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AMMONIUM (METHYLAMMONIUM) TRANSPORT BY *KLEBSIELLA PNEUMONIAE*

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Klebsiella pneumoniae **can accumulate methylammonium up to 80-fold by means of a transport system as indicated by the energy requirement, saturation kinetics and a narrow pH profile around pH 6.8. Methylammonium transport (apparent $K_m = 100$ μM, $V = 40$ μmol/min per g dry weight at 15°C) is competitively inhibited by ammonium (apparent $K_i = 7$ μM). The low K_i value and the finding that methylammonium cannot serve as a nitrogen source indicate that ammonium rather than methylammonium is the natural substrate. Uphill transport is driven by a component of the protonmotive force, probably the membrane potential. The transport system is under genetic control; it is partially repressed by amino acids and completely by ammonium. Analysis of mutants suggest that the synthesis of the ammonium transport system is subject to the same 'nitrogen control' as nitrogenase and glutamine synthetase.**

Introduction

Transport of NH_4^+ across the cell membrane by a specific transport system has been established for a decade in lower eukaryotes (see Ref. 1 for a review). Recently, evidence for specific NH_4^+-transport systems has also been accumulated for several bacteria, including *Escherichia coli* [2] and the N_2-fixing species *Clostridium pasteurianum* [3,4] and *Azotobacter vinelandii* [5,6]. Because of the regulatory function of NH_4^+ in nitrogenase biosynthesis, NH_4^+ transport into N_2-fixing species is of special interest [7]. Here I report the characterization of an ammonium (methylammonium) transport system in the N_2-fixing enterobacterium *Klebsiella pneumoniae*.

Materials and Methods

Growth of organisms. Klebsiella pneumoniae M5al (a gift from Professor R.H. Burris, University of Wisconsin, Madison) was grown either in a continuous culture with N_2 as the sole nitrogen source as described previously [8], or aerobically in batch cultures with different sources of combined nitrogen as will be indicated. Growth was followed spectrophotometrically at 660 nm. Extinctions were adjusted to 0.1–0.3 by dilution.

Mutagenesis. Mutagenesis of *K. pneumoniae* KP5060 ($GlnA^-$ $GlnR^-$ Hut^- Nif^-, a gift from C. Elmerich, Institut Pasteur, Paris) was carried out with ethylmethanesulfonate as follows. Strain KP5060 was grown aerobically overnight with 20 mM glutamine and 20 mM histidine as nitrogen sources; the cells were washed twice with potassium phosphate (100 mM, pH 7.6) and resuspended in the same buffer to a cell density of $1 \cdot 10^8$ cells/ml; then 1% ethylmethanesulfonate was added, and after incubation for 2 h the culture was plated on agar containing the growth medium with 20 mM histidine as the sole nitrogen source (selection of Hut^+ revertants).

Analytical procedures. Dry weight determinations were carried out as described [4]. Intracellu-

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone.

lar volumes were estimated by assuming the cell-bound water to be 1.63-times the bacterial dry weight [9]. Protein was determined by the microbiuret method [10]. Nitrogenase was assayed by the acetylene reduction method as described [8], glutamine synthetase was determined by the ATP-dependent formation of γ-glutamylhydroxamate from glutamate [11].

Assay of $CH_3NH_3^+$ transport. As has been discussed extensively elsewhere [1], uptake of $CH_3NH_3^+$ is a suitable probe for the characterization of an NH_4^+-transport system if two conditions are met: (a) $CH_3NH_3^+$ uptake must be competitively inhibited by NH_4^+; and (b) $CH_3NH_3^+$ should not be metabolized to any large extent during the assay time. Since, as will be shown below, both criteria are met by *K. pneumoniae*, the performed measurements of $CH_3NH_3^+$ transport can be regarded as characterizing an NH_4^+-transport system in this organism.

