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# [$^{14}$C]Methylammonium Transport by *Frankia* sp. Strain CpI1

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**We describe an $NH_4^+$-specific transport system in the $N_2$-fixing symbiotic actinomycete *Frankia* sp. strain CpI1. [$^{14}$C]methylammonium was used as an $NH_4^+$ analog. No specific transport process was detected when cells were grown on high concentrations of $NH_4^+$. A transport system with a high affinity for $CH_3NH_3^+$ was synthesized after 3 to 4 h of nitrogen starvation. Methylammonium transport was not significantly inhibited by a variety of amino acids, primary amines, and polyamines. Ammonium completely eliminated $CH_3NH_3^+$ transport. The $K_m$ for $CH_3NH_3^+$ transport was around 2 ± 1.8 μM with a $V_{max}$ of 4 to 5 nmol/min per mg of protein. The electron transport inhibitors cyanide and azide eliminated uptake, as did the uncoupler carbonyl cyanide-*m*-chlorophenylhydrazone. The sulfydryl reagent *p*-chloromercuribenzoic acid and the heavy metal thallium also inhibited uptake, suggesting the presence of an $NH_4^+$-specific permease. Concentration of $CH_3NH_3^+$ across the membrane was demonstrated by conducting uptakes at low temperature to slow the metabolism of $CH_3NH_3^+$ by glutamine synthetase. At 7°C most of the label was concentrated inside the cells in a form that could be chased from the cells by adding excess $NH_4^+$ to the medium. At 30°C most of the label was present as an impermeant metabolite. Thin-layer chromatography of cell extracts confirmed that the radioactivity inside the cells was mainly in the form of $CH_3NH_3^+$ at 7°C but was present as an unidentified metabolite at 30°C. These studies demonstrate that *Frankia* sp. strain CpI1 has a high-affinity $NH_4^+$ transport system that is synthesized in response to $NH_4^+$ starvation.**

*Frankia* sp. strain CpI1 belongs to a group of slow-growing actinomycetes that form $N_2$-fixing root nodules in association with various nonleguminous plants (9, 31). The resulting symbioses have been termed "actinorhizal," and many actinorhizal plants are important in reclaiming disturbed and nitrogen-poor environments (31, 32). Since most frankiae grow slowly and since isolates have only recently become available, little is known about their nitrogen metabolism and nothing is known about how $NH_4^+$ traverses their cell membrane in symbiosis.

Transport systems specific for $NH_4^+$ have been described in filamentous fungi (6, 11, 15), yeasts (12, 25), and in many bacteria (1, 2, 13, 14, 17–20, 24, 30, 33). Many $N_2$-fixing bacteria have $NH_4^+$-specific transport systems that are synthesized under nitrogen starvation conditions (33). Ammonium transport by $N_2$-fixing organisms involved in plant symbioses is of particular interest because fixed nitrogen is considered to be excreted from these organisms to the plant cytoplasm in the form of $NH_4^+$ (7, 24, 26–28), and the bacterial membrane is the first barrier to the exit of $NH_4^+$ from $N_2$-fixing cells.

For *Rhizobium* spp., $NH_4^+$ excretion has been demonstrated to occur from free-living $N_2$-fixing bacteria (23) and from bacteroids (7). Ammonium excretion has not been directly demonstrated in alder frankiae. However, the concept is supported by the observation of elevated concentrations of glutamine synthetase (GS) antigen in the alder nodule plant cell cytoplasm (16) and by the demonstration that the primary product of $^{13}NH_4^+$ assimilation in alder nodules is glutamine (26, 27). In contrast, GS activity of the endophyte in the root nodules is reportedly low (4).

As part of a study on $NH_4^+$ metabolism in *Frankia* sp., we looked at $NH_4^+$ uptake in free-living *Frankia* sp. strain CpI1 with [$^{14}$C]methylammonium ($^{14}CH_3NH_3^+$) as an $NH_4^+$ analog. We present evidence for the presence of a high-affinity $NH_4^+$ transport system that operates under low concentrations of $NH_4^+$. Our results also suggest that no specific transport system for $NH_4^+$ operates when frankiae are grown with high concentrations of $NH_4^+$.

