

Ammonium Toxicity in Bacteria

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Abstract. Although an excellent nitrogen source for most bacteria, ammonium was—in analogy to plant and animal systems—assumed to be detrimental to bacteria when present in high concentrations. In this study, we examined the effect of molar ammonium concentrations on different model bacteria, namely, *Corynebacterium glutamicum*, *Escherichia coli*, and *Bacillus subtilis*. The studied bacteria are highly resistant to ammonium. When growth was impaired upon addition of molar $(\text{NH}_4)_2\text{SO}_4$ concentrations, this was not caused by an ammonium-specific effect but was due to an enhanced osmolarity or increased ionic strength of the medium. Therefore, it was concluded that ammonium is not detrimental to *C. glutamicum* and other bacteria even when present in molar concentrations.

Ammonium (in this communication, the term “ammonium” is used in a general sense, including NH_4^+ and NH_3 ; distinct ammonium species are indicated by chemical formula) is the preferred nitrogen source for most bacteria and fungi, and also plants acquire ammonium as a nitrogen source from the soil [35]. When present in high concentrations, diffusion of NH_3 , which is in equilibrium with NH_4^+ , across the cytoplasmic membrane into the cell is sufficient to promote growth. In a situation of ammonium limitation, diffusion becomes negligible and special carriers are synthesized in bacteria, fungi, and plants (for a recent review, see [36]).

However, ammonium is a paradoxical nutrient, because it is notorious for its cytotoxic effects. Furthermore, active transport of ammonium can lead to a harmful energy-wasting futile cycling when accumulated ammonium diffuses across the cytoplasmic membrane back into the medium. Sensitivity to ammonium is discussed to be a universal phenomenon; however, it is well investigated only in plant and animal systems [36], whereas the situation in bacteria is less clear.

Our group has been studying nitrogen metabolism and ammonium transport in corynebacteria for several years, with a strong focus on *Corynebacterium glutamicum* [6–8]. This mycolic acids-containing actinomycete is closely related to important pathogens such as *Corynebacterium diphtheriae* and *Mycobacterium tuberculosis*. In contrast to these species, *C. glutamicum* is safe to handle and not pathogenic. Different *C. glutamicum* strains are used for the industrial production of amino acids, e.g., more than 1,500,000 tons of L-glutamate and 560,000 tons of L-lysine in the year 2003 [11]. Because of its industrial importance, *C. glutamicum* is extremely well investigated and established as a model organism for actinomycetes and other bacteria of biotechnological relevance. This fact and the use of ammonium as a nitrogen source as well as a pH regulator (in form of NH_4OH) in fermentation media made it interesting for us to investigate the topic of ammonium toxicity in *C. glutamicum*. In this study, the experiments were additionally extended to other species, namely, the Gram-negative model organism *Escherichia coli* and the Gram-positive *Bacillus subtilis*.

Materials and Methods

Bacterial strains and growth conditions. Strains and plasmids used in this study are given in Table 1. Bacteria were routinely grown in

Table 1. Strains and plasmids used in this study

Strains/plasmids	Relevant genotype/description	Reference/source
<i>C. glutamicum</i>		
ATCC 13032	Wild-type	[1]
LNΔGDH	ATCC 13032 Δ <i>gdh</i>	kindly provided by L. Nolden
MJ6-18	ATCC 13032 Δ <i>amtR</i>	[13]
TMΔ <i>gdh</i> Δ <i>glnA</i>	ATCC 13032 Δ <i>gdh</i> -Δ <i>glnA</i>	This study
<i>E. coli</i>		
MG1655	K12 wild type λ ⁻ F ⁻ <i>rph-1</i>	[15]
<i>B. subtilis</i>		
168	Wild type	DSMZ, Braunschweig
Plasmids		
pK18Δ <i>glnA</i>	Deletion vector pK18mobsacB carrying the flanking regions of <i>C. glutamicum glnA</i> for generation of chromosomal deletions, Km ^R	[12]

Km^R, resistance to kanamycin.

shaking flasks at 30°C (*C. glutamicum* and *B. subtilis*) or 37°C (*E. coli*).