$CH_3NH_3^+$ transport was assayed either with N_2-grown bacteria taken directly from the culture without further processing or with bacteria grown on combined nitrogen after washing and resuspension in 50 mM sodium phosphate (pH 7.0) containing 0.5% glucose and the additions indicated. After incubation at 30°C for 30 min and then for 1–2 h at the specified temperature, transport was started by the addition of 4.2 μM $^{14}CH_3NH_3Cl$ (50 mCi/mmol). 0.1 ml samples were then withdrawn at the intervals specified, and rapidly filtered through a polycarbonate filter with 0.6 μm pores (Nuclepore, Pleasanton, CA). Nonspecific binding of radioactivity was less than 5% of that usually retained by the cells.

Intracellular labeled compounds were extracted and separated by thin-layer chromatography as described [4].

Materials. $^{14}CH_3NH_3Cl$ was purchased from Rohstoff-Einfuhr (Düsseldorf, F.R.G.). $^{14}C_2H_5NH_3Cl$ was from CEA (Gif-sur-Yvette, France), $(^{14}CH_3)_2NH_2Cl$ from Amersham International (Amersham, England), *N,N'*-dicyclohexylcarbodiimide (DCCD) from Fluka (Buchs, Switzerland), and carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) was a gift from E. Komor.

Results

Uptake kinetics and gradient formation.

The uptake of $^{14}CH_3NH_3^+$ at the growth temperature (30°C) was too fast to be determined by our methods. Thus the temperature was lowered to either 12–17°C (after growth on combined nitrogen) or to 2–10°C (after growth on N_2) for kinetic measurements. Fig. 1 shows the uptake of $CH_3NH_3^+$ and $(CH_3)_2NH_2^+$ by a histidine-grown culture, and the reversal of gradient formation by a chase with ammonium acetate (10 mM). An 80-fold $CH_3NH_3^+$ gradient is formed within 1 min, which collapses to more than two-thirds upon the addition of NH_4^+. Uptake of $(CH_3)_2NH_2^+$ was about 40-fold slower at 30°C than $CH_3NH_3^+$ uptake at 16°C. $C_2H_5NH_3^+$ was not taken up at all.

Analysis of intracellular labeled compounds

Analysis of intracellular labeled compounds showed that of the radioactivity 85–95% after 1 min, and 40–60% after 10 min was still present as $CH_3NH_3^+$, while the remainder was incorporated into a less polar metabolite. Since our assay times

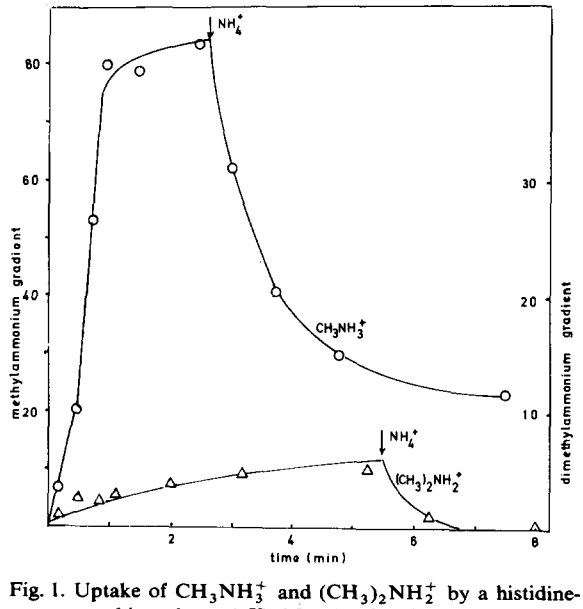

Fig. 1. Uptake of $CH_3NH_3^+$ and $(CH_3)_2NH_2^+$ by a histidine-grown aerobic culture (pH 6.8). At the times indicated by arrows, 10 mM CH_3COONH_4 was added. O——O, $CH_3NH_3^+$ uptake at 16°C; △——△, $(CH_3)_2NH_2^+$ uptake at 30°C.

generally did not exceed 3 min, we can assume that more than 75% of the label accounts for gradient formation of $CH_3NH_3^+$. After hydrolysis in 1 M $HClO_4$ the less polar metabolite lost its radioactivity completely in the form of $^{14}CH_3NH_3^+$. We suppose, that, as in the *C. pasteurianum* case, the less polar metabolite is γ-*N*-methylglutamine. No formation of *N*-methylglutamate could be detected. *K. pneumoniae* was unable to grow aerobically with $CH_3NH_3^+$ as sole nitrogen source.

