Potassium transport in *Corynebacterium glutamicum* is facilitated by the putative channel protein CglK which is essential for pH homeostasis and growth at acidic pH.

Running title: Potassium uptake and impact on *C. glutamicum*

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**Abbreviations:** WT: wild type; cdm: cellular dry matter
Abstract

We studied the requirement for potassium and for potassium transport activity for the biotechnologically important bacterium *Corynebacterium glutamicum* which is used for large scale production of amino acids. In difference to many other bacteria, at alkaline or neutral pH, *C. glutamicum* is able to grow without addition of potassium, resulting in very low cytoplasmic potassium concentrations. In contrast, at acidic pH the ability for growth was found to depend on the presence of K\(^+\). For the first time we provide experimental evidence that a potential potassium channel (CglK) acts as the major potassium uptake system in a bacterium and proved CglK function directly in its natural membrane environment. From the *cglK* gene a full length CglK protein and a separate soluble protein harboring the RCK domain can be translated and both are essential for full CglK functionality. As a reason for potassium dependent growth limitation at acidic pH, we identified the impaired capacity for internal pH homeostasis which depends on the availability and internal accumulation of potassium. Potassium uptake via CglK was found to be relevant for major physiological processes like the activity of the respiratory chain and to be crucial for maintenance of the internal pH as well as for the adjustment of the membrane potential in *C. glutamicum*. 
Introduction

Potassium plays an essential role for bacterial cells and is the most abundant ion in the cytoplasm. Maintenance of high internal potassium concentration and, consequently, potassium uptake is of crucial physiological significance in bacteria and is a key determinant for survival (5). Whereas in the cytoplasm of the Gram-negative bacterium *E. coli* about 400 mM potassium were detected, the Gram-positive *B. subtilis* and *C. glutamicum* contain between 300 and 800 mM potassium, respectively (9, 24, 38). The high potassium content is in general accompanied by high glutamate concentrations, which acts as counter ion (13, 24).

In general, potassium accumulation was described as a first response of bacterial cells towards hyperosmotic stress (6, 29) and a regulatory role in transcription, translation or as secondary messenger for activation of proteins was found (13). Potassium contributes to the global transcription regulation under osmotic stress conditions by interaction with the RNA polymerase and affects protein translation in *E. coli* and *Streptococcus faecalis* (12, 15, 17). In *B. subtilis* potassium uptake and accumulation was found to be essential for osmotic stress tolerance because of its impact on protein de novo synthesis (18, 37). For *C. glutamicum* it was demonstrated that potassium specifically activates the glycine betaine carrier BetP under *in vitro* conditions (31).

Beside its role during osmotic stress response in *E. coli*, potassium uptake and accumulation were found to be essential for the maintenance of internal pH as well (5). Under acidic conditions a neutral pH in the cytoplasm can be maintained only if potassium is available. In the absence of potassium the internal pH decreases in dependence of a decreased external pH and pH homeostasis fails. The mechanism of potassium dependent pH regulation and the impact of potassium on pH homeostasis in *C. glutamicum* is unknown.
For these purposes, bacteria are equipped with potassium uptake systems. The kind of potassium transport systems found in a particular organism was proposed to be related to the potassium availability in its natural habitat (34). In *E. coli* three active potassium transport systems (Trk, Kdp and Kup) as well as potassium channels (Kch, Kef) are found (13). Whereas the Trk transporter is a high capacity, constitutively expressed potassium uptake system, Kup and Kdp represent inducible, relatively low capacity uptake systems. The physiological role of potassium channels in *E. coli* is largely unknown (13). In *B. subtilis* two Trk related Ktr-type transporter systems are the major uptake carriers (18). The contribution of channels in potassium ion transport is unknown. For *C. glutamicum* the requirement for potassium and the presence and the properties of potassium carriers have not been investigated so far.

In this study we provide experimental evidence that the potential potassium channel CglK is the only functional potassium uptake system in *C. glutamicum*. We thus prove the physiological significance of potassium transport by a bacterial channel protein in its natural membrane environment for the first time. In addition, we show that under acidic stress uptake of potassium by CglK is essential for pH homeostasis in *C. glutamicum* cells.

**Material and Methods**

*Bacterial strains, growth and mutant construction*

*E. coli* DH5α mcr cells were grown in Luria-Bertani (LB) medium at 37°C and used for molecular cloning procedures. Strain ATCC 13032 served as *C. glutamicum* WT. *C. glutamicum* cells were grown either in Brain Heart Infusion (BHI) medium (Becton-Dickenson, Heidelberg, Germany) or in minimal medium MMI (21) at 30°C. Plates
were prepared by addition of 15 g l\(^{-1}\) agar to the medium. For all experiments \textit{C. glutamicum} cells were precultivated in 5 ml BHI medium for approx. 8 h and subsequently used for inoculation of 20 ml minimal medium of different potassium concentrations. After approx. 20 h the culture was used to inoculate fresh MMI medium of indicated potassium concentrations to an OD\(_{600}\) of 1-2 and experiments were started after entering the exponential growth phase. Whereas BHI contains 10 mM potassium, MMI contains 37 mM potassium. The lowest potassium concentration applied in liquid minimal medium was 10 µM and on agar plates 50 µM due to contamination by other chemicals. All strains were cultivated in Erlenmeyer flasks shaking at 130 rpm or in micro titer plates sealed with a gas permeable membrane in a volume of 200 µl shaking at 1200 rpm. The medium contained the indicated buffer substances (250 mM) in order to maintain the desired pH. If necessary, the medium was supplemented with kanamycine (25 µg ml\(^{-1}\)). Growth was followed by measuring the optical density at 600 nm (OD\(_{600}\)).

