Characterization of Methylammonium/Ammonium Transport in Mutant Strains of *Anabaena variabilis* Resistant to Ammonium Analogues

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A methylamine-resistant mutant strain (4m3) of *Anabaena variabilis* ATCC 29413 was capable of growth at pH 7.0, but not at pH 9.0, in the presence of concentrations of methylamine that totally inhibited growth of the parent strain. Strain 4m3 showed a marked reduction, at pH 7.0 but not at pH 9.0, in the initial rapid and slower second phases of uptake of [^{14}C]methylamine and ammonia, when compared with the parent strain. The rate of inhibition of nitrogenase activity, on adding ammonia at pH 7.0, was lower in strain 4m3 than in the parent strain and was attributable to a defect in the $\text{CH}_3\text{NH}_3^+/\text{NH}_4^+$ transport system. Evidence that the second slower phase of [^{14}C]methylamine uptake is associated with metabolism via glutamine synthetase (GS) was obtained using mutant strains partially deficient in GS. Unlike these GS-deficient strains, strain 4m3 did not liberate ammonia extracellularly unless treated with L-methionine-DL-sulphoximine. Growth of strain 4m3 in medium containing 3 mM-methylamine resulted in a restoration of the first phase of [^{14}C]methylamine uptake, at pH 7.0, but not of ammonia uptake. However, growth in the presence of 3 mM-ammonia did not enhance the uptake of either methylamine or ammonia. Strain 4m3 is apparently deficient in the active $\text{CH}_3\text{NH}_3^+/\text{NH}_4^+$ transport system, but has a CH_3NH_3^+ transport system which can be induced by growth in the presence of methylamine.

INTRODUCTION

The existence of NH_4^+ transport systems in free-living N_2 -fixing organisms is of interest because nitrogenase activity and synthesis are usually inhibited when the organisms are exposed to high concentrations of ammonia (see Murry & Benemann, 1979; Eady *et al.*, 1982; Stewart *et al.*, 1985). Most cyanobacteria resemble other micro-organisms in taking up ammonia in preference to other nitrogen sources (Ohmori *et al.*, 1977).

NH_3 is a small, uncharged molecule which can pass freely through lipid bilayers by passive diffusion, and a possible mechanism for accumulation of ammonia, at external pH values higher than the cytoplasmic pH, could involve simple diffusion followed by trapping through protonation, given a membrane permeable to NH_3 but impermeable to NH_4^+ [the terms ammonia or methylamine are used, throughout this paper, unless the charged (NH_4^+ or CH_3NH_3^+) or uncharged (NH_3 or CH_3NH_2) species are meant specifically]. Such a mechanism could not, however, account for the observed accumulation of ammonia against a concentration gradient at external pH values lower than the cytoplasmic pH.

Methylammonium (CH_3NH_3^+), an analogue of NH_4^+ , has frequently been used as a substrate for studying NH_4^+ transport systems (Alef & Kleiner, 1982; Boussiba *et al.*, 1984a; Rai *et al.*, 1984; Kerby *et al.*, 1986a; for a review see Kerby *et al.*, 1987a). Recent studies on *Anabaena*

Abbreviations: CCCP, carbonylcyanide-*m*-chlorophenylhydrazine; chl *a*, chlorophyll *a*; GS, glutamine synthetase; MSX, L-methionine-DL-sulphoximine.

variabilis have shown that CH_3NH_2^+ uptake is biphasic, with the first phase representing active (probably $\Delta\psi$ -dependent) transport into the cells and the second phase being dependent upon sustained assimilation of methylamine via glutamine synthetase (GS), resulting in γ -methylglutamine formation (Rai *et al.*, 1984; Kerby *et al.*, 1986a). Inhibition of GS by treatment with L-methionine-DL-sulphoximine (MSX) (Stewart & Rowell, 1975) has been employed as a means of distinguishing between methylamine transport into cells and its subsequent metabolism via GS (Boussiba *et al.*, 1984a; Kerby *et al.*, 1986a). Thus, there is evidence that MSX has no effect on the rapid phase of methylamine uptake into the cells but inhibits the second slower phase of uptake associated with the formation of γ -methylglutamine (Boussiba *et al.*, 1984a; Rai *et al.*, 1984; Kerby *et al.*, 1986a). It has been suggested that it is not ammonia *per se* but a product of its assimilation via GS which regulates nitrogenase synthesis (Stewart & Rowell, 1975; Ramos *et al.*, 1985; Reich *et al.*, 1986; Helber *et al.*, 1988). However, Singh *et al.* (1983) and Turpin *et al.* (1984) have proposed that MSX directly inhibits the NH_4^+ transport system, and that NH_4^+ *per se* regulates nitrogenase synthesis. It was further proposed that the two phases of uptake represent two different NH_4^+ transport systems, with the second slower phase being attributed to an MSX-sensitive transport system (Singh *et al.*, 1985).