Effect of pH

The pH dependence of $CH_3NH_3^+$ transport (not shown) exhibited a relatively narrow and symmetrical optimum around pH 6.8 for both the aerobically and the anaerobically grown culture.

Temperature coefficient

The dependence of $CH_3NH_3^+$ transport on the temperature was determined between 0 and 10°C for an N_2 grown culture. From an Arrhenius plot an apparent activation energy of 71 kJ/mol could be calculated. This value is slightly higher than apparent activation energies of 53–55 kJ/mol obtained for lower fungi (see Ref. 1).

Inhibitors

Inhibition of $CH_3NH_3^+$ uptake was effected by various compounds known to block energy metabolism (Table I). The effects of DCCD and CCCP were investigated in more detail, and will be discussed below. In general, $CH_3NH_3^+$ uptake is considerably slowed down by metabolic inhibitors.

TABLE I

THE EFFECT OF VARIOUS INHIBITORS ON $CH_3NH_3^+$ TRANSPORT

Samples of an N_2-grown culture were incubated anaerobically for 5 min with the inhibitors at 10°C, pH 6.8. DCCD and CCCP were added as solutions in ethanol (final ethanol concentration 1%); ethanol alone had no effect. All other compounds were added as aqueous solutions.

Inhibitor	Final concentration (mM)	Residual uptake rate (%)
None	–	100
Azide	5	12
Iodoacetate	5	21
Arsenate	5	15
DCCD	0.1	16
	0.01	20
CCCP	0.01	112

Inhibition of $CH_3NH_3^+$ uptake by NH_4^+

Competitive inhibition of $CH_3NH_3^+$ transport by NH_4^+ has been repeatedly regarded as evidence that the natural substrate of this transport system is NH_4^+ rather than $CH_3NH_3^+$ (see Ref. 1). Plotting our competition data in a Lineweaver-Burk diagram (not shown) we obtained competitive inhibition of $CH_3NH_3^+$ uptake by NH_4^+. The apparent K_m value for $CH_3NH_3^+$ is 100 μM, while the K_i for NH_4^+ is 7 μM. If we assume that this K_i is identical to the K_m for NH_4^+ uptake, it reflects a considerably higher affinity of the carrier for NH_4^+ than for $CH_3NH_3^+$. Both values are in the same range as those for *C. pasteurianum* (150 and 9 μM, respectively, see Ref. 4), but higher than those reported for *A. vinelandii* (25 and 1 μM, see Ref. 6). The V for $CH_3NH_3^+$ transport by *K. pneumoniae* was 40 μmol/min per g dry weight at 15°C for an N_2-grown culture.

Energy coupling.

Many of the experiments performed for the elucidation of the energy source with *C. pasteurianum* [4] could not be done with *K. pneumoniae*. Especially, we were unable to permeabilize the cell walls by established methods [12] for the penetration of valinomycin. Furthermore, our attempts to measure the membrane potential, $\Delta\psi$, by the distribution of the lipophilic cation [^{14}C]triphenylmethylphosphonium yielded unsatisfactory results. Not only the rate, but also the extent of uptake depended on the concentration of tetraphenyl borate. No triphenylmethylphosphonium was taken up in the absence of tetraphenyl borate. The following observations, however, indicate, that $\Delta\psi$ might be the driving force for $CH_3NH_3^+$ accumulation, as has been suggested for most other NH_4^+ carriers [1,6]. At first, the effect of the ATPase inhibitor DCCD depended on whether the metabolic energy was derived solely from glycolysis (anaerobic assay or after blockage of the respiratory chain) or largely from electron transport phosphorylation (aerobic assay). In the first case

the protonmotive force across the membrane has to be established by the action of the membrane-bound H^+ translocating ATPase, while in the second case the protonmotive force is build up by electron transport and is constantly decreased by the ATPase for ATP synthesis.

