Multiplicity of ammonium uptake systems in *Corynebacterium glutamicum*: role of Amt and AmtB

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In *Corynebacterium glutamicum*, a Gram-positive soil bacterium widely used in the industrial production of amino acids, two genes encoding (putative) ammonium uptake carriers have been described. The isolation of *amt* was the first report of the sequence of a gene encoding a bacterial ammonium uptake system combined with the characterization of the corresponding protein. Recently, a second *amt* gene, *amtB*, with so far unknown function, was isolated. The isolation of this gene and the suggestion of a new concept for ammonium acquisition prompted the reinvestigation of ammonium transport in *C. glutamicum*. In this study it is shown that Amt mediates uptake of (methyl)ammonium into the cell with high affinity and strictly depending on the membrane potential. As shown by the determination of K_m at different pH values, ammonium/methylammonium, but not ammonia/methylamine, are substrates of Amt. AmtB exclusively accepts ammonium as a transport substrate. In addition, hints of another, until now unknown, low-affinity, ammonium-specific uptake system were found.

Keywords: ammonium transport, *Corynebacterium glutamicum*, glutamine synthetase

INTRODUCTION

Most micro-organisms use ammonium (NH_4^+) as the preferred nitrogen source. Although its uncharged form ammonia (NH_3) is highly membrane-permeable, energy-dependent transport systems for ammonium have been described in many bacteria (for a review, see Kleiner, 1993). Usually these carriers are inhibited by glutamine or its analogues (Kleiner & Castorph, 1982; Jayakumar *et al.*, 1986). It is proposed that the intracellular pool of this amino acid directly reflects the nitrogen supply of the cell, and therefore regulation ensures that ammonium uptake activity corresponds exactly to the requirements of the organism. In addition, synthesis of ammonium carriers is regulated by the availability of ammonium itself (Kleiner, 1985). As pointed out by

Kleiner (1995), intracellular ammonium accumulation by an uptake system may create an energy-wasting futile cycle, in which energy-dependent uptake is counteracted by diffusion of ammonia out of the cell. Thus, repression of carrier synthesis under conditions of sufficient nitrogen supply ensures that energy is expended for ammonium uptake only when cells are starving for nitrogen.

In contrast to this model of an energy-dependent, membrane-potential-driven ammonium transport, proposed for a variety of ammonium uptake systems in bacteria and in plants (for reviews, see Kleiner, 1993; von Wirén *et al.*, 2000), a different concept of ammonium acquisition was suggested recently (Soupene *et al.*, 1998). It was proposed that AmtB/Mep proteins simply increase the rate of equilibration of uncharged ammonia across the cytoplasmic membrane rather than actively transporting and accumulating ammonium.

In *Corynebacterium glutamicum*, a Gram-positive soil bacterium widely used in the industrial production of amino acids, two genes encoding (putative) ammonium uptake carriers have been described. The isolation of *amt* (Siewe *et al.*, 1996) was the first report of the

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Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

sequence of a gene encoding a bacterial ammonium uptake system combined with the characterization of the corresponding protein. Recently, a second *amt* gene with so far unknown function was isolated. This gene was originally designated *amtP*, for *amt* paralogue (Jakoby *et al.*, 1999). Based on a great number of homologues cloned in different bacteria, on sequence similarity analyses and on its genetic organization, this system is now designated *amtB* (Jakoby *et al.*, 2000). The isolation of the *amtB* gene and the new model proposed for the mechanism of ammonium uptake (Soupeine *et al.*, 1998) prompted us to reinvestigate (methyl)ammonium uptake in *C. glutamicum*.