For *C. glutamicum* growth tests, a fresh culture in BHI medium (Difco, Detroit, MI) was used to inoculate minimal medium (per liter 42 g MOPS, 20 g (NH₄)₂SO₄, 5 g urea, 0.5 g K₂HPO₄ × 3 H₂O, 0.5 g KH₂PO₄, 0.25 g MgSO₄ × 7 H₂O, 0.01 g CaCl₂, 50 g glucose, 0.2 mg biotin, 10 mg FeSO₄, 10 mg MnSO₄, 1 mg ZnSO₄, 0.2 mg CuSO₄, 0.02 mg NiCl₂ × 6 H₂O, 0.09 mg H₃BO₃, 0.06 mg CoCl₂ × 6 H₂O, 0.009 mg NaMoO₄ × 2 H₂O; pH adjusted to pH 7.0 using NaOH; [16]) for overnight growth. This culture, with an overnight OD₆₀₀ of approximately 25 to 30, was used to inoculate fresh medium containing different concentrations of either (NH₄)₂SO₄ or (Na)₂SO₄. The medium for glutamine-auxotrophic Δ*gdh*-Δ*glnA* strain TMΔ*gdh*Δ*glnA* was supplemented with 100 mM L-glutamine.

E. coli MG1655 cells were grown in medium described by Davis and Mingioli [9] containing 8.02 g K₂HPO₄ × 3 H₂O, 3.0 g KH₂PO₄, 0.3 g tri-sodium citrate × 2 H₂O, 1.06 g (NH₄)₂SO₄, 0.1 g MgSO₄ × 7 H₂O, and 5.0 g glucose per liter. An inoculation scheme similar to *C. glutamicum* was applied with an LB medium [28] over day culture, an overnight minimal medium culture, and inoculation of fresh minimal medium containing different concentrations of either (NH₄)₂SO₄ or (Na)₂SO₄.

B. subtilis 168 was grown in medium containing 15.0 g peptone, 3.0 g yeast extract, 6.0 g NaCl, and 1.0 g glucose per liter (adjusted to pH 7.5) in the presence of different concentrations of either (NH₄)₂SO₄ or (Na)₂SO₄.

Determination of methylammonium uptake. Methylammonium uptake measurements were carried out as described earlier [31]. In brief, cells grown in minimal medium were harvested by centrifugation, washed with MES/Tris buffer (adjusted with NaOH to pH 8.0), and suspended in the same buffer to a final OD₆₀₀ of 3–4. Uptake measurements were started by the addition of 100 μM or 1500 μM [¹⁴C]-labeled methylammonium. Samples were taken in 15-s time intervals over a period of 2 min. The cells were separated from the surrounding medium by rapid filtration, washed twice, and subjected to scintillation counting.

Ammonium concentration measurements. For determination of intracellular ammonium, 2 mL of a *C. glutamicum* culture (OD₆₀₀ approximately 4–5) were harvested by centrifugation. The weight of the pellet was determined, and the cells were resuspended in 100 μL of an aqueous 0.1% *N*-cetyl-*N,N,N*-trimethylammonium bromide solution.

After incubation for 10 min at room temperature, cell debris was removed by centrifugation. The ammonium concentrations of this supernatant as well as of the culture medium obtained in the first centrifugation step were determined by reversed-phase high-performance liquid chromatography (HPLC) using a protocol based on the method described by Lindroth and Mopper [21]. Ammonium was labeled by pre-column derivatization using *ortho*-phthaldialdehyde (OPA). For separation, the Agilent 1100 system (Agilent, Waldbronn) with a LiChrospher RP 18 (150 × 3 mm, 5 mm) column (Merck, Darmstadt) and 60% methanol as mobile phase with a flow rate of 0.8 mL/min at 40°C were used. Column effluents were monitored with a G1321A Fluorimeter (Agilent, Waldbronn) at an excitation wavelength of 330 nm and a detection wavelength of 455 nm.