## MATERIALS AND METHODS

**Organisms.** *Frankia* sp. strain CpI1 is a derivative of the original *Comptonia peregrina* isolate obtained by Callaham et al. (9). The organism was grown in a defined liquid medium containing the following (per liter of distilled water): 6 g of $K_2HPO_4$, 4 g of $KH_2PO_4$, 0.1 g of $CaCl_2$, 0.2 g of $MgSO_4 \cdot 7H_2O$ 0.43 g of $NH_4Cl$, 5 g of sodium succinate, and 0.5 ml of an Fe · EDTA solution consisting of 10 mg of $FeSO_4$, 0.15 mg of $NaMoO_4$, and 2 mg of $Na_2$ · EDTA per ml. Ammonium chloride was omitted and deionized water was used when preparing N-free medium. Cells were grown with rotary shaking at 30°C in 200 ml of medium in 500-ml flasks. They were harvested after 5 to 6 days either for use in uptake studies or for transfer into fresh medium.

**Methylammonium uptake.** Settled cells were harvested with a 25-ml pipette, homogenized in a 40-ml Ten Broeck homogenizer, and centrifuged at 3,000 × *g* for 5 min. The pelleted cells were suspended and centrifuged three additional times in 40 ml of deionized water. The final pellet was suspended in 30 ml of N-free medium and incubated at 30°C with shaking for 10 to 12 h before uptake experiments were begun. Cells were then centrifuged and resuspended in the same medium having half the normal phosphate concentration. The final cell suspensions were maintained on ice to preserve transport activity for up to 5 to 6 h after the starvation period.

Routine $CH_3NH_3^+$ uptake assays were done with assay mixtures containing 2 ml of N-starved cell suspension and 1.9 ml of deionized water. The assay mixtures were preincubated at 30°C for 5 min before the uptake assay was begun. The uptake assay was initiated by adding $^{14}CH_3NH_3^+$ (spe-

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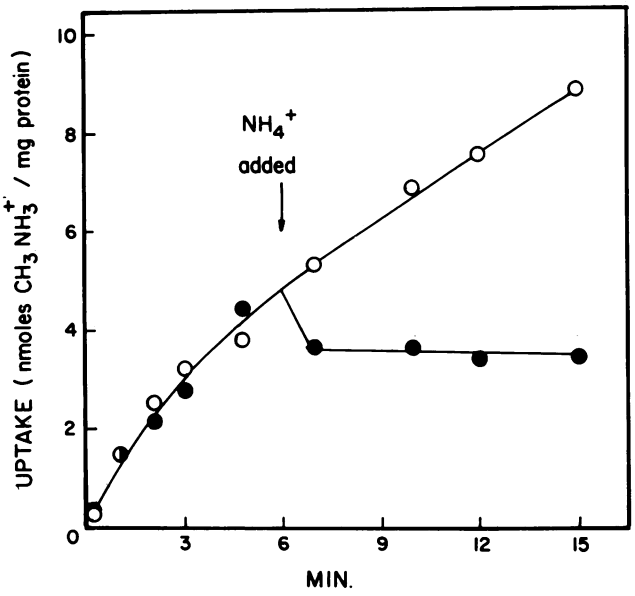


FIG. 1. [$^{14}$C]methylammonium uptake by ammonium-starved CpI1 cells. Control cells (○) were allowed to accumulate methylammonium. Ammonium chloride (32 mM) was added to an identical sample of cells as indicated (●).

cific activity, 10 μCi/μmol) to the assay mixture while it was shaking to a final concentration of 5 μM. At intervals, 0.25- to 0.5-ml samples (sample size depending upon cell density) were rapidly filtered through 0.45-μm-pore-size membrane filters (Millipore Corp., Bedford, Mass.). Cells on the filters were rinsed twice with 3-ml portions of N-free medium. The filters were removed and dissolved in 10 ml of Bray's scintillation fluid for liquid scintillation counting (5).

Rapid sampling techniques were used in some experiments to obtain time points from 1 s to 1 min. Cells in medium (0.2 ml) and deionized water (0.192 ml) were added to disposable polypropylene tubes (17 by 100 mm; Fisher Scientific Co., Medford, Mass.). [$^{14}$C]methylammonium was added to start the uptake assay. Uptake was stopped by adding 5 ml of ice-cold N-free medium, and the cells were collected by filtration as described above.

The intracellular volume of CpI1 cells was estimated by using $^3H_2O$ to determine the total water volume and [$^3$H]polyethylene glycol to measure the extracellular volume. The volume obtained was 2.7 μl per mg of dry cells. This value was verified by taking the difference between pellet wet weight and pellet dry weight (to obtain total water volume) minus the [$^3$H]polyethylene glycol volume (22).