METHODS

Bacterial strains, plasmids and growth conditions. Strains and plasmids used in this study are listed in Table 1. Bacteria were routinely grown in Luria–Bertani (LB) medium (Sambrook *et al.*, 1989) at 30 °C (*C. glutamicum*) or 37 °C (*Escherichia coli*). If appropriate, antibiotics were added at standard concentrations (Ausubel *et al.*, 1987); LB medium for *C.*

glutamicum strains was supplemented with 2% glucose (final concentration). To study the effects of nitrogen starvation under highly comparable conditions, a standard inoculation scheme was applied (Jakoby *et al.*, 2000). A fresh *C. glutamicum* culture in LB medium was used to inoculate minimal medium (Keilhauer *et al.*, 1993) with an excess of nitrogen source (500 mM ammonium) for overnight growth. This culture, with an overnight OD₆₀₀ of approximately 25–30, was used to inoculate fresh minimal medium to an OD₆₀₀ of approximately 0.5, and cells were grown until the exponential-growth phase was reached (OD₆₀₀ approx. 4–5). To induce nitrogen starvation, cells were harvested by centrifugation and the pellet was resuspended in and transferred to prewarmed minimal medium without nitrogen source. The nitrogen-deprived cells were incubated at 30 °C under aeration.

Determination of methylammonium uptake. For measurement of methylammonium uptake, cells were resuspended in minimal medium without nitrogen source to a final OD₆₀₀ of 3–4 for the wild-type or 10 for *amt* deletion strain MJ2-38. After 3 h nitrogen deprivation, uptake was started by the addition of 100 µM (Amt) or 1 mM (AmtB) ¹⁴C-labelled methylammonium (2.5 kBq). Samples were taken at 15 s time

Table 1. Strains and plasmids used in this study

Strain/plasmid	Relevant genotype/description	Reference/source
<i>C. glutamicum</i> strains		
ATCC 13032	Wild-type	Abe <i>et al.</i> (1967)
LN-1.1	Δ <i>amtB</i>	This study
LN-1.2	Δ <i>amt</i> Δ <i>amtB</i>	This study
MJ2-38	Δ <i>amt</i>	This study
MJ4-26	Δ <i>glnA</i>	Jakoby <i>et al.</i> (1997)
<i>E. coli</i> strains		
DH5 <i>α</i> mc	<i>supE44 hsdR17 recA1 endA1 gyrA96 thi1 relA mcrA</i> Δ(<i>mrr</i> – <i>hsdRMS</i> – <i>mcrBC</i>)	Grant <i>et al.</i> (1990)
S17-1	<i>thi-1 endA1 hsdR17 supE44 λ⁻ pro</i>	Simon <i>et al.</i> (1983)
Plasmids		
pGEM3+	<i>E. coli</i> plasmid for <i>in vitro</i> transcription, Ap ^r	Promega
pGEM <i>amt</i>	0.5 kb internal <i>amt</i> fragment in pGEM3+	Jakoby <i>et al.</i> (2000)
pGEM <i>amtB</i>	1.0 kb internal <i>amtB</i> fragment in pGEM3+	Jakoby <i>et al.</i> (2000)
pJC1	<i>E. coli/C. glutamicum</i> shuttle vector, Km ^r	Cremer <i>et al.</i> (1990)
pJCA <i>mtBam</i>	3 kb <i>Bam</i> HI fragment carrying ' <i>ppc secB amt</i> in pJC1	This study
pJCA <i>mtHII</i>	2 kb <i>Hind</i> II/ <i>Bam</i> HI fragment carrying <i>amt</i> in pJC1	This study
pK19 <i>mobsacB</i>	<i>ori</i> pUC, Km ^r , <i>mob sacB</i>	Schäfer <i>et al.</i> (1994)
pK19 <i>mobsacB</i> -del2	Vector for internal deletion in the <i>amtB</i> gene	This study
pK19 <i>mobsacB</i> Δ <i>ppc</i>	2 kb <i>Bam</i> HI fragment with internal <i>amt</i> deletion in pK19 <i>mobsacB</i>	This study
pUCA <i>mtB</i>	pUC18 carrying a 2.4 kb ' <i>ftsY amtB glnK</i> fragment	Jakoby <i>et al.</i> (1999)
pUCA <i>mtB</i> -del2	pUC18 carrying a ' <i>ftsY glnK</i> fragment for an internal deletion in <i>amtB</i>	This study
pUC <i>dppc</i>	3 kb <i>Bam</i> HI fragment carrying ' <i>ppc secB amt</i> in pUC18, Ap ^r	Siewe <i>et al.</i> (1996)
pUCΔ <i>dppc</i>	2 kb <i>Bam</i> HI fragment with internal <i>amt</i> deletion in pUC18, Ap ^r	This study