From the ammonium concentrations measured by HPLC, the intracellular ammonium concentration was calculated. As a prerequisite for this calculation, the ratio of internal cell volume to total pellet volume had to be determined. For this purpose, the distribution of a membrane permeable probe, [³H]-labeled water, and a membrane impermeable one, [¹⁴C]-labeled taurine, was measured. These experiments revealed that 62.0 ± 1.0 % of the cell pellet consisted of surrounding medium.

General molecular biology techniques and construction of a *gdh-glnA* mutant strain. For plasmid isolation, transformation, and cloning standard techniques were used [2, 28]. Competent *C. glutamicum* cells were prepared according to van der Rest and co-workers [34]. In order to generate a *C. glutamicum* strain that is impaired in ammonium assimilation, a *glnA* deletion was introduced in *gdh* deletion strain LNΔGDH using plasmid pK18Δ*glnA* [12] and the protocol for the generation of unmarked genomic gene deletions described by Schäfer and co-workers [29].

RNA isolation and hybridization experiments. 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, Hilden). The RNA was either size-fractionated using agarose gels containing formaldehyde and blotted onto positively charged nylon membranes or spotted directly onto nylon membranes using a Schleicher & Schuell (Dassel) Minifold I Dot Blotter. Hybridization of digoxigenin-labeled RNA probes was detected with a Fuji luminescent image analyzer LAS1000 or Kodak X-OMAT X-ray films using alkaline phosphatase conjugated anti-digoxigenin Fab