As can be seen in Fig. 2, DCCD inhibits $CH_3NH_3^+$ accumulation only when no electron transport phosphorylation occurs (anaerobic assay or inhibition of respiration by CN^-). In the case of uninhibited electron transport, DCCD had no effect or sometimes even stimulated $CH_3NH_3^+$ accumulation. These effects show that the protonmotive force is necessary for driving $CH_3NH_3^+$ transport: if the energy in the organisms is derived from glycolysis, inhibition of the ATPase reduces both protonmotive force and uptake; if the energy is derived from electron transport phosphorylation, inhibition of the ATPase has no inhibitory effect on $CH_3NH_3^+$ transport.

The second question is, which of the components of the protonmotive force drives uphill transport of $CH_3NH_3^+$. As already mentioned,

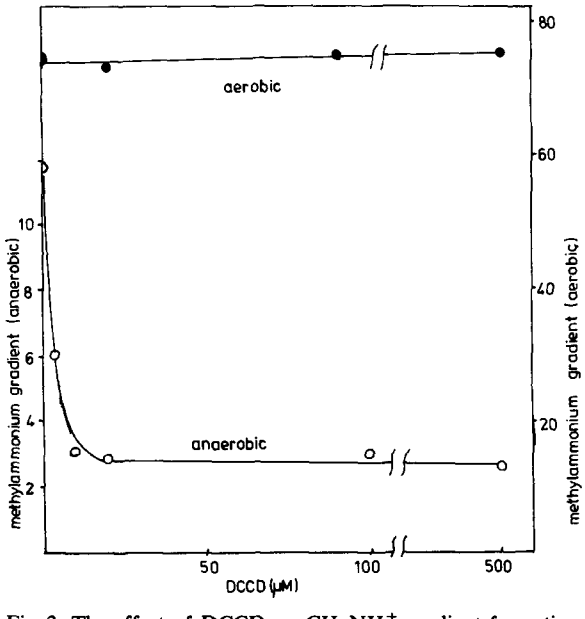

Fig. 2. The effect of DCCD on $CH_3NH_3^+$-gradient formation under aerobic (●———●) and anaerobic (○———○) conditions. Cultures were grown on histidine under air or N_2. The cells were incubated with DCCD for 10 min at 17°C and assayed at the same temperature. 0.5 mM CN^- was added to the anaerobic culture.

TABLE II

THE EFFECT OF CCCP AND K^+ ON $CH_3NH_3^+$ ACCUMULATION

The organisms were grown aerobically on histidine and processed as described. CCCP was added as solution in ethanol.

Compound	Final concentration (mM)	Final gradient obtained
None	–	80
CCCP	0.002	91
	0.007	86
	0.02	83
	0.05	8
	0.1	0
K^+	10	80
	16	77
	50	55

most NH_4^+-transport systems require $\Delta\psi$. We expect this to be the case too for *K. pneumoniae,* but our evidence is still somewhat circumstantial. As can be seen in Table II, relatively high amounts of the proton ionophore CCCP (20 μM), which generally collapse ΔpH and suppress H^+ symport processes in *E. coli* [13–16] to a vast extent, do not affect $CH_3NH_3^+$ uptake. Lower concentrations even prove stimulatory, which we are yet unable to explain. Only at high concentrations does inhibit CCCP $CH_3NH_3^+$ transport. These results suggest that ΔpH is not necessarily involved, and we further have to assume that a considerable part of $\Delta\psi$ is due to cation gradients other than H^+, like in *E. coli* at higher pH (17). Support for $\Delta\psi$-driven transport also comes from the effect of K^+, which at higher concentrations reduces the $CH_3NH_3^+$ gradient (Table II). If we assume that, as in the close relative, *E. coli* [18], the major K^+ transport system in *K. pneumoniae* functions with $\Delta\psi$ as the energy source (analogous to the Trk A system), a massive electrogenic K^+ influx into the cell should reduce $\Delta\psi$ and lead to $CH_3NH_3^+$ efflux, as observed. A decrease in the $CH_3NH_3^+$ gradient formation by K^+ has also been observed in *A. vinelandii* [5].