Ap^r, resistance to ampicillin; Km^r, resistance to kanamycin.

intervals over a period of 1.5 min. The cells were separated from the surrounding medium by rapid filtration, washed twice and subjected to scintillation counting. The effect of mercurials was determined as described by Siewe *et al.* (1996).

Extraction of *C. glutamicum* cells and TLC. Nitrogen-starved cells were incubated in the presence of ^{14}C -labelled methylammonium as described above with the exceptions that a higher cell density was used (OD_{600} approx. 7) and that the specific activity of the approach was increased to 12.5 kBq. Filtered cells were extracted in 4 ml 0.05% SDS/hot 70% ethanol solution (1 h at room temperature) immediately after the washing steps. After 10 min incubation the ethanolic extracts were dried at room temperature under a steady stream of air and subjected to TLC. Samples were separated on cellulose plates with a mixture of n-butanol, acetic acid and water (4:1:1 by vol). The ^{14}C -labelled products were detected either by autoradiography using Kodak X-OMAT films or BAS-MP 2025 imaging plates and a Bio-Imaging Analyzer BAS-1800 (Fuji).

Measurement of ammonium. Decrease of ammonium in the medium was measured using an ammonia-selective electrode (Orion Research). Cells were starved of nitrogen for 3 h to induce the synthesis of Amt and AmtB and to deplete the internal nitrogen pools. Subsequently, 200 μM ammonium chloride was added to the cultures (OD_{600} approx. 5) and at different times cells and culture supernatant were separated by rapid filtration using glass fibre filters (Millipore). The ammonium uptake rate was calculated from the decrease of ammonia determined in the culture filtrate.

Molecular biology techniques. For plasmid isolation, transformation and cloning, standard techniques were used (Ausubel *et al.*, 1987; Sambrook *et al.*, 1989). Southern blotting was carried out with digoxigenin-labelled probes using the DIG-labelling and detection kit as recommended by the supplier (Roche Diagnostics).

RNA preparation and Northern hybridization analysis. Total RNA was prepared after disruption of the *C. glutamicum* cells by glass beads using the RNeasy Mini Kit as recommended by the supplier (Qiagen). The RNA was either size-fractionated using agarose gels containing formaldehyde and blotted onto positively charged nylon membranes (Sambrook *et al.*, 1989), or spotted directly onto nylon membranes using a Schleicher & Schuell Minifold I Dot Blotter. Hybridization of digoxigenin-labelled RNA probes was detected with a Fuji luminescent image analyser LAS1000 or Kodak X-OMAT X-ray films using alkaline phosphatase conjugated anti-digoxigenin Fab fragments and CSPD as light-emitting substrate as recommended by the supplier (Roche Diagnostics).

Construction of deletion mutants. A chromosomal deletion of the *amt* gene was introduced in the *C. glutamicum* genome according to the protocol described by Schäfer *et al.* (1994) using plasmid pK19mobsacB for deletion in *C. glutamicum* and *E. coli* strain S17-1 for conjugation. For this purpose a 1080 bp *AocI/KspI* fragment was removed from plasmid pUCdppc. After treatment with DNA polymerase I (Klenow fragment) and re-ligation, a 2 kb *BamHI* fragment was isolated from plasmid pUC Δ dppc and ligated to *BamHI*-restricted and dephosphorylated pK19mobsacB DNA. The resulting plasmid, pK19mobsacB Δ dppc, was used for the chromosomal deletion via two independent recombination events as described by Schäfer *et al.* (1994).