\textit{C. glutamicum} deletion mutants were constructed as described (32). Standard molecular cloning techniques were used and for amplification of flanking regions of the genes \textit{kup} and \textit{cglK} primers listed in Table 1 were applied. The mutant lacking the \textit{kup} gene (\textit{cg0187}) was named \textit{Δkup}, the mutant lacking the \textit{cglK} gene (\textit{cg0887}) \textit{ΔcglK} and the mutant lacking both \textit{kup} and \textit{cglK} was named \textit{ΔkupΔcglK}. The PCR fragments were cloned into the plasmid pDRIVE (Qiagen, Hilden, Germany) and the correct sequence was confirmed by sequencing (GATC, Konstanz, Germany). After ligation of the PCR fragments into vector pK18mobSacB \textit{C. glutamicum} cells were transformed by electroporation. After two rounds of selection transformants were obtained and deletion of the particular gene was proven by PCR. For complementation the \textit{cglK} gene was amplified by PCR using the primers indicated in Table 1 and cloned into the vector pEKEX resulting in plasmid pEKEX\textit{cglK}. CglK
variants were constructed by replacing the internal start codon at position 137 by a stop codon \( (cglK\_M137\text{Stop}) \) or by a codon for the amino acid isoleucine \( (cglK\_M137\text{I}, \text{Table 1}) \) by application of the Stratagene Quick Site Directed Mutagenesis Kit. They were confirmed by sequencing and after electroporation of \( C. glutamicum \Delta\text{kup}\Delta\text{cglK} \) cells the presence of the plasmid was proven by cultivation on kanamycine containing plates as well as by PCR. The resulting strains are listed in Table 1.

**Measurement of cell volume, internal pH, membrane potential and respiratory activity**

During the exponential phase of growth cells were harvested, washed twice and resuspended in MMI medium buffered at pH 7 (100 mM MOPS) or pH 6 (100 mM MES). Cell volumes were determined by the distribution of \( ^3\text{H}\)-labelled \( \text{H}_2\text{O} \) (0.55 mCi/l) and \( ^{14}\text{C}\)-labelled inulin (0.14 mCi/l) between the cell pellet and the supernatant \( (30) \). The membrane potential was determined by measuring the distribution of \( ^{14}\text{C}\)-labelled TPP (5 \( \mu\text{M} \) final concentration, spec. radioactivity 0.995 Ci/mol) as described \( (30) \). Processing of samples for rapid separation of extra- and intracellular fluids was performed by using silicone oil centrifugation with perchloric acid in the bottom layer \( (30) \). Internal pH was determined by measuring the distribution of \( ^{14}\text{C}\)-labelled benzoic acid (15 \( \mu\text{M} \) final concentration, spec. radioactivity 3.12 Ci/mol). Alternatively, the internal pH was determined using the pH sensitive fluorescent probe 2,7'-bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein (BCECF) as described previously \( (27) \). In short, 10 ml of a potassium free \( C. glutamicum \) suspension (pH 7.5, \( \text{OD}_{600} = 5 \)) was prepared and incubated with BCECF-AM (final conc. 1.2 \( \mu\text{M} \), Sigma-Aldrich, Germany) for 30 min at 30°C in the dark. BCECF-AM is membrane permeable and can be converted into the membrane impermeable BCECF in the cytoplasm. After removing BCECF-AM by washing, BCECF fluorescence was
measured in 1 sec intervals using a Aminco-Bowmann Series2 Spectrometer (Spectronic Instruments, Urbana, IL, USA) at 535 nm after excitation at 450 nm (pH insensitive) or 490 nm (pH sensitive). Calibration was performed by incubation of cells at external pH values between pH 5.5 and pH 7.5 in the presence of a mixture of carbonylcyanide-3-chlorophenylhydrazone (CCCP), valinomycin and nigericin (final concentrations 50, 20 and 5 µM, respectively) in order to equilibrate internal and external pH values. The change of the cytoplasmic pH was followed after addition of cells to a potassium free medium at pH 6 and subsequent addition of potassium.

Rates of oxygen consumption by *C. glutamicum* were measured with a Clark-type electrode (Oxygraph, Hansatech, Reutlingen, Germany) at 30°C in a total volume of 1 ml minimal medium pH 6 (250 mM MES). After precultivation in potassium free medium, exponentially grown cells (minimal medium, pH 7) were suspended at an OD$_{600}$ of 0.3 to 0.5. After incubation for 5 min, constant rates of oxygen consumption were observed. Subsequently potassium chloride (50 mM final conc.) was added and 5 min later valinomycin (20 µM final conc.). All measurements were performed at least in triplicate and standard deviations were calculated.

