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Impact of improved potassium accumulation on pH homeostasis, membrane potential adjustment and survival of *Corynebacterium glutamicum*

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ABSTRACT

Metal ion uptake is crucial for all living cells and an essential part of cellular bioenergetic homeostasis. In this study the uptake and the impact of the most abundant internal cation, potassium, were investigated in *Actinobacteria*, a group of high G+C Gram-positives with a number of prominent biotechnologically and medically important members. Genome analyses revealed a variety of different potassium uptake systems in this monophyletic group ranging from potassium channels common in virtually all *Actinobacteria* to different active carriers that were present predominantly in pathogenic members able to cope with various stress conditions. By applying *Corynebacterium glutamicum* as model system we provide experimental evidence that under optimal conditions a potassium channel is sufficient in bacteria for the maintenance of internal pH and membrane potential ensuring survival of cells under stress conditions. Under potassium limitation, however, viability of *C. glutamicum* was increased under acidic stress or during desiccation when a functional KtrAB potassium transporter from the pathogen *Corynebacterium jeikeium* was heterologously expressed. We provide experimental evidence that the KtrAB mediated enhanced potassium accumulation improved maintenance of internal pH and membrane potential. The results indicate that the occurrence of active potassium transport systems correlates with an improved potassium-dependent bioenergetic homeostasis and survival of bacterial cells under stress conditions.

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1. Introduction

Ion homeostasis is an essential part of life. Since the cell membrane is impermeable for ions, transport systems are mandatory for ion uptake and extrusion and carrier proteins regulate the internal ion content. The alkali metal ion potassium plays an outstanding role in this respect [1]. With concentrations between 0.1 and 0.6 M potassium is the most abundant cation in bacteria, while in general only traces are available in the environment (0.1–10 mM). It is involved in membrane potential adjustment and required for the activity of the respiratory chain [2]. Potassium acts as second messenger for stress signalling and as regulatory element for transcription control as well as counter ion for glutamate and other compounds during osmotic stress response [3]. The impact of potassium for bacterial life is indicated by the diversity of transport systems present for this particular ion. In many enteric bacteria primary active P-type ATPases of the Kdp-type are present in addition to secondary carriers of the Trk-type. Ktr-type systems are related to Trk transporters and are present in many Gram-positive bacteria. A third type of uptake system is the secondary Kup-type transporter that is present in many Gram-positive and Gram-negative bacteria. All secondary active carriers are supposed to use the membrane potential for uptake of potassium [3]. Additionally, several ligand-gated channel proteins are involved in potassium uptake [4,5].

The distribution of potassium transport systems in bacteria was proposed to be correlated with the potassium availability in their natural habitat [6]. Bacteria like *Escherichia coli* or *Bacillus subtilis* harbor different types of potassium carriers and maintain high internal potassium levels under both stress and standard cultivation conditions [7,8], while *Corynebacterium glutamicum*, *Klebsiella pneumoniae* and *Bacillus stearothermophilus* do not require potassium under optimal growth conditions and can be propagated in its virtual absence [1,2]. However, under acidic stress *C. glutamicum* accumulates high cytoplasmic potassium concentrations as well. A Kup-type transporter and a potassium channel encoding gene were found in the *C. glutamicum* genome, respectively. Interestingly, no activity was observed for the secondary active potassium carrier Kup and the potassium channel CglK was found to represent the main uptake system under standard conditions [2]. CglK represents the first example of a bacterial potassium channel for which the activity was proven in its natural membrane environment. Additionally, the analysis of CglK in *C. glutamicum* unravelled for the first time the physiological function of a bacterial potassium channel, namely, the

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decease of the membrane potential and the maintenance of a neutral internal pH under conditions of acidic stress. The CglK channel is sufficient for potassium accumulation in presence of high potassium concentrations or at conditions of low potassium demand. However, if the potassium availability is limited or the requirement for potassium is increased the sole presence of a potassium channel might restrict growth and consequently active carriers might be required [6]. Taxonomically, *C. glutamicum* belongs to the *Actinobacteria* that are widespread in nature and live as soil bacteria, saprophytes, members of the microflora of humans or animal and plant pathogens. Medically important members are *Mycobacterium tuberculosis*, *Nocardia farcinica*, *Propionibacterium acnes*, *Corynebacterium diphtheriae* and *Corynebacterium jeikeium*, while *C. glutamicum*, *Corynebacterium efficiens*, *Bifidobacterium longum* as well as *Streptomyces coelicolor* are important in biotechnological processes [9].