Taken together, these results suggests a $\Delta\psi$-driven uniport of $CH_3NH_3^+$ and NH_4^+ under both

aerobic and anaerobic conditions. Recently, however, Kashket [9] has reported measurements of $\Delta\psi$ in *K. pneumoniae*, and has found a $\Delta\psi$ of zero under anaerobic growth, irrespective of the nitrogen source. This results is not reconcilable with our interpretations. At present we cannot explain the differences. They may be due to different growth conditions (continuous vs. batch cultures) or different ionic compositions of the media (low vs. high Na^+ concentrations). These discrepancies will be investigated further.

Regulation

We repeatedly observed the highest $CH_3NH_3^+$ uptake rates after growth on N_2 as the sole nitrogen source. Generally, histidine, aspartate and glutamate repress the formation of the NH_4^+-transport system between 50 and 75%, while NH_4^+ in concentrations above 10 mM represses its synthesis completely. Under these circumstances, NH_3 diffusion across the membrane is obviously sufficient to support adequate growth. The transition from a culture growing on NH_4^+ to a culture growing on N_2 is accompanied by derepression of the NH_4^+-transport and nitrogenase activities, as shown in Fig. 3. The nitrogenase activity appears about 2 h later than the NH_4^+-transport activity, indicating that upon nitrogen starvation the organism resorts first NH_4^+ scavenging before derepressing the nitrogenase. This is not surprising in view of the much higher energy requirement for N_2 fixation than NH_4^+ transport.

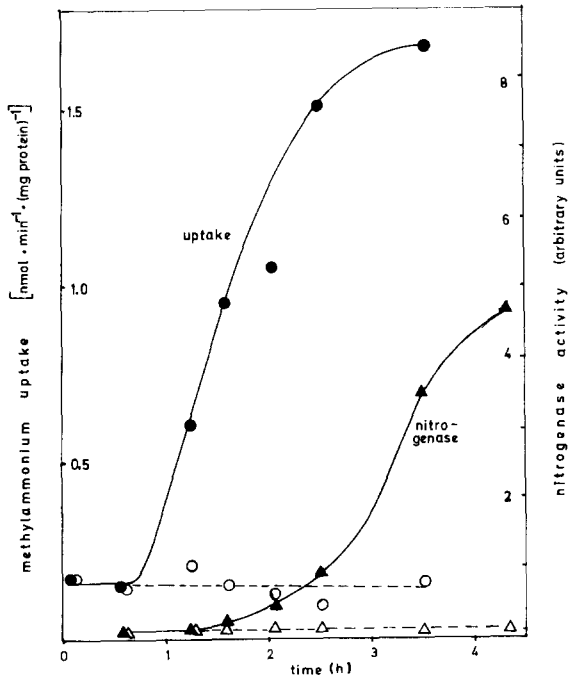

Fig. 3. Derepression of $CH_3NH_3^+$ transport and nitrogenase activity after transition of NH_4^+-grown cells (30 mM) to a medium containing 2 mM aspartate, 1% glucose and trace elements under N_2 as gas phase. The cells were harvested, washed twice and suspended in the new medium which was constantly sparged with N_2. ●——●, $CH_3NH_3^+$ uptake; ▲——▲, nitrogenase activity; ○——○ and △——△, respective controls with 100 mg/l chloramphenicol.

Some hints at the controlling element for the synthesis of the NH_4^+-transport system can be derived from analyzing the properties of a mutant defective in 'nitrogen control' and some revertants. The strain KP5060 bears a mutation in a regulatory gene of the glutamine synthetase region (phenotype $GlnR^-$), which results in pleiotropic depression of several enzymes for the assimilation of various nitrogen sources [19,20], especially glutamine synthetase ($GlnA^-$), nitrogenase (Nif^-), and the enzymes for histidine utilization (Hut^-). Similar mutants in the related enterobacterium *Salmonella typhimurium* have been shown to be deficient in the ability to transport several amino acids [21].

We have found that KP5060 (grown on 2 mM glutamine, 2 mM histidine and 20 mM aspartate) is deficient in NH_4^+ transport, too (we call this phenotype Amt^-).