**Potassium uptake measurements**

Potassium uptake was quantified by monitoring both the external and the internal concentration by flame photometry (ELEX 6361, Eppendorf) or by measuring the initial uptake rates using $^{86}$Rb as tracer. After precultivation in BHI and MMI medium cells were washed three times with MMI medium containing 1 mM K$^+$ and 250 mM HEPPS buffer pH 8.5 and subsequently, inoculated at an OD$_{600}$ of 2 in 100 ml of the same medium. For measurement of the external potassium concentration cells were removed by centrifugation (20,000 g, 30°C, 30 s) and the supernatant was analyzed...
by flame photometry. For measuring the internal potassium concentration cells were collected on filter disks, washed with 5 ml medium and soaked dry by use of a vacuum pump. Resuspension and cell disruption was carried out by adding 2 ml cetyl-trimethyl-ammoniumbromide (CTAB, 0.1%) followed by shaking at room temperature for 30 minutes. After removal of cell debris via centrifugation the potassium concentration in the supernatant was measured. For the cytoplasmic volume 1.8 µl mg\(^{-1}\) cdm was determined as described previously (30).

For determination of initial potassium uptake rates \textit{C. glutamicum} strain DHPF (33) harboring or lacking the \textit{cglK} and/or \textit{kup} gene(s) was applied (Table 1). The cells were precultivated, washed three times and inoculated in potassium free (DHPF, DHPF\(\Delta cglK\), DHPF\(\Delta kup\Delta cglK\)) and potassium containing (100 mM) MMI medium (DHPF) at an OD\(_{600}\) of 1.3. After 12 h at 30°C cells were harvested by centrifugation, washed two times in cold buffer (25 mM NaPi, pH 7.5, 100 mM NaCl, 4°C) and suspended in the same buffer at an OD\(_{600}\) of 6. Transport assays were performed at 30°C at an OD\(_{600}\) of 3 in presence of 20 mM glucose and 2.5 mM \(^{86}\text{RbCl}\) (0.045 mCi/l). The internal accumulation of radioactive label was followed by fast filtration of 200 µl samples through glass fiber filters (APFF02500, Millipore, Schwalbach, Germany) using a manifold filtration device (FH225V, Hoefer, Holliston, USA). The samples were washed twice by 2.5 ml 0.1 M KCl and counted in a liquid scintillation counter (LS-6500, Beckmann Coulter) using Rotiszinth-Plus (Carl Roth, Germany).

\textit{CglK} expression in \textit{E. coli} and purification by Ni-NTA affinity chromatography

The \textit{cglK} gene was amplified by PCR (see Table 1 for primer sequences) cloned into the vector pET52b and expressed in \textit{E. coli} BL21 cells by addition of 1 mM IPTG (Table 1). After 5 h cells were harvested, suspended in buffer A (100 mM NaCl, 100
mM KCl, 5 µg/ml DNaseI, 15 mM imidazol, 50 mM Tris/HCl pH 7.8), broken by French press treatment and the total protein fraction obtained by centrifugation (12500 rpm, 4°C, 60 min) was subjected to batch purification using Ni-NTA-agarose beads (1 ml, Qiagen, Hilden, Germany) according the suppliers protocol. The imidazol concentration of the washing buffer was 30 mM and of the elution buffers 250 (1), 500 (2), 750 (3) and 1000 mM (4). Equal volumes (15 µl) were used for SDS-PAGE, the proteins were transferred onto a membrane and subjected to Immuno Blot analysis using a Anti-His-antibody (Qiagen).

Results

Growth of C. glutamicum is impaired at low pH in a potassium dependent manner

We first addressed the minimal potassium requirement of C. glutamicum. We grew WT cells in minimal medium at potassium concentrations from 50 mM down to 10 µM which represents the lowest potassium concentration resulting from contaminations under standard conditions (MM1 medium, pH 7). Surprisingly, the growth rate of C. glutamicum was not affected by decreasing potassium concentration even after four rounds of inoculation of potassium depleted cells into new medium in the absence of added potassium. Growth rates and final OD-values were not different from those obtained for cells supplemented with 50 mM potassium (data not shown). We concluded that, in contrast to other bacteria like Bacillus subtilis, C. glutamicum tolerates very low potassium concentrations in the medium (9, 18).

For several bacteria a particular requirement for potassium at low pH values was observed (5). Therefore, we inoculated C. glutamicum cells at different pH values on agar plates containing 50, 1 and 0.05 mM potassium, respectively. On
plates at pH 8.5, growth was independent of added potassium and cells exposed to pH 7 were only slightly affected in the presence of the lowest potassium concentration of 0.05 mM. At pH 6, growth was affected even in the presence of 50 mM potassium, and at 0.05 mM potassium no growth at all was observed (Fig. 1). These results demonstrate that *C. glutamicum* does not require external potassium under alkaline or neutral pH conditions, in difference to the situation in an acidic environment.