In this paper we consider the uptake and metabolism of methylamine and ammonia and their effects on nitrogenase activity in a mutant strain of *A. variabilis* resistant to the ammonia analogue methylamine. To our knowledge, this is the first reported characterization of an NH_4^+ transport mutant of a cyanobacterium, providing new information on NH_4^+ transport.

METHODS

Organisms and growth conditions. The parent strain (*Anabaena variabilis* Kütz. ATCC 29413), mutant strains ED81 and ED92 (Polukhina *et al.*, 1982) and the methylamine-resistant mutant strain 4m3 were grown under air in batch culture in BG-11₀ medium (which is free of combined nitrogen) (Rippka *et al.*, 1979) at 25 °C and in continuous light at a photon fluence rate of $15 \mu\text{mol m}^{-2} \text{s}^{-1}$.

Mutagenesis and mutant selection. This was a modification of the method described previously (Kerby *et al.*, 1987b). *A. variabilis* cells were harvested by centrifugation from exponential-phase cultures and were resuspended in 10 mM-HEPES/NaOH (pH 7.0). Cultures were sonicated in a cleaning bath (Cole Parmer, Illinois) until filaments were reduced to an average length of two cells. The cells were washed twice in 10 mM-HEPES/NaOH (pH 7.0) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was added to a concentration of $250 \mu\text{g ml}^{-1}$. The suspension was incubated for 4 h at 30 °C in the light ($15 \mu\text{mol m}^{-2} \text{s}^{-1}$) with shaking. The treatment was terminated by washing the cells three times with BG-11₀ and resuspending in BG-11₀; it resulted in a 1% survival of cells.

In order to isolate mutant strains resistant to methylamine, mutagenized cells were grown at 30 °C at a photon fluence rate of $15 \mu\text{mol m}^{-2} \text{s}^{-1}$ for several generations, sonicated as above, and then plated onto BG-11₀ plates solidified with 1% (w/v) agar containing 10 mM-HEPES/NaOH (pH 7.0) and 1 mM-methylamine. Colonies resistant to methylamine were picked after incubation in the light ($15 \mu\text{mol m}^{-2} \text{s}^{-1}$) at 30 °C to allow colony development and restreaked on selective medium a further five times prior to growth in liquid BG-11₀ containing 10 mM-HEPES/NaOH (pH 7.0) and 1 mM-methylamine.

Chlorophyll *a* determinations. Chlorophyll was extracted in methanol in the dark at 4 °C. Absorbance was measured at 663 nm and the chlorophyll *a* (chl *a*) concentration calculated according to Mackinney (1941).

[¹⁴C]Methylamine uptake. The cyanobacteria were harvested during exponential growth, washed and resuspended in either 10 mM-HEPES/NaOH (pH 7.0) or 10 mM-Tricine/NaOH (pH 9.0) and equilibrated for 30 min at a photon fluence rate of $75 \mu\text{mol m}^{-2} \text{s}^{-1}$ at 25 °C. ¹⁴C-labelled methylamine was then added to the cell suspension (approximately $10 \mu\text{g chl } a \text{ ml}^{-1}$) to a final concentration of $30 \mu\text{M}$ (specific activity 10 kBq ml^{-1}). After periods of incubation, the cells were removed from their bathing medium by microcentrifugation through Dow Corning 550 silicon oil/dinonylphthalate (40:60, v/v) into perchloric acid in water (15%, v/v) (Rai *et al.*, 1984). Samples of the perchloric acid fraction were withdrawn for estimation of ¹⁴C incorporation by liquid scintillation counting using a toluene-based scintillant and a Packard 300 CD scintillation counter. As a result of experimental difficulties in quantifying non-specific binding and/or carry-over of bathing medium, no correction was routinely applied to the ¹⁴C incorporated.

Ammonia uptake. The cyanobacteria were harvested during exponential growth, washed and resuspended, to a concentration of approximately $10 \mu\text{g chl } a \text{ ml}^{-1}$, in 50 ml of either 10 mM-HEPES/NaOH (pH 7.0) or 10 mM-Tricine/NaOH (pH 9.0) and equilibrated for 30 min at a photon fluence rate of $75 \mu\text{mol m}^{-2} \text{s}^{-1}$ at 25 °C. The uptake of ammonia was initiated by addition of a known concentration of NH_4Cl to the cell suspension. After periods of incubation the cells were removed from their bathing medium by centrifugation. NaOH (final concentration 0.1 M) was added to the bathing medium to convert NH_4^+ to NH_3 ($pK_a = 9.25$) and NH_3

concentration was determined using an NH_3 gas sensing electrode (Russell pH, Auchtermuchty, Scotland). The electrode was calibrated using a range of NH_4Cl concentrations.