In this study we analyzed the monophyletic group of *Actinobacteria* regarding the equipment with potassium transport systems. We show that all pathogenic strains, supposed to be exposed to challenging environmental limitations and stress conditions, harbor active potassium transport systems in addition to potassium channels. We addressed the impact of the mode of potassium transport by active carriers and/or channels on growth and survival from a bioenergetic point of view. Improvement of potassium transport of *C. glutamicum* harboring a functional potassium channel by additional presence of the transporter KtrAB from *C. jeikeium* led to an increased potassium content and was beneficial for the maintenance of membrane potential and internal pH accompanied by improved growth and survival of *C. glutamicum* under stress conditions.

2. Materials and methods

2.1. Bacterial strains, growth conditions and construction of mutants

C. glutamicum strain ATCC 13032 served as wild type and was grown either in brain heart infusion (BHI) medium (Becton-Dickenson, Heidelberg, Germany) or in minimal medium MMI [10] at 30 °C in Erlenmeyer flasks shaken at 130 rpm or in microtiter plates sealed with a gas-permeable membrane in a volume of 200 µl shaken at 1200 rpm. Plates were prepared by the addition of 15 g l^{-1} agar to the medium. For all experiments precultivation was performed as described [2] and experiments were performed with cultures entered the exponential growth phase. Whereas BHI contains 10 mM potassium, MMI contains 37 mM potassium. The pH was adjusted by appropriate buffer substances (250 mM) as described [11]. If necessary, the medium was supplemented with kanamycin (25 µg ml^{-1}) and for induction of expression with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Growth was followed by measuring the OD_{600}. Standard molecular cloning techniques were applied using *E. coli* DH5αMCR cells grown in Luria-Bertani (LB) medium at 37 °C.

For amplification of the *ktr* locus of *C. diphtheriae* ISS3319 or *C. jeikeium* K411, primers listed in Table 1 were applied. The genomic fragments, harboring both *ktrB* and *ktrA* as well as 500 bp upstream of *ktrB*, including the putative promoter region and the ribosome binding site (Fig. S1), were cloned into the plasmid pDRIVE (Qiagen, Hilden, Germany) and the sequence of the resulting vectors pDRIVE_*ktrBA_Cd* and pDRIVE_*ktrBA_Cj* was confirmed (GATC, Konstanz, Germany). The PCR primers were designed in order to introduce a Strep-tag coding sequence at the 3′ end of the *ktrA* gene. Subsequently, the *ktrBA* fragments were cloned into the vector pEKEX2 mediating the IPTG inducible expression of genes under the control of the lac promoter [12], resulting in plasmids pEKEX2_*ktrBA_Cd* and pEKEX2_*ktrBA_Cj*. After electroporation of the *C. glutamicum* cells, the presence of the plasmids was proven by cultivation on kanamycin-containing plates, as well as by PCR. The resulting strains are listed in Table 1. Additionally, expression of the Strep-tagged KtrA protein was proven by Western blot analysis as described previously [2] using a Strep tag antibody (Sigma).

2.2. Measurement of external and internal potassium concentration as well as bioenergetic parameters

Monitoring of both external and internal potassium concentration was performed by flame photometry (ELEX 6361; Eppendorf, Germany) as described previously [2]. In short, after precultivation