Subsequently, we addressed the transporter(s) which is (are) responsible for potassium uptake. Inspection of the *C. glutamicum* genome revealed the absence of well-known high capacity systems like Trk or Ktr and that of a high affinity Kdp-system. Instead, genes encoding a Kup-type transporter (*cg0817*) and a putative potassium channel (*cg0887*), respectively, were found. The putative Kup-protein in *C. glutamicum* consists of 624 amino acids, harbors 12 predicted transmembrane domains (TMD) and a C-terminal cytoplasmic domain of 181 amino acids. Its sequence is highly similar to the Kup transporter of *E. coli* (E-value e\textsuperscript{-133}), which probably functions as proton symporter and was found to be important during osmotic stress at low pH (36). The putative potassium channel, which we named CglK (*C. glutamicum* K\textsuperscript{+} channel), is a protein of 353 amino acids and is highly similar to the MthK channel of *Methanobacterium thermoautotrophicum* for which the 3D structure was solved (10). Like the MthK protein, CglK harbors two TMDs and the P-loop region contains the canonical selectivity filter sequence TVGYGD. The C-terminal region of the CglK protein contains a RCK domain which was proposed to be involved in regulation of channel opening and closing (26). For the MthK channel a homotetramer of the full length protein was observed in the crystal structure and an additional ring of four separately expressed RCK domains (19). In order to characterize the contribution of the putative transport systems Kup and CglK in
potassium uptake of \( C. \) glutamicum we constructed the mutants \( \Delta \text{kup} \), \( \Delta \text{cglK} \) and \( \Delta \text{cglK}\Delta \text{kup} \) (Table 1).

**Loss of the \text{cglK} gene causes increased pH sensitivity**

Growth of the mutants \( \Delta \text{kup} \) and \( \Delta \text{cglK} \) was studied on agar plates at different pH values. For cells lacking the \text{kup} gene no difference in comparison to WT cells was found (data not shown). Growth of the mutant \( \Delta \text{cglK} \) was also comparable to WT cells at pH 8.5 and high potassium concentration, however, at pH 7 cells of the \( \Delta \text{cglK} \) mutant required the presence of 50 mM potassium for growth. At 1 mM and without addition of potassium, growth was hardly detectable. At pH 6 cells were not able to grow even in presence of 50 mM potassium (Fig. 1). These results indicate that not Kup but CglK is of major importance for potassium dependent growth of \( C. \) glutamicum at low pH values. The mutant lacking both the \text{cglK} and the \text{kup} gene was identical to cells of the \( \Delta \text{cglK} \) mutant with respect to their potassium dependent pH sensitivity, indicating that the two transport systems can not replace each other and that the two mutations were not additive in terms of potassium dependent pH sensitivity (data not shown). These growth experiments were repeated in liquid medium and comparable results were obtained (data not shown). At a pH of 5.5 the growth rate of the \( \Delta \text{cglK} \) strain was reduced by 50% in comparison to the WT and the \( \Delta \text{kup} \) mutant, respectively, even in presence of 50 mM potassium.

**The potential potassium channel CglK is the major uptake system for potassium in \( C. \) glutamicum**

In order to study the function of the Kup and CglK protein as potassium uptake systems under physiological conditions, we set up a potassium transport assay in \( C. \) glutamicum.
*glutamicum* cultures. After depletion for potassium at pH 8.5, cells were transferred into medium containing 1 mM potassium, both internal and external potassium concentration were determined by flame photometry, and growth was also monitored. As expected at alkaline pH, no difference between WT and mutants was observed (Fig. 2). During growth (8 h) cells containing a functional *cglK* gene (WT, \(\Delta kup\)) exhausted nearly all potassium from the medium, whereas the potassium concentration was found to stay unchanged in cultures of mutants lacking *cglK* (\(\Delta cglK\), \(\Delta kup\Delta cglK\), Fig. 2). This observation proves the function of CglK in potassium transport. The \(\Delta cglK\) and \(\Delta kup\Delta cglK\) cells contained lower initial amounts of potassium (200 mM) in comparison to WT and \(\Delta kup\) cells (270 mM). In all strains the internal potassium concentration was found to decrease in the course of the experiment, indicating that potassium uptake was not sufficient to maintain the initial concentration and that the remaining potassium content was lowered due to cell proliferation. The decrease of the internal potassium concentration was faster in \(\Delta cglK\) mutants resulting in a potassium concentration of about 20 mM at the end of the growth phase (8 h). The internal potassium content of WT and \(\Delta kup\) cells, harboring the functional potential potassium channel CglK, decreased slower, consequently the final potassium concentration was about 100 mM (Fig.2). These results again show that CglK is required for potassium uptake in *C. glutamicum*. To confirm that inactivation of the *cglK* gene is responsible for the observed phenotype, we constructed a plasmid for the constitutive expression of the *cglK* gene in *C. glutamicum*. After transformation of the mutant \(\Delta kup\Delta cglK\) with the plasmid pEKEX*cglK*, these cells (\(\Delta kup\Delta cglK\) pEKEX*cglK*) were able to accumulate potassium like WT cells and consequently maintained a higher internal potassium concentration in comparison to the parental strain \(\Delta kup\Delta cglK\) (Fig. 2).
In order to prove the direct participation of CglK in potassium transport we followed the uptake of radioactively labeled rubidium (\(^{86}\)Rb), which can substitute potassium in transport assays. We determined Rb\(^+\) uptake for cells grown at 50 mM potassium or depleted for potassium, respectively. For potassium depleted cells a higher uptake rate and a higher accumulation of imported Rb\(^+\) was found in comparison to cells precultivated at high potassium concentration (Fig. 3). The double mutant lacking \(cglK\) and \(kup\) was not able to take up Rb\(^+\) after depletion of potassium during precultivation (Fig. 3). Deletion of \(cglK\) alone caused the same effect on Rb\(^+\) uptake activity after depletion of potassium, again indicating that the Kup-type transport system does not contribute to potassium uptake under these conditions (Fig. 3).