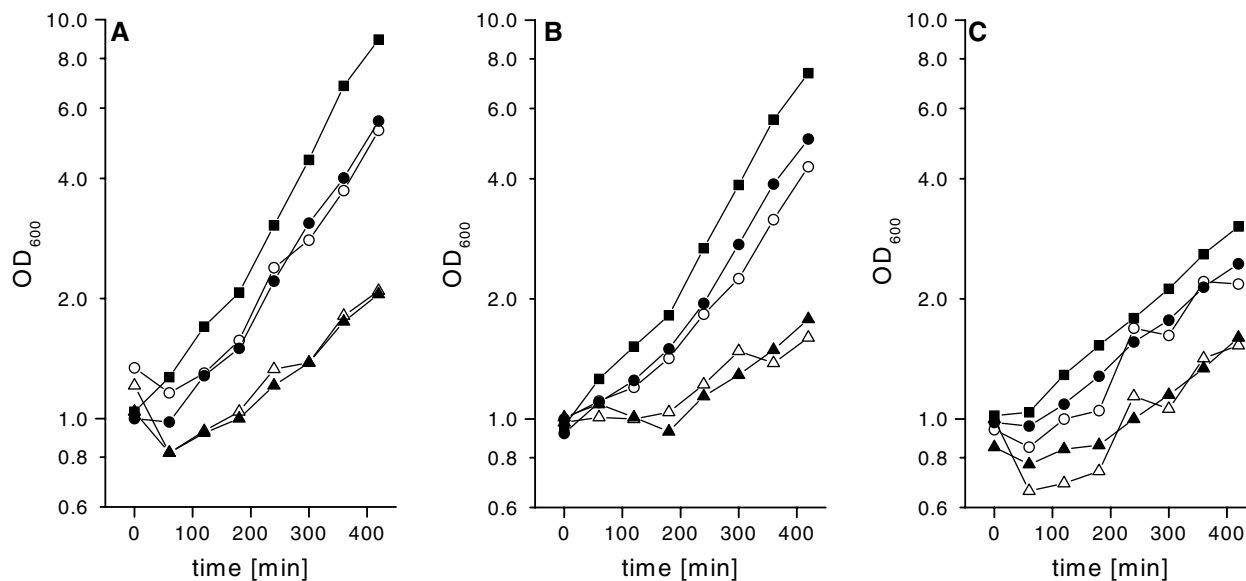


Fig. 1. Effect of increasing solute concentrations on growth of different *C. glutamicum* strains. (A) *C. glutamicum* wild-type ATCC 13032, (B) *amtR* deletion strain MJ6-18, (C) Δ *gdh*- Δ *glnA* deletion strain TM Δ *gdh* Δ *glnA*. Cells were grown in MOPS-buffered minimal medium with 0.25, 0.5, and 1.0 M $(\text{NH}_4)_2\text{SO}_4$ (closed square, circle, and triangle), 0.25 M $(\text{NH}_4)_2\text{SO}_4$ + 0.25 M $(\text{Na})_2\text{SO}_4$ (open circle), and 0.25 M $(\text{NH}_4)_2\text{SO}_4$ + 0.75 M $(\text{Na})_2\text{SO}_4$ (open triangle). The medium of Δ *gdh*- Δ *glnA* deletion strain TM Δ *gdh* Δ *glnA* was additionally supplemented with 100 mM L-glutamine. Experiments were carried out at least in duplicate. Standard errors were below 10%.

fragments and CSPD as light-emitting substrate as recommended by the supplier (Roche Diagnostics, Mannheim).

Results and Discussion

Rising ammonium concentrations impair growth of *C. glutamicum*. A prerequisite for a specific toxic effect of ammonium is its presence in the cell. Evidence for rapid flux of NH_3 across biomembranes was already obtained about 100 years ago (for review, see [18, 20]), and high NH_3 diffusion rates were determined in different bacteria, e.g., in *Bacillus firmus*, *Rhodobacter spheroides*, and *Synechococcus* R-2 [26, 27]. *C. glutamicum* has, compared to these already investigated species, a more complex cell wall structure with a mycolic-acid layer functioning as an additional permeability barrier [25].

The concentration of ammonium inside the *C. glutamicum* cytoplasm was determined. Because rapid diffusion was expected, a centrifugation assay was chosen. When the ammonium concentration in the wild type grown in minimal medium (151 mM ammonium) was tested, 142 mM ammonium was determined inside the cell, which corresponds almost to an equilibrium. *Vice versa*, a rapid diffusion of ammonia from the cytoplasm into the medium was observed, when cells were transferred from nitrogen-rich to ammonium-free medium (data not shown). Therefore, it was concluded that the *C. glutamicum* cell envelope is no barrier for ammonium

flux, and high external ammonium concentrations correspond to a high internal ammonium pool in this organism.

To test the ammonium susceptibility of *C. glutamicum*, the strains ATCC 13032, MJ6-18, and TM Δ *gdh* Δ *glnA* were chosen for growth experiments (Fig. 1A–C). Type strain ATCC13032, used as wild type control, has an intact nitrogen regulation and assimilates ammonium mainly via the glutamate dehydrogenase pathway when grown in nitrogen-rich minimal medium. Ammonium transporter-encoding genes are not expressed under the experimental conditions used. Strain MJ6-18 carries a genomic *amtR* deletion [13], which leads to the lack of the master regulator of nitrogen control, AmtR. As a consequence, genes encoding the enzymes of the glutamine synthetase-glutamate synthase pathway (GS/GOGAT) [3, 24] and ammonium carriers AmtA and AmtB [23, 31] are constitutively expressed. Double deletion strain TM Δ *gdh* Δ *glnA* is lacking the ammonium assimilation enzymes glutamate dehydrogenase and glutamine synthetase (GS I). This leads to a permanent situation of nitrogen limitation resulting in the expression of ammonium transporter-encoding genes even in the presence of externally added L-glutamine (Fig. 2). Cultures of these strains were supplemented with increasing amounts of $(\text{NH}_4)_2\text{SO}_4$ and their growth was monitored (Fig. 1A–C). The standard minimal media for *C. glutamicum* already contain between