**Analytical methods.** Ammonium was measured by the indophenol method after microdiffusion (8, 10). Protein was measured by the method of Lowry et al. (21), using bovine serum albumin as the standard. GS was determined by the γ-glutamyl transferase method described by Bender et al. (3) for whole cells at pH 6.8.

Thin-layer chromatography was carried out on silica gel plates (Analab, North Haven, Conn.) with *n*-butanol saturated with 2 N HCl as the solvent system. Cells on filters were extracted with 2 ml of 70% ethanol at room temperature. The extract was centrifuged and the supernatant was evaporated down to ca. 0.5 ml under a stream of nitrogen. The concentrated extract (20 to 50 μl) was spotted on the thin-layer chromatography plates. The location of radioactive compounds was determined by autoradiography. Radioactivity in each spot was quantitated by scraping the gel from the plates and counting in Bray's solution.

**Chemicals and radioisotopes.** [$^{14}$C]methylammonium hydrochloride was obtained from ICN Pharmaceuticals, Inc., Irvine, Calif. $^3H_2O$ and (1,2-$^3$H)polyethylene glycol were obtained from New England Nuclear Corp., Boston, Mass. All other chemicals were from Sigma Chemical Co., St. Louis, Mo.

## RESULTS

**Methylammonium uptake in unstarved cells.** In initial experiments we determined that *Frankia* sp. strain CpI1 could use $NH_4^+$ as a nitrogen source. Cells grown on $NH_4^+$ efficiently removed $NH_4^+$ from the medium down to undetectable levels (<0.5 μM) even in the presence of Casamino Acids. This observation suggested that CpI1 had a mechanism for scavenging trace amounts of $NH_4^+$ from the medium.

Cells grown on 8 mM $NH_4^+$ and washed with N-free medium did not take up $CH_3NH_3^+$ when incubated with low concentrations (5 μM) of $CH_3NH_3^+$. At higher concentrations (0.1 to 4.0 mM), labeled $CH_3NH_3^+$ appeared to equilibrate rapidly across the cell membrane, but the influx could not be inhibited by $NH_4^+$, various metabolic inhibitors, amino acids, or primary amines. Repeated washing led to the release of most of the label from the cells. The apparent uptake at high concentrations was attributed to nonspecific binding. We cannot rule out passive diffusion into cells as a possible explanation.

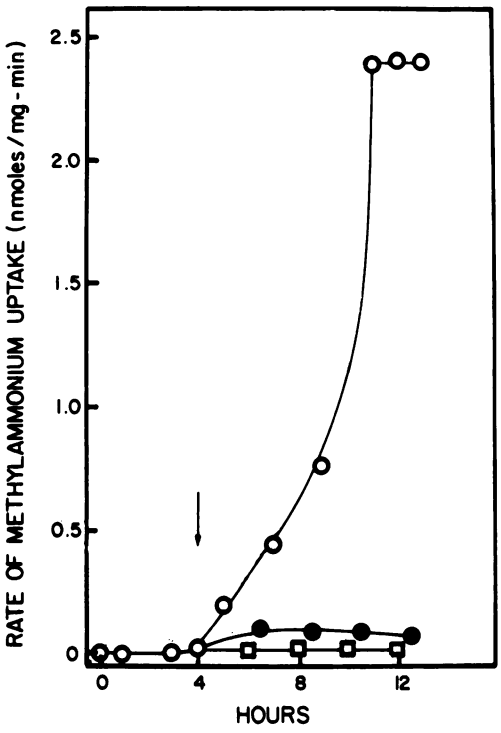


FIG. 2. Time course of appearance of [$^{14}$C]methylammonium uptake activity during nitrogen starvation. Cells growing on 8 mM ammonium were prepared as described in the text and were split into three flasks. The control flask (○) received no additions. Rifampin (50 μg/ml) (●) or chloramphenicol (50 μg/ml) (□) was added to the other flasks at the time indicated by the arrow.

**High-affinity $NH_4^+$ ($CH_3NH_3^+$) transport.** *Frankia* sp. strain CpI1 cells that were incubated in N-free medium for 10 to 12 h took up $^{14}CH_3NH_3^+$ at high rates when uptake assays were done with low concentrations (5 μM) of $CH_3NH_3^+$ (Fig. 1). The uptake was stopped by adding $NH_4^+$ to the reaction mixture.