Ammonia liberation. Ammonia in the growth medium was determined using the method of Solorzano (1969), after removal of cells by centrifugation.

Methylamine and ammonia metabolism. Measurement of methylamine and ammonia incorporation into amino acids was carried out by incubating cells ($200\text{ }\mu\text{g chl } a$) in 10 mM-HEPES/NaOH ($\text{pH } 7.0$) containing a known concentration of either methylamine or ammonia at $25\text{ }^\circ\text{C}$ and a photon fluence rate of $75\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$. The cells were harvested and amino acid extraction and analysis performed as before (Kerby *et al.*, 1986a). For calculation of amino acid pool concentrations, the intracellular volume was determined by the method of Reed *et al.* (1987).

Glutamine synthetase (GS) activity. Cell-free extracts were prepared by sonication in 50 mM-Tris/HCl ($\text{pH } 7.5$), $2\text{ mM-Na}_2\text{EDTA}$, $3\text{ mM-dithiothreitol}$ and 5 mM-MgCl_2 followed by centrifugation at 10000 g . GS Mg^{2+} -dependent biosynthetic activity was assayed as described previously (Kerby *et al.*, 1986a).

Nitrogenase activity. This was determined using the acetylene reduction assay (Stewart *et al.*, 1967).

Protein determination. Protein concentrations of cell-free extracts were determined by the method of Bradford (1976).

Chemicals. [^{14}C]Methylamine hydrochloride was obtained from Amersham. All other chemicals were obtained from Sigma or BDH.

RESULTS

Methylamine-resistant mutant strains of *Anabaena variabilis* were obtained by selecting for growth at $\text{pH } 7.0$ in the presence of 1 mM-methylamine , a concentration which completely inhibited growth of the parent strain. The kinetics of uptake of [^{14}C]methylamine were determined for each of 10 methylamine-resistant strains, in order to identify any strains that might be deficient in CH_3NH_3^+ transport and/or metabolism (Rai *et al.*, 1984; Kerby *et al.*, 1986a). Six of the strains tested showed kinetics of [^{14}C]methylamine uptake, at an external pH of 7.0 , which were similar to those of the parent strain or even showed enhanced uptake (data not shown), when compared with the parent strain. Four strains showed a reduced [^{14}C]methylamine uptake at $\text{pH } 7.0$, and one of these (4m3), which showed the most marked reduction, was further characterized with respect to the uptake and assimilation of methylamine and ammonia.

The kinetics of growth of the parent strain and strain 4m3 at $\text{pH } 7.0$ were similar in N-free medium or in medium containing 3 mM-ammonia (doubling time $110\text{--}135\text{ h}$), although strain 4m3 showed a longer lag phase in the presence of ammonia. The growth of strain 4m3 (doubling time 135 h) was not inhibited in medium containing 3 mM-methylamine at $\text{pH } 7.0$ whereas growth of the parent strain was totally inhibited. Neither the parent strain nor strain 4m3 was capable of growth in medium containing 1 mM-methylamine at $\text{pH } 9.0$.

Kinetics of methylamine uptake by strain 4m3

As previously demonstrated (Rai *et al.*, 1984; Kerby *et al.*, 1986a), the parent strain showed a typical biphasic uptake of [^{14}C]methylamine at external pH values of 7.0 and 9.0 (Fig. 1). At $\text{pH } 7.0$ (Fig. 1a), strain 4m3, grown in N-free medium, showed a reduced first phase of methylamine uptake and a slower second phase of uptake than the parent strain, whereas at $\text{pH } 9.0$ (Fig. 1b), the kinetics of uptake were similar to those of the parent strain. The reduced first phase of uptake at $\text{pH } 7.0$ indicates a deficiency in CH_3NH_3^+ transport which is not apparent at $\text{pH } 9.0$, where methylamine can accumulate internally by passive diffusion of CH_3NH_2 followed by ion trapping in response to the pH gradient (lower inside, assuming a cytoplasmic pH of approximately 7.5 : see Hawkesford *et al.*, 1983). Since the rate of uptake during the first phase is too rapid for accurate measurement, only the extent of uptake in this phase was determined.