To inactivate *amtB*, an internal fragment of this gene was removed from plasmid pUCamtB by *EcoRV/BsmI* restriction.

After blunting of the linearized plasmid DNA by mung bean nuclease treatment and re-ligation, a DNA fragment carrying the flanking regions of the deletion was isolated from the corresponding plasmid, pUCamtB-del2, by *EcoRI/BamHI* restriction and used further as described above. The different deletions were verified by PCR using primers annealing at flanking regions (data not shown). In addition, chromosomal DNA of the deletion strains was amplified via PCR and sequenced using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit and an ABI 310 automated sequencer (PE Applied Biosystems) to verify that the flanking DNA regions of the different deletions were not impaired during the recombination events.

RESULTS

Characterization of the *C. glutamicum* Amt system

To reinvestigate the transport activity of Amt in *C. glutamicum*, an *amt* deletion strain, MJ2-38, was constructed. When this strain was characterized in transport experiments using low concentrations of methylammonium (final concentrations 100 μM and lower), only a very low uptake rate of approximately 0.5 nmol (mg dry weight) $^{-1}$ min $^{-1}$ was detected. A similar result was obtained by Siewe *et al.* (1996), when methylammonium uptake in the *amt* gene disruption strain Amt $^{-}$ was studied. This background activity was also observed in de-energized or heat-inactivated cells (data not shown) and corresponds to passive diffusion. Uptake of methylammonium in strain MJ2-38 could be restored by transformation with plasmids pJCAmtBam and pJCAmtHII, carrying the *C. glutamicum* *amt* gene (Fig. 1), whilst transformation with plasmid pJC1, the vector without the *amt* gene, had no effect. Taken

Fig. 1. Methylammonium uptake rates of different *C. glutamicum* strains. To induce expression of the *amt* gene, cells were incubated for 3 h in minimal medium without nitrogen source. Uptake was started by the addition of [^{14}C]methylammonium (100 μM final concentration). 1, Wild-type; 2, MJ2-38; 3, MJ2-38 pJCAmtBam; 4, MJ2-38 pJCAmtHII; 5, MJ2-38 pJC1.

Fig. 2. Kinetic analysis of methylammonium transport depending on pH. (a) pH 6.0, (b) pH 8.5. The experiments were carried out as described by Sieve *et al.* (1996).

together, these results indicate that Amt is the only high-affinity methylammonium uptake system in *C. glutamicum*.

Kinetic analysis of (methyl)ammonium uptake by Amt

Soupe *et al.* (1998) proposed that ammonia (NH₃), but not ammonium (NH₄⁺) is transported by Amt/Mep systems. To define the transported substrate species for the *C. glutamicum* Amt system, methylamine/ammonia or (methyl)ammonium, the *K_m* of the Amt transporter for methylammonium was determined at pH 6.0 and 8.5 (Fig. 2). Since methylammonium (CH₃NH₃⁺) has a p*K* of 10.65, the concentration of methylamine (CH₃NH₂) changes in this range by a factor of 300, whereas that of methylammonium remains more or less constant. As a consequence, the apparent *K_m* of the transporter as related to the total amount of methylamine and methylammonium, respectively, should change drastically in the case of (methyl)amine transport, whereas it should remain constant in the case of (methyl)ammonium being the substrate. An apparent *K_m* of Amt for methylammonium of 53 ± 11 μM was directly calculated by non-linear fitting from the uptake kinetics at pH 6.0 and of 47 ± 8 μM at pH 8.5. These results are identical to those obtained by Sieve *et al.* (1996) who reported a *K_m* of 44 ± 7 μM at pH 7.0. Since no change in the apparent *K_m* was observed, it was concluded that (methyl)-

Fig. 3. Influence of CCCP on methylammonium uptake. Δ, control culture without addition of CCCP; ▲, response of methylammonium uptake upon addition of CCCP (indicated by an arrow).

ammonium and not (methyl)amine is transported by the corynebacterial Amt system.