We have produced revertants of KP5060, which were able to grow on histidine as the sole nitrogen source (Hut^+). Two spontaneous revertants were obtained, forming small (KP5060RS) and large colonies (KP5060RL), and three more revertants were selected after ethylmethanesulfonate mutagenesis (KP5060R1, KP5060R2, KP5060R3). As can be seen from Table III, all revertants had regained the ability to synthesize glutamine, to fix N_2, to degrade histidine and to transport NH_4^+. We suppose that these revertants have regained the $GlnR^+$ genotype.

These results strongly support the hypothesis that the NH_4^+-transport system in *K. pneumoniae* is under the same 'nitrogen control' as many other enzymes for the assimilation of nitrogenous com-

TABLE III

K. PNEUMONIAE STRAINS AND THEIR CHARACTERISTICS

EMS, ethylmethanesulfonate.

Strain	Phenotype	Source
M5a1	Wild type	R.H. Burris
KP5060	$His^- GlnA^- GlnR^- Nif^- Hut^- Amt^-$	18
KP5060RL	$His^- GlnA^+ GlnR^+$(?)$Nif^+ Hut^+ Amt^+$	spontaneous from KP5060
KP5060RS	same	spontaneous from KP5060
KP5060R1	same	EMS of KP5060
KP5060R2	same	EMS of KP5060
KP5060R3	same	EMS of KP5060

pounds, especially nitrogenase and glutamine synthetase.

Discussion

The studies described demonstrate the occurrence of an NH_4^+-transport system in *K. pneumoniae*. The system is characterized as follows:

(1) $CH_3NH_3^+$ is absorbed against a concentration gradient by an energy-requiring process; inhibitors of energy metabolism inhibit transport.

(2) $CH_3NH_3^+$ transport is competitively inhibited by NH_4^+ with the K_i of NH_4^+ (7 μM) being considerably lower than the K_m for $CH_3NH_3^+$ (100 μM). $(CH_3)_2NH_2^+$ is transported very slowly, and $C_2H_5NH_3^+$ not at all. These results indicate a high specificity for NH_4^+.

(3) The $CH_3NH_3^+$ transported is slowly converted to a less polar compound, which on hydrolysis yields quantitatively $CH_3NH_3^+$, and no further conversion occurs. Growth experiments showed that *K. pneumoniae* cannot grow on $CH_3NH_3^+$ as the sole nitrogen source. Therefore the evolution of a specific $CH_3NH_3^+$ carrier would be useless, and we can assume that the system characterized operates with NH_4^+ as the natural substrate.

(4) Transport shows saturation kinetics and a distinct pH profile.

(5) Mutants deficient in transport can be produced.

(6) The energy source for uphill transport is a component of the protonmotive force, probably $\Delta\psi$.

(7) The synthesis of the transport systems seems to be regulated by the same 'nitrogen control' mechanism as other nitrogen assimilatory pathways, e.g., N_2 fixation, glutamine synthesis, and amino acid degradation.

The transport system is repressed by its own substrate NH_4^+ in high concentrations (above 10 mM). This implies that at these concentrations diffusion of NH_3 is adequate to support growth. By using rather crude assumptions of the organism's surface area and its intracellular NH_4^+ concentration (see Ref. 8), we calculate a lower limiting value for the permeability coefficient for NH_3 of $P = 5 \cdot 10^{-5}$ cm/s. This P value is about 10–100-times lower than the permeability coefficient for water through bilayer lipid membranes [22]. Nonetheless, it is high enough to allow considerable leakiness of the membrane towards NH_3. This must also apply to the reverse case: that is, if the extracellular NH_3 concentration is below the intracellular level, as in the case for N_2 fixation. The fact that under N_2-fixing conditions the free-living bacteria *K. pneumoniae* [8], *C.pasteurianum* [11] and *A. vinelandii* [23] do not release appreciable amounts of NH_3 into the medium, despite the maintenance of up to 100-fold NH_4^+ gradients across the membrane [3,8,23], may now be explained by the assumption that the NH_4^+-transport systems discovered in these strains do not serve only for NH_4^+ scavenging, but also for cyclic retention of NH_3 lost by diffusion. We are currently trying to quantitate these postulated fluxes of NH_3 and NH_4^+ during N_2 fixation and their energy costs.

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