The rates of the second phase of methylamine uptake (Fig. 1) were 20 and $23\text{ pmol min}^{-1}(\text{ }\mu\text{g chl } a)^{-1}$ at $\text{pH } 7.0$ and $\text{pH } 9.0$, respectively, for the parent strain, and 4 and $19\text{ pmol min}^{-1}(\text{ }\mu\text{g chl } a)^{-1}$ at $\text{pH } 7.0$ and $\text{pH } 9.0$, respectively, for strain 4m3. When strain 4m3 was grown in medium containing 3 mM-methylamine , the first phase of uptake of methylamine at $\text{pH } 7.0$ (Fig. 1a) was increased to a level identical to that of the parent strain grown in N-free medium,

Fig. 1

Fig. 2

Fig. 1. Methylamine uptake at pH 7.0 (a) and pH 9.0 (b) by the parent strain ($\circ$) and mutant strain 4m3 ($\square$), grown in BG-11₀ medium at pH 7.0 (a) and pH 9.0 (b), and by strain 4m3 ($\blacksquare$) grown in the same medium containing 3 mM-methylamine at pH 7.0. Media were buffered at the appropriate pH using 10 mM-HEPES/NaOH (pH 7.0) or 10 mM-Tricine/NaOH (pH 9.0). $^{14}\text{CH}_3\text{NH}_3\text{Cl}$ was added to give a concentration of 30 μM at zero time. Lines were fitted to data points from 1 to 10 min by linear regression. In this and other figures, the results are means of at least three replicates, with standard errors being less than $\pm 10\%$ of the mean.

Fig. 2. Effects of the uncoupler CCCP on methylamine uptake at pH 7.0 by the parent strain ($\circ$, $\bullet$) and mutant strain 4m3 ($\square$, $\blacksquare$) grown in BG-11₀ medium at pH 7.0. Prior to adding $^{14}\text{CH}_3\text{NH}_3\text{Cl}$ (to 30 μM) at zero time, cell suspensions in 10 mM-HEPES/NaOH (pH 7.0) were incubated either in the light at a photon fluence rate of 75 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in the absence of CCCP ($\circ$, $\square$) or in the dark with 100 μM -CCCP ($\bullet$, $\blacksquare$) for 3 h.

although growth in the presence of 3 mM-ammonia (data not shown) did not have this effect. These findings indicate the presence of a methylamine-induced CH_3NH_3^+ transport system. The rate of the second phase of uptake [$8 \text{ pmol min}^{-1} (\mu\text{g chl } a)^{-1}$] by strain 4m3 grown in the presence of methylamine was lower than that for the parent strain. Since the second phase of uptake of methylamine is considered to be dependent on the metabolism of methylamine via GS, the data indicate a lower rate of methylamine metabolism in strain 4m3 than in the parent strain. GS biosynthetic activities in cell-free extracts of the parent strain and strain 4m3 were similar [73 and $63 \text{ nmol product formed min}^{-1} (\text{mg protein})^{-1}$, respectively]. This suggests that the reduced second phase of methylamine uptake is not due to a reduction in GS activity under substrate-saturating conditions. As we have previously shown (Kerby *et al.*, 1986b), addition of 3 mM-ammonia to *A. variabilis* at pH 7.0 caused a rapid efflux of radioactivity and prevented further [^{14}C]methylamine uptake. In both the parent strain and the mutant strain, the magnitude of the efflux of radioactivity was similar to that of the first phase of uptake of methylamine (data not shown) and probably represents displacement of the internal free pool of methylamine together with some non-specifically bound CH_3NH_3^+ . To check the extent to

Table 1. *Effects of ammonia and methylamine on amino acid pools of the parent strain and mutant strain 4m3*

Free pools of glutamate, glutamine and γ -methylglutamine (MeGln) were determined before (control) and after adding 50 μM - NH_4Cl (20 min incubation) or 30 μM - $\text{CH}_3\text{NH}_3\text{Cl}$ (30 min incubation) at pH 7.0. Cells grown in batch culture in BG-11₀ medium were harvested by centrifugation, resuspended in 10 mM-HEPES/NaOH (pH 7.0) and allowed to equilibrate for 30 min at 25 °C and a photon fluence rate of 75 $\mu\text{mol m}^{-2} \text{s}^{-2}$, prior to adding NH_4Cl or $\text{CH}_3\text{NH}_3\text{Cl}$. ND, not detectable.