**Potassium uptake by CglK is important for energy homeostasis and cell physiology in \(C.\ glutamicum\)**

We were interested in the specific requirement of \(C.\ glutamicum\) for potassium at acidic pH. A decrease of the external pH affects the electrochemical proton potential at the cytoplasmic membrane, the proton motive force (pmf). The pmf consists of the chemical gradient for protons \(\Delta p\text{H}\) and the electric potential \(\Delta \Psi\) (inside negative) according to the equation \(\text{pmf} = -Z \Delta p\text{H} + \Delta \Psi\). A decrease of the external pH causes an increase of the pH gradient, proton influx is accelerated and could impact the pmf. In order to maintain constant pmf values, either the membrane potential or the internal pH value must be lowered. However, since the cell aims to maintain a neutral cytoplasmic pH (pH homeostasis), \(\Delta \Psi\) must be lowered and this may be achieved by potassium influx via CglK in \(C.\ glutamicum\).

In order to further elucidate the impact of potassium on membrane potential and internal pH regulation we performed pH shifts from pH 7 to pH 6 either with (50
mM) or without addition of potassium and determined the components of the pmf in C. glutamicum cells. In WT cells at pH 7 we measured under our experimental conditions an internal pH of 7.4 ± 0.02 and a membrane potential of 170 mV, resulting in a pmf of about 200 mV. Upon a shift to pH 6 in the absence of added K⁺, the membrane potential was found to increase to values of 180 to 190 mV at a significantly decreased internal pH of 6.1 (Fig. 4A). The same was found for mutants lacking the kup and/or cglK gene (data not shown). In the presence of 50 mM potassium the membrane potential was significantly lowered to 130 mV, and the internal pH was maintained at pH 7.0 ± 0.08 in the WT. In all cell lines harboring a cglK gene (Δkup, ΔcglK_pEKEXcglK) the same adjustment of ΔΨ and ΔpH was found. However, in cells lacking cglK (ΔcglK, ΔkupΔcglK) in the presence of 50 mM potassium a lower internal pH of 6.53 ± 0.03 and an almost unchanged membrane potential of 168 to 173 mV was determined (Fig. 4A). Interestingly, the resulting pmf values were in all cases maintained at approximately 200 ± 8 mV, irrespective of the cell type or the experimental condition. These results indicate that upon a decreasing external pH the adjustment towards a decreasing membrane potential as well as the maintenance of a neutral internal pH depends on the availability of potassium and on a functional CglK protein in C. glutamicum.

For validation and a direct (online) quantification of the impact of potassium on internal pH regulation, we followed the change of the internal pH by using the fluorescent pH indicator BCECF after a pH shift from 7 to pH 6 in the absence of potassium and after subsequent addition of potassium, respectively (Fig. 4B). For WT cells we observed an immediate increase of the internal pH upon potassium addition, whereas for ΔcglK mutant cells no change of the pH was observed within 5 min. This indicates that ΔcglK cells were not able to regulate the internal pH in a potassium dependent manner. The experiments were also performed with the
mutants $\Delta kup$ and $\Delta cglK$-pEKEX$cglK$, resulting in the same increase of the internal pH as observed for WT cells (data not shown). In order to prove that adjustment of the internal pH is in fact closely related to potassium uptake by CglK, we performed the same experiment as shown in Fig. 4A and simultaneously followed potassium uptake. Cells were subjected to pH 6 in the presence of 1 mM potassium, the cytoplasmic pH was measured by radioactive probes and potassium uptake was quantified. Within five minutes an increase of the internal pH by $0.22 \pm 0.02$ units was observed in WT cells whereas in $\Delta cglK$ cells no increase of the internal pH was found as was shown before using the BCECF probe (Fig. 4B). Concomitant potassium accumulation in WT cells was $225 \pm 21$ nmol K$^+$ (mg cdm)$^{-1}$, whereas $\Delta cglK$ cells accumulated negligible amounts of potassium ($0.05 \pm 0.02$ nmol K$^+$ (mg cdm)$^{-1}$) within five minutes (Fig. 4C). These results again indicate that potassium uptake by CglK is crucial for maintenance of the internal pH in $C. glutamicum$.

Furthermore, we analyzed the impact of potassium uptake at low pH values on other processes. The respiratory chain is the major energy conversion process in bacterial membranes and was supposed to be sensitive to changes of membrane potential and pH (23). We measured the oxygen consumption in WT and $\Delta cglK$ cells upon a sudden shift of the external pH. In the absence of potassium, the activity of the respiratory chain was strongly reduced in both types of cells (Fig. 5). After addition of 50 mM potassium the oxygen consumption of WT cells increased and reached a level of $94$ nmol O$_2$ (min ml OD$_{600}$)$^{-1}$, whereas for $\Delta cglK$ mutant cells the value did not change and $31$ nmol O$_2$ (min ml OD$_{600}$)$^{-1}$ were measured. When the membrane potential was decreased by the potassium ionophore valinomycin in presence of 50 mM potassium, oxygen consumption of the WT and the $\Delta cglK$ mutant was found to be $83$ and $76$ nmol O$_2$ (min ml OD$_{600}$)$^{-1}$, respectively. These results
indicate that the activity of the respiratory chain during response of *C. glutamicum* cells to acidic stress conditions depends on potassium and a functional CglK protein.