Inhibition of (methyl)ammonium uptake by the uncoupler CCCP

A linear dependence of methylammonium transport on the actual electrical potential in the range from 25 to 150 mV was found previously (Sieve *et al.*, 1996) by applying potassium diffusion potentials in the presence of valinomycin. To further elaborate the energy dependence of the transport reaction catalysed by Amt, we investigated the influence of the protonophor carbonyl cyanide *m*-chlorophenylhydrazine (CCCP). The observed instant uptake inhibition after its addition (Fig. 3), favours a membrane-potential-driven mechanism of (methyl)ammonium transport by Amt rather than facilitated diffusion. It was previously shown for *C. glutamicum* that under the experimental conditions used, CCCP primarily leads to inhibition of secondary transport, whilst the ATP pool of the cells and primary transport systems stay unaffected for several minutes (Krämer & Lambert, 1990; Krämer *et al.*, 1990).

Metabolism of methylammonium

When methylammonium is transported into the cell via Amt, the question arises whether it is metabolized in the cytoplasm, in particular since Soupe *et al.* (1998) proposed metabolism as being the only driving force for methylammonium uptake. While Tachiki *et al.* (1983) observed a small but significant turnover of methylammonium in *in vitro* measurements, we found none (Sieve *et al.*, 1996). In the latter study, however, an acid cell lysis method was used, which may have led to the degradation of products of methylammonium metab-

Fig. 4. TLC of ethanolic extracts of nitrogen-starved *C. glutamicum* cells incubated with [^{14}C]methylammonium. Lanes: 1–3, cells extracted after 0.5, 1 and 1.5 min incubation with [^{14}C]methylammonium, respectively; 4, control [^{14}C]methylammonium.

olism. To avoid acid treatment, nitrogen-starved wild-type cells incubated with [^{14}C]methylammonium were extracted in 70% ethanol, and the extract was dried under a steady air stream at room temperature. More than 90% of label from the extracts was recovered using this method, whilst lyophilization or heating of the sample led to a loss of label. In contrast to the previous studies (Tachiki *et al.*, 1983; Siewe *et al.*, 1996), we found that methylammonium is immediately metabolized by *C. glutamicum*, as detected by autoradiography (Fig. 4). In *E. coli*, methylammonium was shown to be converted to 5-*N*-methylglutamine, most likely by glutamine synthetase (Soupene *et al.*, 1998). In *C. glutamicum*, glutamine synthetase I is at the least not the only enzyme converting this compound. When thin-layer chromatograms of cell extracts from *glnA* deletion strain MJ4-26 incubated with [^{14}C]methylammonium were analysed, metabolism of this compound was still observed (data not shown), although this strain is auxotrophic for glutamine and exhibits no glutamine synthetase activity (Jakoby *et al.*, 1997). Due to these results, and in contrast to our previous model (Siewe *et al.*, 1996), an accumulation of (methyl)ammonium in *C. glutamicum* cannot be deduced from an accumulation of label.

Inhibition of methylammonium transport by different amines

Since methylammonium was found to be metabolized in *C. glutamicum*, the question concerning the physiological range of substrates of Amt arises. Therefore,

Fig. 5. Inhibition of [^{14}C]methylammonium uptake by ammonium. Cells were incubated as described in Methods with the exception that 0, 0.5, 1, 2, 5, 10, 20, 50, 100 or 200 μM ammonium was added together with 50 μM [^{14}C]methylammonium.

competition assays were carried out. When 50 μM methylammonium was used in the uptake assay together with increasing amounts of ammonium, the methylammonium uptake rate decreased depending on the ammonium concentration. An inhibition of 50% was reached when 10 μM ammonium was added under these conditions and transport was almost completely abolished in the presence of 50 μM ammonium (Fig. 5). These data indicate that ammonium is the natural substrate of Amt.