Amino acid	Intracellular concentration (mM)					
	Parent strain			Strain 4m3		
	Control	+ NH_4Cl	+ $\text{CH}_3\text{NH}_3\text{Cl}$	Control	+ NH_4Cl	+ $\text{CH}_3\text{NH}_3\text{Cl}$
Glu	33.9	24.2	16.5	11.2	7.9	10.1
Gln	3.8	32.8	3.3	9.0	21.4	5.7
MeGln	ND	ND	9.0	ND	ND	4.9

which the uptake of [^{14}C]methylamine was due to non-specific binding to the cells and/or carry-over of bathing medium, uptake was examined (Fig. 2) at pH 7.0 in the dark in cells pretreated for 3 h with the uncoupler carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) at a concentration of 100 μM (see Hawkesford *et al.*, 1983). Under these conditions, where both the transport and metabolism of methylamine should be inhibited, the first phase of uptake of methylamine by the parent strain was reduced by about 75%. The residual uptake of methylamine presumably represents non-specific binding to the cells. In strain 4m3, grown in the absence of methylamine, pretreatment with CCCP resulted in only a slight reduction (approximately 30%) in the first phase of uptake of methylamine, indicating that most of the apparent methylamine uptake at pH 7.0 does not represent the accumulation of an endogenous free pool of methylamine, but perhaps results from the non-specific binding of CH_3NH_3^+ to the cells.

Relationship between methylamine uptake and the synthesis of γ -methylglutamine

It has previously been shown that the pretreatment of *A. variabilis* with MSX, to wholly or partially inactivate GS, results in a corresponding reduction in the second phase of methylamine uptake and also inhibits the intracellular accumulation of γ -methylglutamine (Rai *et al.*, 1984; Kerby *et al.*, 1986a). To further examine the relationship between the second phase of uptake of methylamine and GS activity, [^{14}C]methylamine uptake and γ -methylglutamine accumulation over a 30 min period were compared. In strain 4m3 the rate of [^{14}C]methylamine uptake increased to 9 $\text{pmol min}^{-1} (\mu\text{g chl } a)^{-1}$ after 30 min, resulting in a higher mean rate of uptake over a 30 min period than over the 10 min period shown in Fig. 1, whereas in the parent strain, the rate decreased to 14.5 $\text{pmol min}^{-1} (\mu\text{g chl } a)^{-1}$ after 30 min. Therefore, the second phase of uptake corresponded to a total uptake between 1 and 30 min of 0.48 and 0.24 nmol methylamine ($\mu\text{g chl } a)^{-1}$ for the parent strain and strain 4m3, respectively. The free pools of γ -methylglutamine (Table 1), 30 min after adding 30 μM -methylamine, were 0.49 and 0.27 nmol ($\mu\text{g chl } a)^{-1}$ for the parent strain and strain 4m3, respectively. Thus, the amount of methylamine taken up corresponds with the amount of γ -methylglutamine formed, confirming that the second phase of uptake depends on metabolism via GS and that γ -methylglutamine is not further metabolized, as we have previously shown for the parent strain (Rai *et al.*, 1984; Kerby *et al.*, 1986a). Strain 4m3 accumulated γ -methylglutamine intracellularly [12 nmol ($\mu\text{g chl } a)^{-1}$; (approximately 220 mM in chemostat cultures at pH 7.0 with 3 mM-methylamine in the growth medium)], although no significant further metabolism was detected.

Kinetics of methylamine uptake by mutant strains deficient in GS

Strains ED81 and ED92 are mutant strains of *A. variabilis* resistant to ethylenediamine (1,2-diaminoethane), which have reduced GS biosynthetic activity and which liberate ammonia extracellularly (Kerby *et al.*, 1986b). As Fig. 3 shows, the first phases of [^{14}C]methylamine

Fig. 3. Methylamine uptake at pH 7.0 by the parent strain (○) and mutant strains ED92 (□) and ED81 (■) grown in BG-11₀ medium at pH 7.0. For other experimental details, see legend to Fig. 2.

uptake by ED81 and ED92 at pH 7.0 were similar to that of the parent strain, whereas the second phases of uptake were reduced by 60% and 63% in ED81 and ED92, respectively. Thus ED81, which has a catalytically deficient GS, and ED92, which synthesizes reduced levels of GS protein and mRNA (Hien *et al.*, 1988), do not show a deficiency in the $\text{CH}_3\text{NH}_3^+/\text{NH}_4^+$ transport system. The fact that both of these mutant strains have a reduced second phase of uptake of methylamine is again consistent with its dependence on an active GS.