**Lack of the separate RCK protein causes growth deficiency at low pH**

The protein sequence and domain structure of *C. glutamicum* CglK was found to be similar to that of the potassium channel MthK from *Methanobacterium thermoautotrophicum* (24% identity, 41% similar, E value $7 \times 10^{-12}$). The *mthK* mRNA is translated into a full length protein and a separate soluble RCK protein from an internal start codon (aa position 107) resulting in the formation of an octameric ring structure at the cytoplasmic side of the membrane (19). In the *C. glutamicum* CglK protein a methionine is present at position 137 which could represent an alternative start position. For a protein consisting of amino acids 137 to 353 we calculated a theoretical molecular mass of 23 kDa in comparison to 38 kDa for the full length CglK protein. After cloning the full length *cglK* gene into the pET52b vector and expression in *E. coli*, protein bands of 40 kDa and 25 kDa were observed in SDS page and Western Blot analyses after purification (Fig. 6). Identification of proteins by peptide mass fingerprinting revealed for the 25 kDa protein a peptide pattern covering solely the RCK domain of CglK, whereas for the 40 kDa protein peptides covering the complete CglK protein including the membrane part were found (data not shown). This indicates that the *cglK* gene of *C. glutamicum* can be translated as full length protein and a cytoplasmic protein harboring the RCK domain only.

In order to further elucidate the function of the RCK domain in *C. glutamicum* we constructed different variants of CglK for expression in the ∆*kup*∆*cglK* mutant background. Beside the full length *cglK*, a gene was cloned encoding the membrane part of the channel only by changing Met137 into a stop codon. Furthermore, Met137 was changed into Ile in order to prevent the formation of the additional cytoplasmic
RCK protein (Table 1). The resulting *C. glutamicum* mutants were grown in liquid medium at pH 6.5, i.e. under conditions where potassium uptake was found to be essential. At potassium concentrations of 10 and 5 mM the WT and the mutant expressing the full length *cglK* gene were able to grow (Fig. 7). Mutants lacking CglK or harboring the plasmide encoding the membrane part only were either hardly able to grow (10 mM potassium) or could not grow at all (5 mM potassium). For the mutant expressing the *cglK* M137I variant we found an intermediate phenotype. The growth rates were lower than for WT cells but significantly higher than for cells lacking *cglK*. The difference was more evident at 5 mM potassium because mutants lacking *cglK* were not able to grow at this potassium concentration whereas mutants harboring CglKM137I did so. At very low potassium concentrations all strains were impaired in growth and only for WT cells significant growth was observed. These results indicate that the loss of *cglK* can be fully complemented by expression of plasmid encoded full length *cglK* and partially by *cglK* M137I demonstrating the requirement of the cytoplasmic RCK protein for functionality of the potential channel CglK in *C. glutamicum*.

**Discussion**

In contrast to many other bacteria *C. glutamicum* tolerates potassium deficiency and low internal potassium concentrations

Surprisingly, we observed growth of *C. glutamicum* at neutral or alkaline pH values in the absence of added potassium, even after several rounds of inoculation in nearly potassium free medium. In WT cells grown under potassium supplementation, cytoplasmic potassium decreased from 300 to 100 mM upon growth in the presence of 1 mM external potassium, which was almost completely exhausted by the cells.
Upon growth in the presence of 10 µM potassium the internal potassium concentration dropped to about 20 mM. Consequently, internal potassium can vary in *C. glutamicum* from 800 mM (24) down to 20 mM, or even lower values during continuing growth under potassium limitation. This represents a reduction to 2.5% or even lower amounts in comparison to the usual potassium content in *C. glutamicum*. *E. coli* cells contain up to 450 mM potassium but are not able to grow in the presence of internal potassium concentrations lower than 150 mM (9). In *B. subtilis* 300 mM potassium was found as cytoplasmic concentration (38). However, in spite of the presence of highly effective Ktr-type potassium uptake systems, at external potassium concentrations below 0.4 mM no growth was observed (18). We conclude that, in contrast to *E. coli* and *B. subtilis*, *C. glutamicum* tolerates potassium limitation because it can cope with very low internal potassium content at neutral or alkaline pH. In *Klebsiella pneumoniae* and *Bacillus steathermophilus* a comparable phenotype was observed (7). In both strains the potassium content decreased drastically at higher pH values and *B. steathermophilus* was able to grow in the absence of added potassium (10 µM) at a pH of 8.5. Under these conditions, the internal potassium content was decreased to 1.4% of that observed in cells growing at 0.5 mM external potassium. However, the presence of ammonium was required, indicating that potassium can be replaced by ammonium in a pH dependent manner (7). This could be the case in *C. glutamicum* as well. As a matter of fact, we found that growth of the mutant Δ*cglK* on agar plates or in liquid medium in the absence of ammonium chloride was much more potassium dependent (data not shown).