Uptake began within 4 h after cells were suspended in N-free medium (Fig. 2). Uptake rates increased until 10 to 12 h after suspension in N-free medium. The protein synthesis inhibitor chloramphenicol eliminated the onset of uptake activity when added at 4 h. Chloramphenicol added at 9 h did not inhibit existing uptake activity but stopped further increases in transport rates (data not shown). Rifampin, a transcription inhibitor, also prevented the onset of most of the uptake activity when added at 4 h (Fig. 2). The small increase in uptake activity in the rifampin-treated cells presumably occurred because some mRNA coding for a permease had been synthesized by 4 h but was subsequently degraded.

**Specificity of methylammonium uptake.** To demonstrate that the uptake system being synthesized by cells starved for $NH_4^+$ was in fact specific for $NH_4^+$, we tested the effect of various compounds on $CH_3NH_3^+$ uptake. Several amino acids at concentrations 200-fold in excess of the $CH_3NH_3^+$ concentration and amines and polyamines present at 10-fold-greater concentration than $CH_3NH_3^+$ had no effect or only a marginal effect on $CH_3NH_3^+$ transport (Table 1). Only $NH_4^+$ completely eliminated $CH_3NH_3^+$ transport at all concentrations tested.

The addition of $NH_4^+$ to the assay delayed the onset of $CH_3NH_3^+$ uptake in proportion to the amount of $NH_4^+$ added (Fig. 3), indicating that virtually all $NH_4^+$ must be removed before $CH_3NH_3^+$ is transported. The uptake curves for $CH_3NH_3^+$ were typically biphasic with a rapid initial rate in the first 1 to 2 min followed by a slower linear rate. The rapid initial rate became less pronounced with increasing $NH_4^+$ concentrations.

From the delay in $CH_3NH_3^+$ uptake, we calculated that $NH_4^+$ can be taken up at a rate of about 15 nmol/min per mg of protein. This rate is four- to fivefold higher than the rapid initial rate of $CH_3NH_3^+$ transport and 37-fold higher than the overall rate of $CH_3NH_3^+$ uptake. Because of the high preference of the transport system for $NH_4^+$ and the rapid change in $NH_4^+$ concentrations under our assay conditions, we could not accurately determine a $K_i$ for $NH_4^+$ on $CH_3NH_3^+$ uptake.

TABLE 1. Inhibition of methylammonium uptake by amines[a]

| Compound | Uptake rate (% control[b]) |
|---|---|
| Amino acids (1 mM) | |
| Alanine | 75 |
| Glutamate | 100 |
| Asparagine | 105 |
| Glutamine | 80 |
| Lysine | 105 |
| Amines and polyamines (50 μM) | |
| Cadaverine | 90 |
| Spermidine | 90 |
| Putrescine | 91 |
| Dimethylamine | 130 |
| Ethylamine | 104 |
| Hydroxylamine | 77 |
| Propylamine | 139 |
| Triethylamine | 109 |
| Ammonium chloride (50 μM) | 0 |
| Control | 100 |

[a] Cells were starved for nitrogen as described in the text. All additions except for ammonium were made 2 to 3 min before addition of methylammonium. Ammonium was added at the same time as methylammonium. Final concentration of $^{14}CH_3NH_3^+$ was 5 μM.

[b] The control rate for $CH_3NH_3^+$ uptake was 1.82 nmol/min per mg of protein.