Kinetics of ammonia uptake by the parent strain and strain 4m3

Ammonia uptake at pH 7.0 and pH 9.0, at external concentrations of 50 μM -ammonia (Fig. 4) and 1 mM-ammonia (Fig. 5), showed a rapid initial uptake followed by a slower linear uptake. Ammonia uptake, at all time points measured, clearly represents both transport and the subsequent assimilation (Rowell *et al.*, 1977). The slower linear rate of uptake appears to be dependent on the rate of assimilation via GS and not on the rate of transport. At pH 9.0, where ammonia uptake may occur by diffusion of NH_3 , as described above for methylamine, both strains showed similar kinetics of ammonia uptake, with a larger initial uptake occurring at pH 9.0 than at pH 7.0. At pH 7.0 ammonia uptake was markedly reduced in the mutant strain grown in N-free medium (Figs 4 and 5) or in the presence of methylamine (data not shown), indicating that the NH_4^+ transport system is absent or reduced and confirming the above findings for CH_3NH_3^+ transport.

At an external concentration of 1 mM-ammonia the initial uptake was greater than at 50 μM -ammonia. In cells pretreated with 100 μM -CCCP for 3 h the ammonia uptake over this initial 5 min period at pH 7.0 was reduced by 76% in the parent strain and by 34% in strain 4m3, and there was no further uptake (data not shown). The residual uptake of ammonia, in the presence of CCCP, may be attributed to non-specific binding of NH_4^+ to the cells.

The rates of the linear phase of ammonia uptake (assimilation), at an external concentration of 50 μM -ammonia, were 98 and 90 $\text{pmol min}^{-1} (\mu\text{g chl } a)^{-1}$ at pH 7.0 and pH 9.0, respectively, for the parent strain and 21 and 65 $\text{pmol min}^{-1} (\mu\text{g chl } a)^{-1}$ at pH 7.0 and pH 9.0, respectively, for the mutant strain. At an external concentration of 1 mM-ammonia the rates were 62 and 54 $\text{pmol min}^{-1} (\mu\text{g chl } a)^{-1}$ at pH 7.0 and pH 9.0, respectively, for the parent strain and 17 and 60 $\text{pmol min}^{-1} (\mu\text{g chl } a)^{-1}$ at pH 7.0 and pH 9.0, respectively, for the mutant strain. Thus ammonia assimilation occurs at similar rates at external pH values of 7.0 and 9.0 and at external ammonia concentrations of 50 μM and 1 mM, except in the case of strain 4m3, where the rate of assimilation at pH 7.0 was markedly lower. The rates of ammonia assimilation are well within the range of extractable GS activities [an ammonia assimilation rate of 60 $\text{pmol min}^{-1} (\mu\text{g chl } a)^{-1}$ converts to 2.4 $\text{nmol min}^{-1} (\text{mg protein})^{-1}$] and are approximately three times higher than the corresponding rates of methylamine assimilation, which may be related to the fact that GS of *A. variabilis* has a higher K_m for methylamine than for ammonia (Kerby *et al.*, 1987a).

Fig. 4

Fig. 5

Fig. 4. Ammonia uptake at pH 7.0 (○, □) and pH 9.0 (●, ■) by the parent strain (○, ●), and mutant strain 4m3 (□, ■) suspended in 10 mM-HEPES/NaOH (pH 7.0) or 10 mM-Tricine/NaOH (pH 9.0). NH_4Cl was added, to a concentration of $50 \mu\text{M}$ at zero time, and the disappearance of ammonia from the medium was measured using an NH_3 -specific electrode.

Fig. 5. Ammonia uptake at pH 7.0 (○, □) and pH 9.0 (●, ■) by the parent strain (○, ●) and mutant strain 4m3 (□, ■), over a 5 h period (a) or a 30 min period (b). Experimental details are as given in the legend to Fig. 4, except that NH_4Cl was added to a concentration of 1 mM at zero time.

Effects of ammonia and methylamine on nitrogenase activity

Strain 4m3 had an identical heterocyst frequency (5.7% of total cells) and nitrogenase activity [$5.6 \text{ nmol C}_2\text{H}_2 \text{ reduced h}^{-1} (\mu\text{g chl a})^{-1}$] to the parent strain when grown in N-free medium, although the heterocyst frequency and nitrogenase activity were slightly lower [4.7% and $5.1 \text{ nmol C}_2\text{H}_2 \text{ reduced h}^{-1} (\mu\text{g chl a})^{-1}$, respectively] for strain 4m3 grown at pH 7.0 in medium containing 3 mM-methylamine, which inhibits growth of the parent strain. However, the addition of ammonia or methylamine affected the nitrogenase activities of the parent strain and strain 4m3 differently. On adding 3 mM-ammonia to N_2 -fixing cultures at pH 7.0 (Fig. 6a), the inhibition of nitrogenase activity was more rapid in the parent strain than in strain 4m3, whereas at pH 9.0, nitrogenase activities decreased at similar rates in both strains. Addition of ammonia at a lower concentration ($50 \mu\text{M}$) at pH 7.0 (Fig. 7) similarly resulted in a decrease in nitrogenase activity, although when the ammonia concentration fell below approximately $20 \mu\text{M}$, nitrogenase activity recovered. On adding 3 mM-methylamine at pH 7.0 (Fig. 6b) there was an initial rapid decrease followed by a recovery of nitrogenase activity in the parent strain whereas that of strain 4m3 was virtually unaffected. At pH 9.0 methylamine addition, which inhibited growth of both strains, resulted in a more rapid decrease in nitrogenase activity in the parent strain than in strain 4m3.