**Regulation of CglK depends on the RCK domain**

Based on the high sequence similarity of the CglK and the MthK protein and on the fact that we detected two different proteins after expression of the *cglK* gene in *E.
coli, we assume that the functional potential potassium channel CglK consists of four full length proteins and four additional soluble proteins harboring the RCK domain. The selectivity filter sequence of CglK resembles that of MthK (TVGYGD) indicating its function as a specific potassium pore (19). For regulation of MthK activity, binding of Ca\(^{2+}\) ions at Glu210, Glu212 and Asp184 of the RCK domain was described and the dynamic oligomerization of the soluble RCK proteins in dependence on Ca\(^{2+}\) binding was proposed (19, 26). In *E. coli* cells, however, MthK function was shown to be Ca\(^{2+}\) independent indicating that other parameters may be important under in vivo conditions (28). The eukaryotic SloI potassium channel contains a RCK domain as well, is activated by low internal pH values in the virtual absence of Ca\(^{2+}\), and histidine residues were proposed to participate in this process (3). In spite of the high sequence similarity of MthK and CglK the residues responsible for Ca\(^{2+}\) binding in MthK are not conserved in the CglK sequence. However, three histidine residues are present in RCK domain of CglK at positions 140, 246 and 308 and might be involved in internal pH sensing.

Upon a decrease of the external pH, protons enter the cell along their electrochemical gradient, and this is likely to activate CglK via the RCK domain. The impact of the RCK domain for CglK function at low pH in *C. glutamicum* was proven (Fig. 7) and resembles observations made for the HpKch potassium channel of *H. pylori*. A HpKch variant lacking the separate RCK protein was, on the one hand, found to be more sensitive towards potassium limitation than the WT, but less than a mutant lacking the complete HpKch channel, on the other (34). Further studies are required in order to unravel the mechanism of signal perception by the *C. glutamicum* RCK domain and the signal transduction to the membrane anchored pore.
Potassium transport via CglK is essential for *C. glutamicum* acidic stress response

For a number of bacteria from different habitats it has been shown that at acidic pH values the presence of K$^+$ is required for maintenance of a neutral internal pH, amongst others for *Lactococcus lactis* (22), *E. coli* (25), *Enterococcus faecalis* (4), *Bradyrhizobium* sp. Strain 32H1 (14), *Rhodobacter sphaeroides* (2) and *Streptococcus mutans* (8). This also holds true for *C. glutamicum*. At pH 6 in the medium the internal pH of WT cells was found to be pH 7 in the presence of K$^+$ and was strongly decreased to pH 6.2 in the absence of K$^+$. Growth experiments at different pH values in the absence of potassium confirmed its importance under acidic conditions. At pH 6, addition of at least 1 mM K$^+$ was necessary to support growth. In the presence of sufficient K$^+$ (50 mM) the $\Delta$cglK mutant grows at alkaline and neutral pH similar to the WT, but growth was significantly impaired at acidic pH. We demonstrated that the loss of K$^+$ transport impaired the capacity for pH homeostasis. This proves that the K$^+$ dependent pH homeostasis in *C. glutamicum* is mediated by CglK which is therefore essential for growth at acidic conditions.

Measurement of the components of the proton motive force revealed a direct correlation between the adjustment of the internal pH value and the membrane potential in *C. glutamicum*. A decreased pH gradient at pH 6 during potassium limitation or in mutants lacking the cglK gene was correlated with an increased membrane potential. After addition of potassium a significant pH gradient could be established by cells harboring the cglK gene and the membrane potential was found to be decreased concomitantly. The extent of $\Delta$pH and $\Delta\Psi$ adaptation was complementary resulting in constant pmf values of approximately 200 mV. Due to this correlation between adjustment of $\Delta\Psi$ and increase of cytoplasmic pH, it is likely that lowering of the membrane potential may be required for effective pH
homeostasis as suggested previously for *E. coli* (25). A sudden decrease of the external pH causes an increase of ΔpH and thereby an increase of the electrochemical proton potential. Consequently, the driving force for the influx of protons is increased which results in a decreased internal pH. WT cells are able to respond by decreasing the membrane potential by the influx of positively charged potassium ions via the potential channel CglK thus keeping the electrochemical proton potential constant. Regulation of ΔΨ and maintenance of an internal pH close to neutral are mandatory for many physiological processes like the activity of the respiratory chain. On the other hand, in the absence of potassium or in mutants lacking CglK, ΔΨ cannot be decreased, the inwardly directed electrochemical proton potential is increased and, as a consequence, the internal pH decreases and causes limitation of growth or cell death.

