46] Gruswitz, F., O'Connell III, J. and Stroud, R.M. (2007) Inhibitory complex of the transmembrane ammonia channel, AmtB, and the cytosolic regulatory protein, GlnK, at 1.96 Å. Proc. Natl. Acad. Sci. USA 104, 42–47.
[47] Ma, H., Boogerd, F.C. and Goryanin, I. (2009) Modelling nitrogen assimilation of *Escherichia coli* at low ammonium concentration. J. Biotechnol. 144, 175–183.
[48] Buurman, E.T., Teixeira de Mattos, M.J. and Neijssel, O.M. (1991) Futile cycling of ammonium ions via the high affinity potassium uptake system (Kdp) of *Escherichia coli*. Arch. Microbiol. 155, 391–395.
[49] Buurman, E.T., Teixeira de Mattos, M.J. and Neijssel, O.M. (1989) Nitrogen-limited behaviour of micro-organisms growing in the presence of large concentrations of ammonium ions. FEMS Microbiol. Lett. 58, 229–232.
[50] Müller, T., Walter, B., Wirtz, A. and Burkovski, A. (2006) Ammonium toxicity in bacteria. Curr. Microbiol. 52, 400–406.
[51] Kleiner, D. (1985) Bacterial ammonium transport. FEMS Microbiol. Rev. 32, 87–100.
[52] Soupene, E., Chu, T., Corbin, R.W., Hunt, D.F. and Sydney Kustu, S. (2002) Gas channels for NH_3: proteins from hyperthermophiles complement an *Escherichia coli* mutant. J. Bacteriol. 184, 3396–4000.
[53] Blakey, D., Leech, A., Thomas, G.H., Coutts, G., Findlay, K. and Merrick, M. (2002) Purification of the *Escherichia coli* ammonium transporter AmtB reveals a trimeric stoichiometry. Biochem. J. 364, 527–535.
[54] Jensen, P.R., Westerhoff, H.V. and Michelsen, O. (1993) Excess capacity of H^+-ATPase and inverse respiratory control in *Escherichia coli*. EMBO J. 12, 1277–1282.
[55] Blauwkamp, T.A. and Ninfa, A.J. (2002) Physiological role of the GlnK signal transduction protein of *Escherichia coli*: survival of nitrogen starvation. Mol. Microbiol. 46, 203–214.