Ammonium (methylammonium) transport by heterocysts and vegetative cells of *Anabaena variabilis*

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Abstract

Transport of the ammonium analogue [14C]methylammonium was similar in non-growing, fully differentiated heterocysts as compared to vegetative, multiplying cells of the filamentous cyanobacterium *Anabaena variabilis*. NH₄⁺ inhibited uptake into the cells and released accumulated methylammonium from the cells. These observations suggest that the main function of ammonium transport in heterocysts may not be NH₄⁺ acquisition but cyclic retention of ammonia produced by nitrogenase. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Although present only in low levels in many environments, ammonium (NH₄⁺) is the preferred nitrogen source for most bacteria and cyanobacteria [1,2]. Most microorganisms living in terrestrial and aquatic environments express specific ammonium carriers for the energy-dependent and concentrative uptake of this compound. Expression of these carriers is repressed in ammonium-rich media, since then diffusion of NH₃ apparently is sufficiently fast to satisfy the metabolic demands [2]. Derepression in general is observed under nitrogen-limiting conditions, where ammonium carriers are suggested to serve in cyclic retention of NH₃/NH₄⁺ which is produced intracellularly, e.g. by degradation of amino acids or fixation of N₂, and to a large extent is lost to the outside by passive diffusion as NH₃ [2]. This cyclic retention hypothesis was challenged [3]. Support, however, was derived from genetically undefined mutants of the N₂-fixing enterobacterium *Klebsiella* M5al, which do not express ammonium carriers and constantly excrete NH₃ [4,5].

Activity of ammonium transport (Amt) systems is generally studied by determining uptake of the non-metabolizable ammonium analogue [14C]methylammonium (MA), necessary controls including inhibition by ammonium and the ability of ammonium to chase unmetabolized methylammonium from the cells.

Amt, as evidenced from [14C]MA uptake, has been reported in cyanobacteria [1]. An especially interest-
ing group are the filamentous cyanobacteria which can form differentiated cells, i.e. akinetes and heterocysts [6]. Akinetes are non-growing, cyst-like cells which are formed under adverse conditions like drought or severe phosphate limitation. Heterocysts are the sites of $N_2$ fixation. They are formed upon nitrogen deprivation as a result of major physiological and morphological changes, affecting expression of about 1000 genes (see e.g. [8]). It is unknown to what extent membrane functions like transport are changed during this transition.

Singh et al. [9] isolated mutants which were defective in both Amt and heterocyst formation. This indicates a regulatory link between these two characteristics.

So far Amt has only been determined in vegetative cells and in akinetes [7].

Here we report that heterocyst formation does not affect ammonium transport in *Anabaena variabilis*, and discuss the biological significance for these non-growing cells.

2. Materials and methods

*A. variabilis* ATCC 29413 was obtained from P. Wolk (Michigan State University). The strain, maintained as described [10], was grown asynchronously in 2 l nitrate-containing BG11 medium [11] at 30°C and under continuous illumination. At mid-exponential phase (generally with a chlorophyll content of around 1 mg l$^{-1}$), the bacterial filaments were collected, washed twice with BG11 $0$ (nitrate-free), resuspended in BG11 $0$ and incubated under the same growth conditions for 3 days to form mature heterocysts.

Heterocysts were isolated by a modification of the methods of Golden et al. [12] and Fay [13]: filaments containing these cells were resuspended in 50 ml of isotonic buffer and homogenized briefly using a glass homogenizer. After addition of lysozyme (1 mg ml$^{-1}$), the suspensions were lightly shaken for 2 h at 30°C. The digestion mixture was then diluted 5 times with the same buffer and 0.5% (w/v) sodium dodecyl sulfate was added. After centrifugation for 5 min at 3000×g, the pellet was resuspended in 250 ml of the same buffer and centrifuged at 400×g for 5 min. Microscopic inspections showed that these preparations contained more than 95% heterocysts. In addition, after plating these cells on BG11 agar plates, no growth was observed after 3 days, indicating the absence of viable vegetative cells. Controls with whole filaments showed good growth under these conditions.