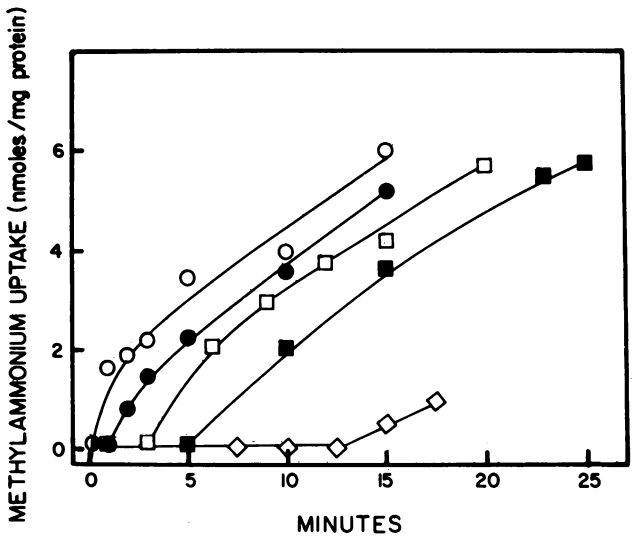


FIG. 3. Delay in the onset of [$^{14}$C]methylammonium uptake with increasing concentrations of ammonium. Uptakes were performed as described in the text except that $^{14}CH_3NH_3^+$ was added immediately after the addition of ammonium chloride. Ammonium was added to final concentrations of 0 (○), 10 (●), 25 (□), 50 (■), and 100 μM (◇).

**Affinity of the transport system for $CH_3NH_3^+$.** Rapid sampling techniques were used for determining initial rates of $CH_3NH_3^+$ uptake at various concentrations of $CH_3NH_3^+$. Figure 4 is a Lineweaver-Burk plot of $CH_3NH_3^+$ uptake done with concentrations between 1 and 30 μM $CH_3NH_3^+$. Time points for rate determinations were taken at 10, 20, and 30 s. Uptake exhibited typical saturation kinetics (Fig. 4, insert). The $K_m$ from six separate determinations was 2 ± 1.8 μM. The $V_{max}$ was between 4 and 5 nmol/min per mg of protein.

**Effect of inhibitors on $CH_3NH_3^+$ transport.** Several energy poisons were tested for their effect on $CH_3NH_3^+$ transport. The electron transport inhibitors cyanide (0.2 mM) and azide (30 mM) eliminated $CH_3NH_3^+$ uptake, as did the uncoupler carbonyl cyanide-*m*-chlorophenylhydrazone (0.2 mM). The ATPase inhibitor *n,n*-dicyclohexylcarbodiimide (0.1 mM) lowered activity by 50% at the concentration used.

The heavy metal thallium has been reported to be an inhibitor of $NH_4^+$ permeases (1). Monovalent thallium inhibited uptake by 90% at a concentration of 0.5 mM, providing evidence for the existence of an $NH_4^+$-specific permease in CpI1. In addition, the sulfhydryl reagent *p*-chloromercuribenzoic acid (1.0 mM) eliminated uptake, again suggesting the presence of a permease.

**Effect of ionic strength.** We tested the effect of ionic strength on $CH_3NH_3^+$ transport by diluting the N-free incubation medium (100 mM total solute concentration) to lower the ionic strength and by adding KCl to raise the ionic strength. The optimum concentration for uptake studies was

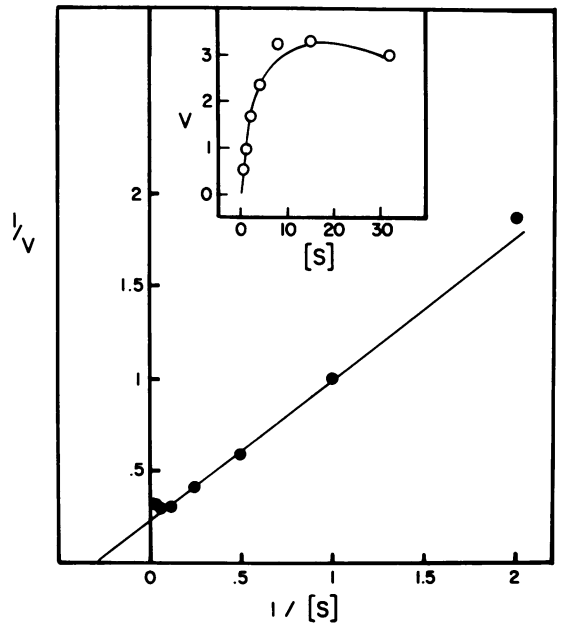


FIG. 4. Lineweaver-Burk plot of the kinetics of $^{14}CH_3NH_3^+$ uptake. Inset shows the saturation curve for uptake from which the double-reciprocal plot was derived. Methylamine concentration ([S]) is expressed in micromolars; rate ($v$) is expressed as nanomoles per minute per milligram of protein.

below 25 mM (Table 2). Higher solute concentrations had a marked effect on $CH_3NH_3^+$ uptake with only 25% of the activity remaining at 50 mM solute concentration.