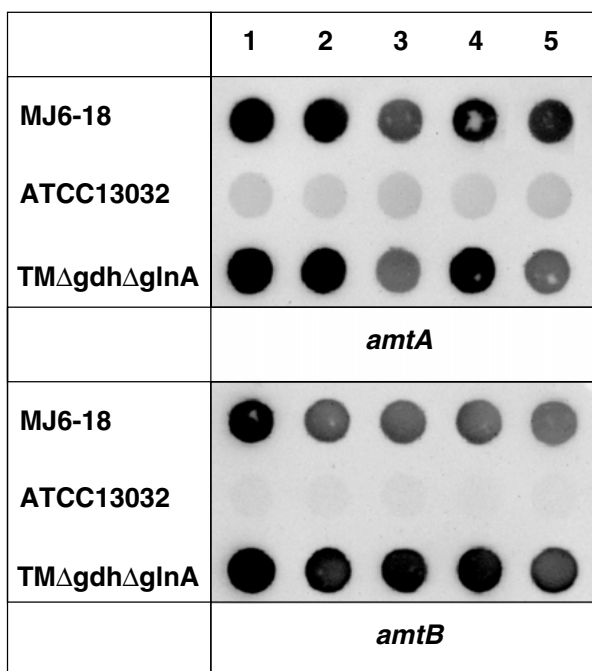


Fig. 2. Expression of ammonium transporter-encoding genes in different *C. glutamicum* strains. Equal amounts (2.5 µg) of total RNA prepared from cells grown in minimal medium with 0.25, 0.5, and 1.0 M (NH₄)₂SO₄ (1–3) and a mixture of 0.25 M (NH₄)₂SO₄ + 0.25 M (Na)₂SO₄ as well as 0.25 M (NH₄)₂SO₄ + 0.75 M (Na)₂SO₄ (4, 5) were spotted on nitrocellulose membranes and probed for *amtA* and *amtB* expression. Whereas expression of these genes is repressed by the global nitrogen regulator AmtR in the wild-type ATCC 13032, constitutive *amtA* and *amtB* transcription is observed in *amtR* deletion strain MJ6-18 and strain TMΔgdhΔglnA.

200 and 500 mM ammonium, indicating a high ammonium resistance. Thus, a slight effect on growth of the different strains was observed in the presence of 1 M ammonium (0.5 M (NH₄)₂SO₄). Addition of 2 M ammonium (1 M (NH₄)₂SO₄) resulted in a lag-phase of approximately 3 h and a decreased growth rate. Although wild-type ATCC 13032 and Δ*amtR* strain MJ6-18 showed comparable doubling times, growth of ammonium assimilation-defect mutant TMΔgdhΔglnA was always reduced. Obviously, this strain has a general growth problem even when supplemented with 100 mM L-glutamine, most likely because the relatively slow L-glutamine uptake system [30]. Nevertheless, in all three strains, concentration-dependent growth impairment in response to ammonium addition was observed.

The ammonium effect on *C. glutamicum* is the result of an increasing osmolarity or ionic strength of the medium. In principle, differences in the cellular capacity to metabolize ammonium, e.g., in the wild type and glutamine synthetase–glutamate dehydrogenase

mutant TMΔgdhΔglnA, were expected to be reflected by different abilities to cope with high ammonium concentrations. Because such strain-specific differences were not observed, we assumed that the growth retardation caused by the addition of ammonium sulfate was the result of a secondary effect, for example, of ionic or osmotic stress. In fact, adding rising amounts of sodium sulfate to the cells led to identical growth rates compared with the effect of the corresponding ammonium sulfate concentrations (Fig. 1A–C). Consequently, the growth retardation observed upon addition of (NH₄)₂SO₄ cannot be attributed to a specific toxicity of ammonium.

The effect of a putative futile cycling of ammonium is negligible in *C. glutamicum*. As indicated by the growth experiments presented above, ammonium has no specific toxic effect, independent of the genetic background of the strains used. This is astonishing, because synthesis of ammonium transporters AmtA and AmtB is deregulated in *amtR* deletion strain MJ6-18 [13]. In strain TMΔgdhΔglnA, the situation is even more extreme, because *amtA* and *amtB* are transcribed (Fig. 2) while the ammonium assimilating enzyme glutamine synthetase and glutamate dehydrogenase are lacking. This is a putative dangerous situation for the two strains, because intracellular ammonium accumulation by an uptake system may create a harmful energy-wasting futile cycle, in which energy-dependent uptake is counteracted by diffusion of NH₃ out of the cell, leading to a breakdown of the membrane potential [19].