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

Strain or plasmid	Related genotype or description[a]	Reference
E. coli		
DH5αMCR	*endA1 supE44 recA1 gyr96 relA1 deoR U169 ϕ80ΔlacZΔM15 mcrA* Δ (*mrr-hsdRMS-mcrBC*)	Grant et al. [22]
***C. glutamicum* strains**		
ATCC 13032	Wild type	Abe et al. [23]
Δ*kup*	Deletion of *kup* (*cg0187*) in ATCC 13032	Follmann et al. [2]
Δ*cglK*	Deletion of *cglK* (*cg0887*) in ATCC 13032	Follmann et al. [2]
Δ*cglK*Δ*kup*	Deletion of *kup* and *cglK* in ATCC 13032	Follmann et al. [2]
*Cg*_p	ATCC 13032 harboring plasmid pEKEX2	This study
*Cg*_p*ktrBA*	ATCC 13032 harboring plasmid pEKEX2_*ktrBA_Cj*	This study
*Cg*_p*ktrBA*	ATCC 13032 harboring plasmid pEKEX2_*ktrBA_Cd*	This study
Δ*cglK*Δ*kup*_p*ktrBA_Cj*	Δ*kup*Δ*cglK* harboring plasmid pEKEX2_*ktrBA_Cj*	This study
Plasmids		
pDRIVE	A-T cloning vector (Km^R, Ap^R)	Qiagen, Hilden, Germany
pDRIVE_*cglK_Cd*	pDRIVE harboring an internal fragment of gene DIP0724 of *C. diphtheriae*	This study
pDRIVE_*ktrB_Cd*	pDRIVE harboring an internal fragment of gene DIP1931 of *C. diphtheriae*	This study
pDRIVE_*ktrBA_Cj*	pDRIVE harboring the *ktrBA* genomic locus of *C. jeikeium*	This study
pDRIVE_*ktrBA_Cd*	pDRIVE harboring the *ktrBA* genomic locus of *C. diphtheriae*	This study
pEKEX2	*E. coli*–*C. glutamicum* shuttle expression vector (Km^R)	Eikmanns et al. [12]
pEKEX2_*ktrBA_Cj*	pEKEX2 harboring the *ktrBA* genomic locus of *C. jeikeium*	This study
pEKEX2_*ktrBA_Cd*	pEKEX2 harboring the *ktrBA* genomic locus of *C. diphtheriae*	This study
Primer[b]		
Pre500_*ktrB*_Cjeik_5`	5′-GC**CTGCAG**GGAGACTCAGCCCGTGCTGCGTTTGC-3′ (PstI)	This study
*ktrA*_CStrep_Cjeik_3′	5′-GC**GAATTC**GCCTA*TTTTTCGAACTGCGGGTGGCTCCA*GGAATCCGCAAACTTATCCAGGT-3′ (EcoRI)	This study
Pre500_*ktrB*_Cdiph_5′	5′-GC**CTGCAG**GAAACTCAGGCGGTGTTGGCGTTGCTGC-3′ (XhoI)	This study
*ktrA*_CStrep_Cdiph_3′	5′-C**GAATTC**GCCTA*TTTTTCGAACTGCGGGTGGCTCCA*-ACCAATGATCAGCCTTTCTACAGG-3′ (EcoRI)	This study

[a] Abbreviations: Ap^R, ampicillin resistance; Km^R, kanamycin resistance.
[b] Letters in bold indicate the recognition site for the restriction enzyme given in parentheses, the stop codon is underlined and the Strep tag-coding sequence is shown in italic letters.

cells were washed three times with MMI medium without potassium and then inoculated in MMI medium containing 1 mM KCl at a defined pH and an OD_{600} of 2 in 100 ml of medium. External potassium concentration was measured after removal of cells by centrifugation in the supernatant. For detection of internal potassium concentrations, cells of 2 ml culture were collected by centrifugation and the pellet was washed twice with potassium-free MMI. Cell disruption was carried out by ultrasonication 80% (Sonorex Digital DK 512 P; Badelin, Berlin) at 85 °C in 1.7 ml H_2O for 20 min. After centrifugation the potassium concentration in the supernatant was measured.

The cytoplasmic volume was determined by the distribution of ^{3}H-labeled H_2O (0.55 mCi l^{-1}) and ^{14}C-labeled inulin (0.14 mCi l^{-1}) between the cell pellet and the supernatant to be 1.8 µl mg^{-1} cell dry matter (CDM) as described previously [2]. 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 [2]. The membrane potential was determined by measuring the distribution of tetraphenylphosphonium (TPP) (final concentration 5 µM, specific radioactivity 0.995 Ci mol^{-1}) as described previously [2]. Internal pH was determined by measuring the distribution of ^{14}C-labeled benzoic acid (final concentration 15 µM, specific radioactivity 3.12 Ci mol^{-1}) as described [2].