**Fate of intracellular $CH_3NH_3^+$.** In other systems, $CH_3NH_3^+$ is reportedly metabolized by GS to γ-*N*-methylglutamine upon transport into the cell (2, 13, 20). To determine if the CpI1 GS was metabolizing $CH_3NH_3^+$ upon entry into the cell, we used the GS inhibitors L-methionine-DL-sulfoximine (MSX) and methionine sulfone (MSF) to block incorporation. No effect on incorporation was seen at concentrations up to 10 mM MSX or MSF, and little radioactivity could be chased from the cells by adding $NH_4^+$ to the uptake medium. Since MSX and MSF inhibited GS purified from CpI1 cells, we concluded that the inhibitors were not being taken up by the cells.

To determine if $CH_3NH_3^+$ could be concentrated inside the cell as a free pool, we performed uptake experiments at two different temperatures. Preliminary experiments showed that transport proceeded at a temperature of 7°C, whereas GS transferase activity of permeabilized cells was reduced to less than 25% of the activity at 30°C (data not shown). Uptakes were done at 7 and 30°C, and cells were allowed to accumulate $CH_3NH_3^+$ for 4 and 6 min, respectively, until the amount of radiolabel was approximately equal in cells incubated under each condition. At those times, ammonium chloride was added to a final concentration of 12.5 mM, and the release of internal $CH_3NH_3^+$ was followed. Cells incubated at 30°C released 35% of their radioactivity, whereas cells incubated at 7°C released 85% of the accumulated label (Fig. 5). From this experiment and knowing the intracellular volume to be 2.7 μl/mg of dry cell weight, we calculated that $CH_3NH_3^+$ is concentrated at least 44-fold at 7°C and 15-fold at 30°C.

By repeating the experiment at 7°C and allowing $CH_3NH_3^+$ accumulation to proceed for a longer time period,

TABLE 2. Effect of solute concentration on methylammonium uptake[a]

| Total solute concn (mM)[b] | Uptake (% maximum[c]) |
|---|---|
| 10 | 100 |
| 25 | 96 |
| 50 | 24 |
| 100 | 16 |
| 250 | 6 |
| 500 | 2 |
| 1,000 | 0 |

[a] Cells were allowed to preincubate for 5 min before $^{14}CH_3NH_3^+$ was added.
[b] Solute concentrations were adjusted as described in the text.
[c] Maximum $CH_3NH_3^+$ uptake rate was 0.51 nmol/min per mg of protein.

we found that virtually all of the added radioactivity was concentrated in the cells, with 95% of it in a form that could be chased from the cells by the addition of $NH_4^+$ (Fig. 6). Adding an equivalent amount of KCl gave no release of radioactivity. Adding 50 μM KCN before $CH_3NH_3^+$ eliminated uptake, whereas adding 50 μM KCN after cells had accumulated $CH_3NH_3^+$ led to the release of 25% of the radioactivity. The addition of carbonyl cyanide-*m*-chlorophenylhydrazone resulted in the release of 90% of the radioactivity.

Thin-layer chromatography of cell extracts showed that 85% of the radioactivity in cells incubated at 30°C was present in a metabolite that migrated separately from $^{14}CH_3NH_3^+$ (Table 3). On the other hand, most of the radioactivity in extracts prepared from cells incubated at 7°C migrated with the same $R_f$ value as $^{14}CH_3NH_3^+$ with only 3% of the radioactivity in a spot corresponding to the major metabolite seen at 30°C. The internal concentration of $CH_3NH_3^+$ at 7°C was therefore several orders of magnitude