To define the substrate specificity of Amt in more detail, methylammonium uptake was determined in the presence of a 50-fold excess of different putative competitors. The uptake rates for [^{14}C]methylammonium decreased to $3.0 \pm 0\%$ when unlabelled methylammonium was added as a control. Addition of a 50-fold excess of dimethylamine led to a reduction of $65 \pm 2\%$, whilst trimethylamine and tetramethylamine were not inhibitory. When the influence of ethylamine, propylamine and butylamine on methylammonium uptake was tested, only the addition of ethylamine reduced transport rates significantly. Propylamine and butylamine led to partial permeabilization of the cell membranes. Under the growth conditions tested, none of these compounds was used as sole nitrogen source in *C. glutamicum* (data not shown). In addition, methylammonium was not metabolized by *C. glutamicum* as sole carbon and energy source as also shown for other bacteria (Large, 1980). Further studies might address the question of whether these substances can be used as a co-substrate together with other nitrogen or carbon sources.

Regulation of *amt* transcription depending on the medium pH

As shown recently, *amt* transcription is derepressed upon nitrogen starvation (Jakoby *et al.*, 2000). On the assumption that *C. glutamicum* Amt facilitates trans-

Fig. 6. Expression of the *amt* genes. Northern hybridizations were carried out, using total RNA prepared from strain ATCC 13032, 5 min before the onset of nitrogen starvation (1), or 20 min after the beginning of starvation in medium adjusted to pH 6.0 (2) and pH 7.0 (3), respectively. A, *amt* anti-sense probe; B, *amtB* anti-sense probe; C, 16S rRNA probe as control.

port of ammonia rather than catalysing ammonium transport, its expression should be increased in low pH medium, since diffusion of ammonia across the membrane would become limiting under these conditions. Northern hybridization experiments were carried out using total RNA isolated from nitrogen-starved cells incubated at pH 6.0 and 7.0. When hybridized against an *amt* antisense probe, RNA isolated from cells incubated at pH 6.0 showed only a faint signal as compared with RNA prepared from cells incubated at pH 7.0, whilst the amount of 16S rRNA in these samples stayed constant (Fig. 6). These data do not indicate an increased expression of *amt* at low pH and are thus in accord with the model that Amt works as a (methyl)ammonium- rather than a (methyl)amine-uptake system.

Role of the AmtB system

We recently isolated and sequenced a paralogue of the *amt* gene, *amtB* (Jakoby *et al.*, 1999). When we tested transcription of the *amtB* gene, we found that its expression is, as with *amt* transcription, repressed under nitrogen excess (Jakoby *et al.*, 2000) and derepressed upon nitrogen starvation (Fig. 6). In contrast to *amt* transcription, no influence of the external pH was observed.

From the measurements obtained during the comparison of wild-type and *amt* deletion strain MJ2-38, it was assumed that *amtB* might encode a low-affinity permease, since no high-affinity methylammonium uptake was observed in strain MJ2-38. In this case, methylammonium would be transported in the *C. glutamicum* cells via both a high-affinity uptake system, Amt, and a low-affinity carrier, AmtB, a scenario which is similar to the situation in the cyanobacterium *Synechocystis* PCC 6803 (Montesinos *et al.*, 1998).

To investigate the basic kinetic parameters of AmtB and

a possible regulation depending on the nitrogen availability, cells of strain MJ2-38 were starved for nitrogen sources and the rate of methylammonium uptake was determined using 1 mM [14 C]methylammonium. However, methylammonium uptake could never be observed in strain MJ2-38 either without or with nitrogen starvation for up to 4 h (data not shown).