It should be noted that, in contrast with the effects of ammonia and methylamine, addition of nitrate to N_2 -fixing cultures at pH 7.0 resulted in a greater inhibition of nitrogenase activity in strain 4m3 than in the parent strain (Table 2).

Fig. 6

Fig. 7

Fig. 6. Effects of (a) 3 mM- NH_4Cl and (b) 3 mM- $\text{CH}_3\text{NH}_3\text{Cl}$, added at zero time, on nitrogenase activity of the parent strain ($\circ$, $\bullet$) and mutant strain 4m3 ($\square$, $\blacksquare$) grown in BG-11₀ medium at pH 7.0 and incubated in the same medium at pH 7.0 ($\circ$, $\square$) and pH 9.0 ($\bullet$, $\blacksquare$). Nitrogenase activities at zero time were 6.8 and 6.9 nmol C_2H_2 reduced h^{-1} ($\mu\text{g chl } a$) $^{-1}$ for the parent strain and strain 4m3, respectively.

Fig. 7. Effects on nitrogenase activity of adding 50 μM - NH_4Cl , at zero time, to the parent strain ($\circ$) and mutant strain 4m3 ($\square$) grown in BG-11₀ medium at pH 7.0 and incubated in the same medium. (a) Nitrogenase activity; (b) NH_4Cl concentration in the medium. Nitrogenase activities at zero time were 5.4 and 5.6 nmol C_2H_2 reduced h^{-1} ($\mu\text{g chl } a$) $^{-1}$ for the parent strain and strain 4m3, respectively.

Table 2. *Effects of MSX on nitrogenase activity and ammonia liberation by the parent strain and mutant strain 4m3*

Cultures, grown in BG-11₀ medium at pH 7.0, were harvested by centrifugation, washed and resuspended in BG-11₀ medium supplemented with 10 μM -MSX and 20 mM- NaNO_3 , where indicated. After incubating at a photon fluence rate of 75 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 24 h, the acetylene reduction rates and concentrations of ammonia in the media were measured. All data are the mean $\pm$ SE for at least five replicates. Figures in parentheses represent percentages of the respective control values. ND, Not detectable.

Strain	Additions to medium	Acetylene reduction [nmol h^{-1} ($\mu\text{g chl } a$) $^{-1}$]	Ammonia liberated [nmol ($\mu\text{g chl } a$) $^{-1}$]
Parent	None	5.59 $\pm$ 0.36 (100)	ND
	NO_3^-	4.31 $\pm$ 0.30 (77)	ND
	MSX	6.58 $\pm$ 0.53 (122)	27.4 $\pm$ 3.9
	NO_3^- , MSX	5.13 $\pm$ 0.21 (95)	28.1 $\pm$ 2.0
4m3	None	5.59 $\pm$ 0.41 (100)	ND
	NO_3^-	3.19 $\pm$ 0.25 (57)	ND
	MSX	8.40 $\pm$ 0.75 (150)	37.4 $\pm$ 1.9
	NO_3^- , MSX	7.05 $\pm$ 0.84 (126)	70.8 $\pm$ 3.6

Extracellular liberation of ammonia

Since it has been proposed that an NH_4^+ transport system is important for the retention of ammonia generated internally (Castorph & Kleiner, 1984; Kleiner, 1985) by, for example, N_2 -fixation, strain 4m3 was tested for ammonia liberation. The data in Table 2 show that strain 4m3, like the parent strain, only liberated ammonia extracellularly following treatment with MSX to inactivate GS (Stewart & Rowell, 1975). However, nitrogenase activity was enhanced to a greater extent and the amount of ammonia liberated, over the 24 h experimental period, was greater for strain 4m3 than for the parent strain. Additionally, ammonia liberation by strain 4m3, following MSX treatment, was greater in the presence of nitrate.