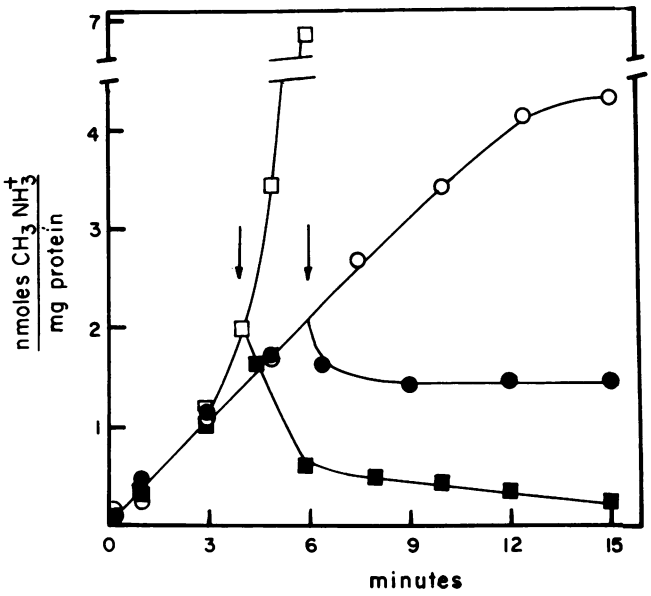


FIG. 5. Uptake of $^{14}CH_3NH_3^+$ at 7 and 30°C and release by an ammonium pulse. Uptakes were performed by the rapid sampling technique as described in the text. Control cells at 7°C (□) and 30°C (○) received no ammonium. Ammonium chloride was added to a final concentration of 12.5 mM at the times indicated by the arrows to cells held at 7 (■) and 30°C (●).

higher than the external concentration, and we concluded that the transport process we were observing could be characterized as active transport.

## DISCUSSION

Ammonium transport systems have been found in a variety of fungi and bacteria (1, 13, 14, 17–20, 24, 30, 33). To our knowledge, this is the first report of an $NH_4^+$-specific transport system in a euactinomycete and only the second report of such a system in a gram-positive bacterium (20).

Although similar in many respects to $NH_4^+$ transport systems from other bacteria, the *Frankia* system is distinguished by its extremely high affinity for $CH_3NH_3^+$ and even higher affinity for $NH_4^+$. The $K_m$ for $CH_3NH_3^+$ was around 2 μM and the $K_m$ for $NH_4^+$ was even lower, possibly in the nanomolar range. This value is much lower than that obtained for other bacteria. The $K_m$ of $NH_4^+$ transport systems for $CH_3NH_3^+$ has been estimated at 61 μM (14) or 25 μM (1) for *Azotobacter vinelandii*, 100 μM for *Klebsiella pneumoniae* (19), and 150 μM for *Clostridium pasteurianum* (20). The high affinity of the *Frankia* system probably reflects the ability of actinomycetes in general to scavenge nutrients from the environment.

The conclusion that $NH_4^+$ is the natural substrate for the transport system is supported by several lines of evidence. First, no $CH_3NH_3^+$ uptake was detected in the presence of $NH_4^+$. Second, amino acids, primary amines, and polyamines had little effect on uptake even when present in great excess over the $CH_3NH_3^+$ concentration. Finally, cells of CpI1 could not use $CH_3NH_3^+$ as a source of carbon or nitrogen for growth (data not shown).

Distinguishing uptake (transport plus metabolism) of $CH_3NH_3^+$ from transport alone has been difficult in other systems because $CH_3NH_3^+$ is metabolized by GS to γ-*N*-methylglutamine (1, 2, 13, 14, 20). We found that most of the $CH_3NH_3^+$ transported by CpI1 cells was quickly converted to an impermeant metabolite at 30°C. This observation suggested that rapid metabolism of $CH_3NH_3^+$ coupled with either passive diffusion or facilitated diffusion rather than active transport could be responsible for the concentration of $CH_3NH_3^+$ inside CpI1 cells. Passive diffusion plus rapid metabolism was ruled out because a permease was clearly involved. Protein synthesis was necessary before high-affinity $NH_4^+$ ($CH_3NH_3^+$) transport commenced in CpI1 cells, as demonstrated by the elimination of the onset of $CH_3NH_3^+$ uptake by chloramphenicol and rifampin. Further evidence for the involvement of a permease was provided by the observation that thallium, an inhibitor of $NH_4^+$ permeases (1), and *p*-chloromercuribenzoic acid, a sulfhydryl group reagent, both acted to inhibit transport.

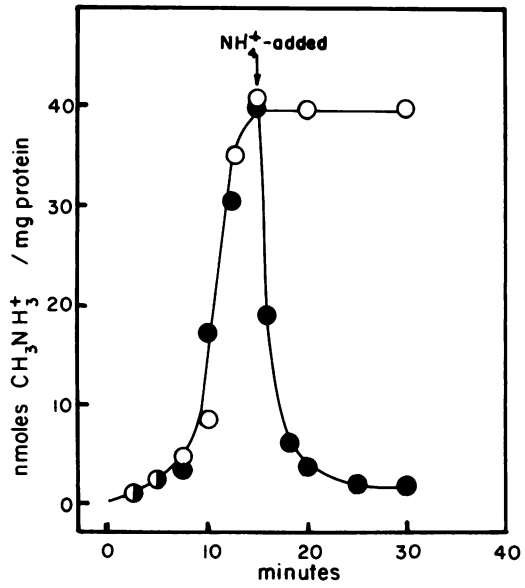


FIG. 6. Concentration of $^{14}CH_3NH_3^+$ inside *Frankia* sp. strain CpI1 cells at 7°C and release with ammonium. Uptake assays were performed by the rapid sampling technique as described in the text. Control cells (○) received no ammonium. Ammonium chloride was added to a final concentration of 12.5 mM to a parallel culture (●) at the time indicated.