Rates of oxygen consumption by *C. glutamicum* cells were measured by 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) with cells harvested at the exponential phase and resuspended at an OD_{600} of 0.3 to 0.5 in MMI medium. Steady-state oxygen consumption rates were determined in absence or presence of potassium (50 mM).

2.3. Determination of pH and desiccation tolerance of C. glutamicum

For the drop assays, cells were depleted of potassium by precultivation in minimal medium without potassium supplementation. Subsequently, cultures were diluted in minimal medium without potassium to the OD values indicated. Four microliters of each cell suspension was dropped onto minimal medium agar plates with a pH of 7.5 and 7.0 (250 mM MOPS) as well as pH 6.5 and 6.0 (250 mM MES) and the indicated potassium concentrations. After 48 h of incubation, results were documented by photography. Desiccation tolerance was analyzed with cells depleted of potassium diluted in minimal medium with 5 mM potassium to an OD_{600} of 1. In a well of a microtiter plate 100 µl of each cell suspension was exposed to desiccation by shaking at 1200 rpm at 30 °C. After 24 and 48 h dried cells were resuspended in 100 µl NaP_i -puffer, pH 7.4 and dilutions were made as indicated. Subsequently, 4 µl of each cell suspension was dropped onto BHI agar plates containing 100 mM KCl. After 24 h of incubation, results were documented by photography.

3. Results

3.1. Occurrence of different potassium transport systems in Actinobacteria

Addressing the question whether the existence of active potassium transport is correlated with a particular bacterial life style, a bioinformatic analysis of (putative) potassium transport systems in *Actinobacteria* including the non-pathogenic species *C. efficiens* and *C. glutamicum* strain R and the pathogens *Corynebacterium aurimucosum*, *C. diphtheriae*, *C. jeikeium*, *Corynebacterium urealyticum* and *Corynebacterium kroppenstedtii* was performed. Outside the genus *Corynebacterium*, the closely related species *M. tuberculosis*, *Mycobacterium abscessus*, *N. farcinica*, *P. acnes* and *Clavibacter michiganensis* were included, which are prominent human or plant pathogens as well as the non-pathogenic and biotechnologically important strains *B. longum*, *Streptomyces avermitilis* and *S. coelicolor* [9]. The sequence of (subunits of) the known *E. coli* potassium transport systems Trk, Kup and Kdp, the potassium exporter KefB as well as the potassium channel Kch was applied as query beside the potassium channel KcsA of *Streptomyces lividans*. Sequences highly similar to the known potassium transport systems were employed for a cluster analysis including the sequence of the *E. coli* sodium transporter NhaA as outgroup. As seen in Fig. 1, with either KcsA or CglK all strains harbor at least one potassium channel protein, while in *N. farcinica* and *S. coelicolor* both types of potassium channels are present. In Gram-