Deletion of the *amtB* gene

Since AmtB was obviously not directly involved in methylammonium transport, we speculated that this protein might have a regulatory function. Recently, a sensor function was discussed for the yeast (methyl)ammonium transporter Mep2 (Lorenz & Heitman, 1998). Moreover, an interaction of the different yeast Mep systems was proposed (Marini *et al.*, 2000). These observations led to the hypothesis that *C. glutamicum* AmtB might interact with Amt to regulate transport activity. However, when methylammonium uptake was determined in the *amtB* deletion mutant LN-1.1, no difference between wild-type and *amtB* deletion mutant was observed. Upon nitrogen starvation, a methylammonium uptake rate of 10.8 ± 1.3 nmol (mg dry weight) $^{-1}$ min $^{-1}$ was observed in the wild-type ATCC 13032 and 11.0 ± 1.0 nmol (mg dry weight) $^{-1}$ min $^{-1}$ in *amtB* deletion strain LN-1.1. Also, regulation of the level of Amt activity was not impaired. When glutamine was added, in both strains a decrease in transport rate of 14% was observed within 10 min (data not shown). Together, these results demonstrate that AmtB is not involved in Amt regulation.

Effect of pH on growth at different ammonium concentrations

As a more physiological approach to studying the function of Amt and AmtB and to investigate which of the different concepts for ammonium acquisition holds true for *C. glutamicum*, we carried out growth experiments. Based on the fact that at low pH the concentration of the unprotonated ammonia is extremely low, diffusion and uptake should become limiting if ammonia is the transported substrate species (Soupene *et al.*, 1998). As a result, a difference in growth of wild-type and *amt* deletion mutants is expected in the case of ammonia being transported as proposed for *E. coli* AmtB (Soupene *et al.*, 1998). *C. glutamicum* wild-type strain ATCC 13032, *amt* deletion strain MJ2-38, *amtB* deletion LN-1.1 and *amt/amtB* double deletion LN-1.2 were grown at various ammonium concentrations (0.1, 0.5, 1.0 and 5.0 mM NH $_4$ Cl) in minimal medium adjusted to different pH values (pH 6.0, 6.5, 7.0, 7.5). No significant difference in the growth rate of the different *C. glutamicum* strains depending on pH and ammonium concentration was detected, although the final optical densities were found to depend directly on the amount of ammonium added (data not shown). This result led us to investigate whether ammonium depletion results in a decreased growth rate in *C. glutamicum* at

Fig. 7. Ammonium uptake in *C. glutamicum* depending on the presence of Amt-type transporters: $\square$, wild-type ATCC 13032; $\circ$, *amt* deletion strain MJ2-38; $\triangle$, *amtB* deletion strain LN-1.1; $\blacktriangledown$, *amt/amtB* double deletion LN-1.2. Experiments were carried out in duplicate and mean values are shown.

all. In fact, when cells were incubated in the absence of any nitrogen source, for a short time a similar growth rate as compared to nitrogen-supplemented cells was observed before cell division ceased completely. Obviously, *C. glutamicum* is able to circumvent the effects of ammonium limitation on the growth rate found in other bacteria (e.g. *E. coli*; see Soupene *et al.*, 1998) by using intracellularly stored nitrogen sources like glutamate and glutamine. Up to 200 mM internal glutamate (Krämer & Lambert, 1990) and 50 mM internal glutamine (Tesch *et al.*, 1999) have been determined in *C. glutamicum* cells grown in minimal medium. As a consequence, growth experiments seem not to be an appropriate tool to discriminate the physiological role of Amt and AmtB.

Measurement of ammonium uptake

To circumvent the problems arising from the high internal glutamate and glutamine pools in *C. glutamicum*, depletion of ammonium was directly determined in the culture supernatants of strains ATCC 13032, MJ2-38, LN-1.1 and LN-1.2 (Fig. 7). Ammonium chloride (200 μ M) was added to nitrogen-starved cells (OD_{600} approx. 5) and ammonium concentrations were determined. From the linear part of the decrease, an uptake rate of approximately 11 nmol (mg dry weight) $^{-1}$

min $^{-1}$ was calculated for the wild-type, which perfectly correlates with the methylammonium uptake rates measured. The transport rates obtained for *amt* mutant strain MJ2-38 and *amtB* mutant LN-1.1 were found to be about 10 and 9 nmol (mg dry weight) $^{-1}$ min $^{-1}$, respectively, and thus not significantly lower. Interestingly, for the double mutant a biphasic decrease of ammonium in the supernatant was observed. First, ammonium decreased with a rate of approximately 8 nmol (mg dry weight) $^{-1}$ min $^{-1}$ until uptake ceased at a concentration of 70 μ M ammonium. This result cannot be explained by diffusion alone, since diffusion should depend linearly on the transmembrane concentration difference, and therefore hints of the presence of an additional ammonium uptake system.