**Equipment with potassium transporters is different in apathogenic and pathogenic representatives of the Actinomyces**

*C. glutamicum* possesses the potential MthK-type channel CglK as the sole functional potassium uptake system. At least under the conditions tested, the Kup-type transporter is not functional. In other *Actinomyces*, homologs of CglK are present, e.g. *C. jeikeium*, *C. diphtheriae*, *C. efficiens* and *Mycobacterium tuberculosis*. Beside a potential channel, *C. efficiens* possesses a Kup-type protein like *C. glutamicum*. In the genome sequences of *C. jeikeium* and *C. diphtheriae* additional Ktr-type transporter encoding genes are present, and in the genomes of *C. jeikeium* and *M. tuberculosis* Kdp-type transporter subunits are encoded as well. In conclusion, the apathogenic strains *C. efficiens* and *C. glutamicum* harbor a potential potassium channel as well as a Kup-type transporter encoding gene, whereas pathogenic strains possess various active carriers in addition. A correlation of the
bacterial life style and the occurrence of different potassium transport systems was proposed already previously (34). The multiplicity of active potassium transport systems could represent a prerequisite for pathogenic Actinomyces to survive under potassium limiting and/or acidic conditions during host infection, an ability, not necessary for the soil bacterium C. glutamicum.

Acknowledgement

We would like to thank Anja Wittmann for excellent technical assistance, Henrik Strahl and Evert Bakker for helpful discussions and the Bundesministerium für Bildung und Forschung (BMBF) as well as Evonik-Degussa for financial support (SysMap program).

References


Table 1: Strains, plasmids and primers used in this study.

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* Abbreviations: Ap⁺, ampicillin resistance; Km⁺, kanamycin resistance

** letters in bold indicate the recognition site for the restriction enzyme given with the primer sequence, underlined letters indicate

the codon ATG at position 137 that was changed in order to modify the protein sequence of CglK from Met137 to Ile (ATC) or Stop (TAG), respectively.
Figure Legends

Fig. 1: Potassium dependent growth of C. glutamicum WT and ∆cglK mutant at different pH values. Cells were depleted of potassium by precultivation in minimal medium without potassium supplementation and subsequently diluted to the indicated OD values. Four µl of each cell suspension was dropped on agar plates with adjusted pH values and potassium concentrations. After 48 h of incubation results were documented by photography.

Fig. 2: Growth and Potassium uptake by C. glutamicum WT (circle), ∆kup (triangle down), ∆cglK (square), ∆kup∆cglK (diamond) and ∆kup∆cglK pEKEXcglK cells (triangle up). Cells were washed and suspended in MMI medium containing 1 mM potassium at a pH of 8. Subsequently, growth was followed by measuring OD_{600} (A) and the external (B) as well as internal (C) potassium concentrations were determined.

Fig. 3: Initial rates of potassium transport in C. glutamicum DHPF, (circles) DHPF∆cglK (squares) and DHPF∆kup∆cglK (triangles). Cells were depleted of potassium during precultivation (open symbols) or grown at standard potassium concentration of 50 mM (filled symbols). Potassium uptake was followed by the accumulation of ^{86}Rb as radioactive tracer.

Fig. 4: Impact of potassium on energetic parameters in C. glutamicum. (A) Steady state values of the pmf (black bars) were determined after a shift from pH 7 to pH 6 by measuring membrane potential ∆Ψ (light grey bars) and ∆pH (dark grey bars) in WT, ∆cglK and ∆cglK pEKEX_cglK cells in presence (+, 50 mM) or absence (-, 10
µM) of potassium. All values are given in mV and the internal pH is indicated at the right axis. (B) The dynamic change of internal pH was followed for five minutes by application of the pH probe BCECF in cells of the WT (black symbols) and mutant ΔcglK (grey symbols) at an external pH of 6 before and after addition of 150 mM potassium at 70 sec. (C) Potassium accumulation of WT (black bar) and ΔcglK cells (grey bar) after five minutes incubation at pH 6 in presence of 1 mM potassium.

Fig. 5: Dependence of respiration in *C. glutamicum* on potassium. Potassium depleted cells of the WT (white bars) or the mutant ΔcglK (grey bars) were subjected to a shift of the external pH from 7 to 6 in the absence or presence of potassium. Directly after the pH shift, respiration activity was determined as oxygen consumption rate and the potassium ionophore valinomycin was added subsequently.

Fig. 6: Co-purification of the full length CglK protein and the Rck subunit by Ni-NTA affinity chromatography. The *cglK* gene was cloned into the vector pET52b mediating the expression of C-terminal penta-His-tagged proteins in *E. coli* BL21 cells. During batch purification samples were taken for input (I), flow-through (FT), washing (1-3) and elution (1-3) fractions and subjected to SDS-PAGE, blotted and analyzed by Immuno blot using a His-antibody. Bands representing the CglK full length protein and the separately translated Rck subunit are indicated by arrows. (M: Marker)

Fig. 7: Dependence of CglK function on RCK. (A) Schematic representation of CglK protein variants by two subunits of the full length protein harboring the membrane anchored pore region (black stick), the RCK domain (grey ball) and the separate RCK domain (light grey ball) present in WT (black bars), strain ΔkupΔcglK (dark grey bars), strain ΔkupΔcglK pEKEX_cglK (light grey bars), strain ΔkupΔcglK pEKEX_cglK M137Stop (white bars) and
strain $\Delta kup\Delta cglK$ pEKEX$_{cglK}$ M137I (stripped bars). All strains were inoculated in minimal medium at pH 6.5 containing the indicated potassium concentrations in MTP plates at a cell density of (0.1). Growth was followed for 6 h hours and growth rates were determined (B).