A simple explanation for the ability of MJ6-18 and TMΔgdhΔglnA to survive exposure to high ammonium concentrations would be a downregulation of ammonium transporters at the level of activity. To study this hypothesis, transport rates were determined using the [¹⁴C]-labeled ammonium analog methylammonium. Although the wild-type ATCC 13032 and strain MJ6-18 revealed no differences in growth (Fig. 1), transport measurements revealed that ammonium uptake systems are inactive in the wild-type ATCC 13032 but active in strain MJ6-18 (Fig. 3). Although no uptake of methylammonium at all was determined for strain ATCC 13032, transport rates of 0.9 and 1.1 nmol (mg dry weight)⁻¹ min⁻¹ were determined for MJ6-18 at 100 µM and of 8.5 and 12.1 nmol (mg dry weight)⁻¹ min⁻¹ at 1500 µM [¹⁴C]-labeled methylammonium. The absence of any growth deficit of strain MJ6-18 compared to the wild type suggests that ammonium futile cycling is not occurring in the studied *C. glutamicum* strain or its putative negative effect is negligible. However, because ammonium transport was measured

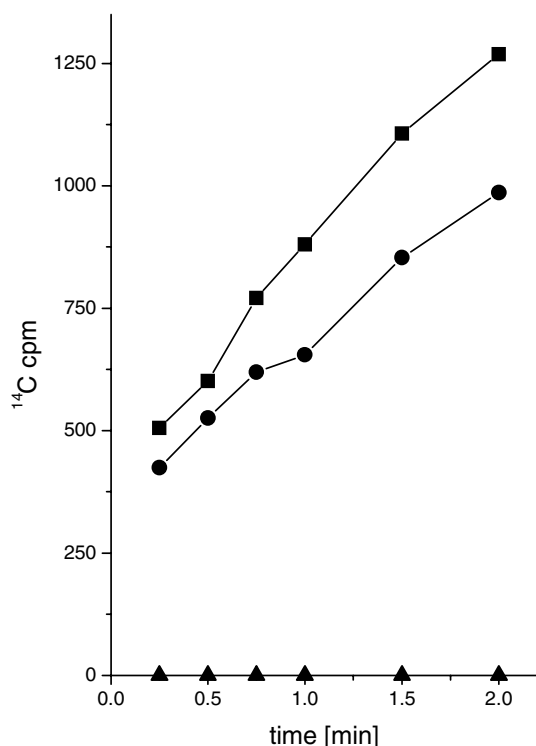


Fig. 3. Methylammonium uptake of *C. glutamicum* wild-type ATCC13032 and *amtR* deletion strain MJ6-18. Cells were grown in nitrogen-rich minimal medium, and uptake measurements were carried out in the presence of 100 or 1500 μM [^{14}C]-labeled methylammonium. Although methylammonium transport was not detectable in wild-type cells grown under these conditions (triangles), a considerable uptake was observed in *C. glutamicum* strain MJ6-18 (100 μM [^{14}C]-methylammonium: circles, 1500 μM [^{14}C]-methylammonium: squares). Experiments were carried out in duplicate with identical results.

indirectly, an activity regulation of ammonium transport cannot be excluded.

Because of the lack of the ammonium-assimilating enzymes glutamate dehydrogenase and glutamine synthetase, strain $\text{TM}\Delta\text{gdh}\Delta\text{glnA}$ expresses *amtA* and *amtB* even during ammonium surplus conditions. As a consequence, this strain showed methylammonium uptake (data not shown), but no indications for an ammonium futile cycle were detected. The interpretation in comparison to the wild type is less clear as in strain MJ6-18 because of the general growth defect of these cells.

Ammonium toxicity in *E. coli* and *B. subtilis*. To investigate whether *C. glutamicum* is especially resistant to ammonium or whether the lacking toxicity is a general phenomenon found in bacteria, the effect of high ammonium concentrations was studied in the Gram-negative model bacterium *E. coli* and in the Gram-positive model organism *B. subtilis*.