Chlorophyll was assayed according to [14], total protein concentration was determined after sonication (2 min for vegetative cells, 5 min for heterocysts) according to [15]. Transport assays with $[^{14}C]$MA were carried out as described in [16], using a substrate concentration of 30 μM. For best results, heterocysts had to be incubated in the isotonic isolation buffer for 2–3 h in daylight without shaking.

3. Results and discussion

Typical data as shown in Fig. 1 clearly indicate that concentrative ammonium uptake in *A. variabilis* remains largely unaffected during formation of heterocysts. If we assume that – similar to *Escherichia coli* [17] – the dry mass contains about 50% protein, and that 70% of the cell is water, we arrive at an approximately 80-fold concentration gradient of $[^{14}C]$MA across the membrane of both vegetative cells and heterocysts. This is in the range previously reported for a number of bacterial and cyanobacte-

![Fig. 1. Transport of $[^{14}C]$MA by vegetative cells (filled symbols) and heterocysts (empty symbols) of *A. variabilis* ATCC 29413. At 4.5 min a chase with 5 mM $NH_4Cl$ was performed. Circles show the activities of untreated cells, triangles show the activities of cells treated with 5 mM $NH_4Cl$ 5 min prior to the start of the uptake assay.](https://academic.oup.com/femsle/article-abstract/181/12/303/73620)
Both the inhibition of $[^{14}\text{C}]\text{MA}$ transport and chase by ammonium strongly indicate [1,2,18] that the activities determined are due to an ammonium transport system.

This is also corroborated by the results in Fig. 2, where 5 and 10 µM NH$_4$Cl were added together with $[^{14}\text{C}]\text{MA}$: as noted previously [19,20], uptake is preceded by a lag phase, the length of which is proportional to the amount of ammonium added. This suggests that $[^{14}\text{C}]\text{MA}$ uptake started when ammonia was exhausted from the uptake medium.

After extended time intervals, vegetative cells exhibited a second, slower uptake phase, as had been noted previously [1,7,9]. In contrast, akinetes [7], heterocysts did not show this second uptake phase (results not shown). This slower phase had been attributed to metabolism by glutamine synthetase or to diffusion into the intrathylakoid space [1].

$[^{14}\text{C}]\text{MA}$ uptake by proteobacteria and cyanobacteria has been reported to be inhibited by compounds collapsing the proton motive force like carbonyl cyanide $m$-chlorophenyl hydrazone (CCCP) or triphenylmethyl phosphonium salts (TPMP) [1,2]. As expected, MA uptake in vegetative cells was abolished completely by 0.2 mM CCCP or 0.5 mM TPMP (5 min pre-incubation with the inhibitors). In contrast, these inhibitors did not or only marginally (less than 10%) affect MA uptake by heterocysts, even after pre-incubations up to 1 h. Similar results were obtained with 0.5 mM methionine sulfoximine, which had been found to inhibit MA transport in proteobacteria and cyanobacteria [2]. We suppose that these negative results are due to the inability of these compounds to diffuse across the heterocyst cell wall and to reach the cytoplasmic membrane.

As to the biological significance of ammonium transport by heterocysts, it must be borne in mind that these cells (a) are the oxygen-free sites of ammonia production by N$_2$ fixation under otherwise aerobic conditions and (b) are irreversibly and terminally differentiated and thus unable to grow and divide (see [8]). This implies that they do not require exogenously supplied ammonium for metabolism but rather export fixed N. Therefore we conclude that the ammonium carriers of heterocysts function in cyclic retention of ammonia produced by nitrogenase. Without cyclic retention considerable net diffusion of NH$_3$ through the cytoplasmic membrane to the outside would occur.

A second function may be sensing the extracellular ammonium level, the rise of which leads to rapid switch-off of nitrogenase activity by an unknown post-translational modification [21]. This would stop a considerable energy expenditure [6,8]. However, the experimental conditions triggering this modification (pH 10 and >0.5 mM ammonia) do not necessarily require an ammonium transporter.

In conclusion, heterocysts provide a well defined system to demonstrate the involvement of ammonium carriers in cyclic NH$_3$/NH$_4^+$ retention.

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References


Fig. 2. $[^{14}\text{C}]\text{MA}$ uptake by heterocysts in the presence of 5 µM (filled circles) and 10 µM NH$_4$Cl (open circles).

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