TABLE 3. Thin-layer chromatography of *Frankia* sp. strain CpI1 cell extracts[a]

| Sample | $R_f$ of major spot (% total) | $R_f$ of minor spot (% total) |
|---|---|---|
| $^{14}CH_3NH_3^+$ in water | 0.21 | |
| $^{14}CH_3NH_3^+$ in cell extract | 0.23 | |
| Cell extract (30°C) | 0.34 (85) | 0.23 (15) |
| Cell extract (7°C) | 0.22 (97) | 0.34 (3) |

[a] Samples were prepared as described in the text. Cells were allowed to accumulate label for 15 min at 30°C and for 9 min at 7°C.

Facilitated diffusion coupled with rapid metabolism as a mechanism for transport was also ruled out because $CH_3NH_3^+$ was concentrated inside the cells as a free pool. This was most dramatically shown by conducting uptakes at low temperature to slow the formation of a metabolite. Thin-layer chromatography of extracts from cells incubated at 7 and 30°C confirmed that most of the label in cells incubated at 7°C was in the form of $CH_3NH_3^+$, whereas most of the label in cells incubated at 30°C was present as a metabolite, presumably γ-*N*-methylglutamine formed by GS. Another argument that can be made against the idea that rapid incorporation by GS was responsible for the high-affinity uptake of $CH_3NH_3^+$ is that the $K_m$ for $CH_3NH_3^+$ transport in CpI1 is more than 4 orders of magnitude lower than the $K_m$ for $CH_3NH_3^+$ of GSs described from other organisms (2). Thus, unless the GS from frankiae is radically different from other GSs that have been studied, a specific permease must be responsible for the observed uptake.

The involvement of metabolic energy was shown by the elimination of transport by cyanide, azide, and carbonyl cyanide-*m*-chlorophenylhydrazone. The involvement of an ion gradient was also shown by the ability of the uptake system to concentrate $CH_3NH_3^+$ across the cell membrane. Cells that had concentrated $CH_3NH_3^+$ inside released some of the label when cyanide was added but maintained about 75% inside. We interpret this result to mean that an ion gradient can be maintained for some time even in the presence of cyanide, probably through the operation of an ATPase. On the other hand, carbonyl cyanide-*m*-chlorophenylhydrazone, a proton ionophore, allowed 95% of the label to exit the cell. In this case, carbonyl cyanide-*m*-chlorophenylhydrazone dissipated the proton gradient and the $CH_3NH_3^+$ concentrated inside equilibrated by passive diffusion across the membrane as $CH_3NH_2$.

The ability of *Frankia* sp. strain CpI1 to scavenge $NH_4^+$ down to undetectable levels with a specific $NH_4^+$ permease suggests that such a system does not function to take up $NH_4^+$ when the organism is in symbiosis with a plant. Rather, high-affinity transport is probably important to the

survival of frankiae in the soil. Since CpI1 cells grown with high concentrations of $NH_4^+$ lack a specific $NH_4^+$ transport system, $NH_4^+$ probably equilibrates sufficiently rapidly across the membrane for growth to proceed. In the root nodule, *Frankia* cells exist in a nongrowing or extremely slow-growing state and therefore do not require a large input of $NH_4^+$ for growth. Ammonium from $N_2$ fixation presumably leaves by simple diffusion from the cell. The rate of diffusion from the cell would be accelerated by the presence of high concentrations of plant GS that would rapidly remove $NH_4^+$ or by the presence of acidic conditions in the plant tissue relative to the bacterial cytoplasm. The latter possibility might involve the functioning of proton-pumping ATPases in the plant plasma membrane that would acidify the environment immediately adjacent to the endophyte in symbiosis. Activation of such ATPases have been implicated in certain fungus-induced plant diseases (29).

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