Table 2. Growth rates of *E. coli* strain MG1655 and *B. subtilis* strain 168 in medium with different concentrations of $(\text{NH}_4)_2\text{SO}_4$ and $(\text{Na})_2\text{SO}_4$

Added salt	t_D <i>E. coli</i> [min]	t_D <i>B. subtilis</i> [min]
50 mM $(\text{NH}_4)_2\text{SO}_4$	72/72	48/36
250 mM $(\text{NH}_4)_2\text{SO}_4$	66/72	48/48
50 mM $(\text{NH}_4)_2\text{SO}_4$ + 200 mM $(\text{Na})_2\text{SO}_4$	90/90	42/36
375 mM $(\text{NH}_4)_2\text{SO}_4$	132/102	84/126
50 mM $(\text{NH}_4)_2\text{SO}_4$ + 325 mM $(\text{Na})_2\text{SO}_4$	^{b/p}	78/84
500 mM $(\text{NH}_4)_2\text{SO}_4$	260/186	^{b/600}
50 mM $(\text{NH}_4)_2\text{SO}_4$ + 450 mM $(\text{Na})_2\text{SO}_4$	^{b/p}	^{b/600}

^aAll experiments were carried out in duplicate (biological replicates) and doubling times (in min) are shown.

^bgrowth was recovered after approximately 6 to 8 h lag-phase.

Standard minimal media used for *E. coli* contain ammonium concentrations in the low millimolar range. When these were increased to up to 500 mM ammonium (250 mM $(\text{NH}_4)_2\text{SO}_4$), no detrimental effect on growth was observed. Only when 750 mM ammonium was added, a decrease in growth rate was observed. As in *C. glutamicum*, this is caused by osmotic or ionic stress rather than by ammonium toxicity, as shown by the addition of sodium sulfate to cells, which led to even severe effects (Table 2). It might be speculated that the less drastic effect of ammonium compared to sodium is the result of a faster equilibration due to diffusion of NH_3 and resulting smaller osmotic gradients between medium and cytoplasm.

For *B. subtilis*, a standard complex medium was supplemented with $(\text{NH}_4)_2\text{SO}_4$. Also in this case, a specific ammonium effect was not observed (Table 2). As in *E. coli*, addition of 750 mM and 1000 mM ammonium (375 mM and 500 mM $(\text{NH}_4)_2\text{SO}_4$) impaired growth; however, similar effects were observed when $(\text{NH}_4)_2\text{SO}_4$ was exchanged against $(\text{Na})_2\text{SO}_4$.

In summary, our results indicate that ammonium is not toxic for *E. coli* and *B. subtilis*, and growth retardations observed in the presence of molar concentrations are the result of general osmotic or ionic effects as in the case of *C. glutamicum*.

Conclusions

Ammonium is in contrast to the situation in animal cells (e. g. see [22]) and plants (e.g., [4, 5]) not toxic for the studied model bacteria *C. glutamicum*, *E. coli*, and *B. subtilis*, even in molar concentrations. Because most bacteria prefer ammonium as nitrogen source and some species even produce ammonium, for example, rhizobia

and cyanobacteria by N₂-fixation and proteolytic clostridia by amino acid fermentation, we assume that ammonium resistance is a general phenomenon in bacteria.

In ammonium-rich medium, the diffusion of NH₃ across the cytoplasmic membrane is sufficient to promote bacterial growth, and wild-type cells of *E. coli* [32], *B. subtilis* [10], and *C. glutamicum* [23, 31] synthesize special ammonium transporters only in a situation of nitrogen limitation. As one reason for this strict regulation, prevention of a putative energy-wasting futile cycling of ammonium was discussed. In such a situation, NH₄⁺ would be transported into the cell by an energy-dependent uptake mechanism and would subsequently diffuse across the cytoplasmic membrane back into the medium in the form of NH₃. For *C. glutamicum*, such an effect of ammonium cycling could not be shown. Mutant strains, which showed in contrast to the wild-type methylammonium uptake during ammonium surplus in the medium, were not impaired in growth. A possible explanation of this observation is an energy-independent transport by ammonium channels, which facilitates diffusion, rather than by permeases, which concentrate ammonium inside the cell by an energy-dependent transport mechanism. This kind of transport mechanism was first suggested by Soupene and co-workers for ammonium transporter AmtB from *Salmonella typhimurium* [32, 33] and was further supported by the recently published structure of *E. coli* AmtB [17] as well as by transport data [14].

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