DISCUSSION

The results obtained for the parent strain of *Anabaena variabilis* extend previous findings for N_2 -fixing cyanobacteria (Rai *et al.*, 1984; Kerby *et al.*, 1986*a*, 1987*b*) and for other cyanobacteria, particularly *Anacystis nidulans* (Boussiba *et al.*, 1984*a*, *b*; Boussiba & Gibson, 1985). The kinetics of uptake of methylamine and ammonia by strain 4m3 suggest that it is defective in the active transport of CH_3NH_3^+ and NH_4^+ .

The biphasic uptake of [^{14}C]methylamine by cyanobacteria has been interpreted either as a rapid transport and a subsequent slower metabolism via GS (Rai *et al.*, 1984), such that the rate-limiting step is metabolism, or as two active transport systems (Singh *et al.*, 1983, 1985, 1987), the slower of which is inhibited by MSX. Data from this laboratory (Rai *et al.*, 1984; Kerby *et al.*, 1986*a*, 1987*a*; this paper), which are not consistent with the latter idea that the biphasic uptake represents two transport systems, indicate a clear correlation between GS biosynthetic activity and the rate of the second slower phase of uptake, either in mutant strains with reduced GS activity (ED81 and ED92) (Kerby *et al.*, 1986*b*) or in the parent strain when GS has been inactivated by treatment with MSX. Our data show that the rate of the second phase of uptake of methylamine or ammonia is the same at pH 7.0 as at pH 9.0, indicating that this phase is not limited by the rate of transport, since both compounds can enter rapidly by passive diffusion and accumulate to a higher concentration at pH 9.0. If active transport was rate-limiting, the rate of the second phase would presumably be greater at pH 9.0 than at pH 7.0. The reduction in the rate of the second phase of methylamine uptake by strain 4m3 does not appear to be due to differences in the level or kinetic properties (our unpublished data) of GS, but the possibility that GS activity *in vivo* is limited by substrate availability or that there is a defect at a later step in the pathway of ammonia assimilation cannot be discounted.

Strain 4m3 appears to have a methylamine-inducible CH_3NH_3^+ transport system which is not induced by ammonia. Metabolism of [^{14}C]methylamine by strain 4m3 is limited to the synthesis and accumulation of γ -methylglutamine, as we have previously shown for the parent strain (Rai *et al.*, 1984; Kerby *et al.*, 1986*a*). Resistance of strain 4m3 to methylamine cannot, therefore, simply be ascribed to an inability to synthesize γ -methylglutamine as in some mutant strains of *Escherichia coli* (Servin-Gonzalez *et al.*, 1987).

Mutant strain 4m3 did not liberate ammonia extracellularly unless GS was inactivated by treatment with MSX, in contrast with findings for an NH_4^+ -transport-deficient mutant of *Klebsiella pneumoniae* (Castorph & Kleiner, 1984) but in agreement with findings for an NH_4^+ -transport-deficient mutant of *Chlamydomonas reinhardtii* (Franco *et al.*, 1987), suggesting that an active NH_4^+ -transport system is not essential for the retention of internally-generated ammonia in this cyanobacterium. This is clearly the case whether ammonia is derived via N_2 fixation in heterocysts or via processes such as NO_3^- reduction (Rai & Bergman, 1986) or photorespiratory nitrogen cycling (Bergman, 1984) in vegetative cells. However, the liberation of ammonia by strain 4m3, treated with MSX, is more extensive than that by the parent strain, particularly in the presence of nitrate. Such increased liberation of ammonia by strain 4m3 may be due to the increased nitrogenase activity (Table 2) and, possibly, to an increased nitrate reductase activity. However, the possibilities that it is due to the deficiency in NH_4^+ transport, to an increased uptake of MSX or to an alteration of GS, making it more susceptible to inactivation by MSX, cannot be discounted.

The finding that nitrogenase activity in strain 4m3 is less sensitive to inhibition by exogenous ammonia at pH 7.0 is, presumably, due to the reduced ability, at this pH, to accumulate intracellularly the ammonia and/or a product of its assimilation which are involved in the regulation of nitrogenase synthesis. The inhibitory effect of ammonia on nitrogenase activity at pH 7.0 and pH 9.0 is prevented by MSX, suggesting the involvement of a product of ammonia assimilation (Kerby *et al.*, 1987a). In contrast, the rapid decrease in nitrogenase activity on adding methylamine at pH 9.0 is only partly prevented by MSX treatment and is probably mainly due to uncoupling (Kerby *et al.*, 1987a). Further studies on the ammonia regulation of nitrogenase activity in strain 4m3 and on the interrelationships between NH_4^+ transport and assimilation in *A. variabilis* are in progress.

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