In summary, the data obtained indicated (i) that a defect of Amt can be complemented by AmtB and vice versa, (ii) that AmtB is a high-affinity, ammonium-specific transport system, and (iii) that an additional (low-affinity) ammonium uptake system might be present in *C. glutamicum*.

DISCUSSION

In this work (methyl)ammonium transport via two *C. glutamicum* permeases, Amt and AmtB, was investigated. The data of Siewe *et al.* (1996) and those presented here indicate that the actual transport substrate of Amt in *C. glutamicum* is not methylamine/ammonia but (methyl)ammonium, which is taken up by a membrane-potential-driven mechanism. Five lines of evidence support this idea. First, a linear dependence of methylammonium transport and electrical potential in the range 25–150 mV was found (Siewe *et al.*, 1996), which makes facilitated diffusion of ammonia in *C. glutamicum* unlikely. Second, the instant uptake inhibition after addition of CCCP favours membrane-potential-dependent transport by *C. glutamicum* Amt rather than facilitated diffusion as supposed for the *E. coli* AmtB carrier (Soupene *et al.*, 1998). Third, and most importantly, the apparent K_m of Amt for methylammonium of approximately 50 μ M was not changed when measured at both pH 6.0 and 8.5. The measured K_m is furthermore identical at pH 7.0 to that previously obtained by Siewe *et al.* (1996). This result is only in accord with uptake of (methyl)ammonium and not of methylamine/ammonia. Fourth, the K_m of the *C. glutamicum* glutamine synthetase with respect to ammonium was determined earlier to be in the range 1.1–5.0 mM (Tachiki *et al.*, 1981, 1983). Our own experiments (J. Meier-Wagner, unpublished results) and experiments carried out by Tachiki *et al.* (1983) indicated that the K_m of this enzyme for methylammonium is even higher. The affinity of the metabolizing enzyme is thus at least two orders of magnitude lower than that of the carrier protein Amt, which showed a K_m of approximately 50 μ M for methylammonium. The fact that the kinetic properties of ammonium uptake and glutamine synthetase activity are so dramatically different suggests that action of

glutamine synthetase is not the major driving force for (methyl)ammonium uptake in *C. glutamicum*. This is in accord with the observation that methylammonium is metabolized even in the absence of glutamine synthetase I. Fifth, studies on the regulation of *amt* expression showed that synthesis of Amt is dramatically diminished at pH 6.0, which is in contrast to the expected synthesis of an ammonia facilitator.

Whilst Amt is the main uptake system, transporting (methyl)ammonium with high affinity into the cell, AmtB obviously does not contribute to the uptake of methylammonium but is an ammonium-specific permease. Whether ammonium or ammonia is the actual substrate species transported by AmtB was not determined. A similar set of ammonium transport proteins was found in *Rhodobacter sphaeroides*, where an ammonium-specific uptake system together with a methylammonium/ammonium permease was found (Cordts & Gibson, 1987), and in *Arabidopsis thaliana* where AMT1 proteins transport (methyl)ammonium (Gazzarrini *et al.*, 1999) whilst AtAMT2 is ammonium-specific (Sohlenkamp *et al.*, 2000). Furthermore, the kinetic results of ammonium uptake studies indicated the presence of an additional, low-affinity ammonium transport system in *C. glutamicum*.

In summary, membrane-potential-driven transport of (methyl)ammonium was shown for the *C. glutamicum* Amt system. This type of uptake mechanism might also hold true for other bacteria. In physiological terms, moreover, energy-dependent uptake of ammonium makes sense especially for organisms living in acid environments, in which almost no ammonia is present.

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