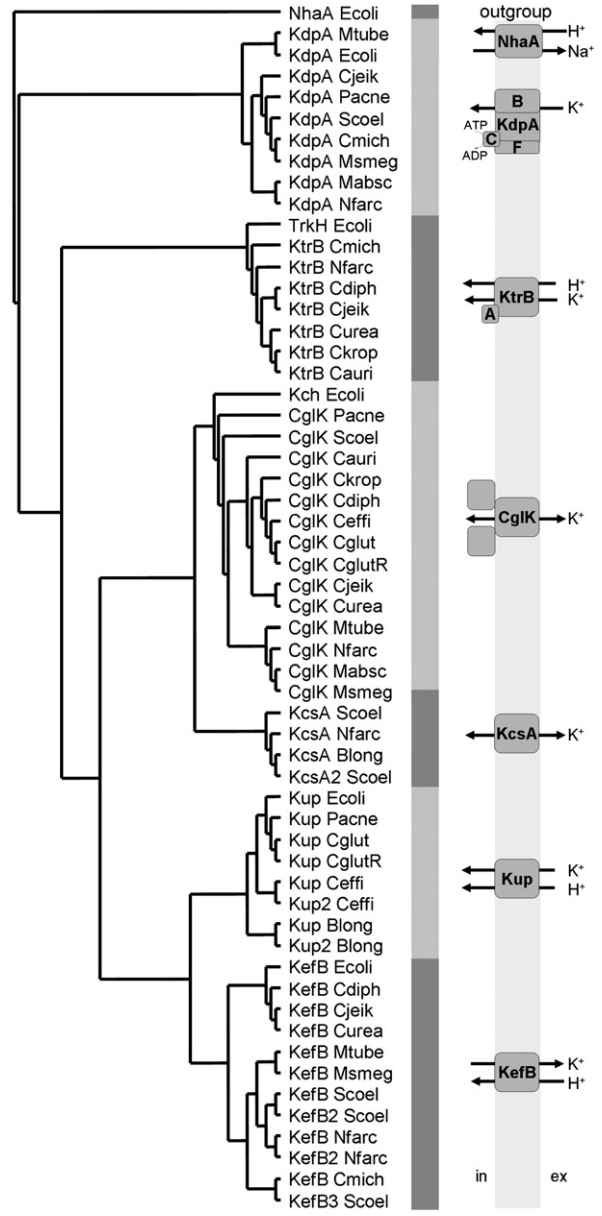

Fig. 1. Occurrence of different types of potassium transporters in *Actinobacteria*. Proteins similar to known potassium transporter subunits in *E. coli* (Ecoli, Kch, KefB, TrkH, KdpA) or *S. coelicolor* (Scoel, KcsA) were identified by genomicBlast searches against the genomes of *Corynebacterium aurimucosum* (Cauri), *Corynebacterium diphtheriae* biotype gravis strain NCTC13129 (Cdiph), *C. efficiens* strain YS-314 (Ceffi), *Corynebacterium glutamicum* strain ATCC 13032 (Cglut), *Corynebacterium glutamicum* strain R (CglutR), *Corynebacterium jeikeium* K411 (Cjeik), *C. urealyticum* DSM 7109 (Curea), *C. kroppenstedtii* DSM 44385 (Ckrop), *Mycobacterium tuberculosis* strain H37RV (Mtube), *M. abscessus* ATCC 19977 (Mabsc), *Nocardia farcinica* strain IFM 10152 (Nfarc), *Propionibacterium acnes* strain KPA171202 (Pacne), *S. avermitilis* strain MA-4680 (Saver), *S. coelicolor* strain A3(2) M145 (Scoel), *B. longum* strain NCC2705 (Blong) and *C. michiganensis* subsp. *michiganensis* NCPPB 382 (Cmich). The sequences were grouped according to a cluster analysis performed by using ClustalX and visualised by TreeView [24,25]. The sequence of the *E. coli* sodium transporter NhaA was used as an outgroup. The (putative) subunit composition and the mode of transport for each subgroup are indicated on the right.

positive bacteria homologs of the *E. coli* Trk transporter, designated Ktr in this group, and the Kdp-type uptake system are present almost exclusively in pathogenic species, whereas the Kup-type transporter is found in non-pathogenic strains predominantly. The KefB carrier is mostly present in pathogenic strains. In conclusion, non-pathogenic strains harbor potassium channels and Kup-type potassium uptake systems whereas pathogenic strains are equipped with Ktr- and Trk-type potassium uptake systems. Because pathogenic strains are exposed to numerous stress conditions like acidic pH and/or low potassium availability the presence of appropriate potassium transport systems might be beneficial for survival of pathogenic *Actinobacteria* [13,14].

We hypothesized that the exclusive presence of a potassium channel may represent a bottleneck for the maintenance of bioenergetic parameters that can be overcome by an additional active transport system. To test this hypothesis experimentally *C. glutamicum* was chosen as model system because mutants lacking particular potassium transporters are available and a variety of methods is established to address the impact of potassium transport on bioenergetic parameters and survival. Our strategy was to improve potassium accumulation in *C. glutamicum* by expression of *ktrAB* genes from *C. diphtheriae* or *C. jeikeium*. Since no expression of genes derived from *C. diphtheriae* in *C. glutamicum* was detected (data not shown) we focused on the impact of KtrAB from *C. jeikeium* on *C. glutamicum*.

3.2. Expression of functional C. jeikeium ktrBA in C. glutamicum

The Ktr transport system of *C. jeikeium* putatively consists of two subunits which are encoded by the genes *ktrB (jk0347)* and *ktrA (jk0348)*. For the KtrB subunit eight transmembrane domains, a molecular weight of 49 kDa is predicted, whereas the KtrA subunit is a soluble 23 kDa protein harboring a putative NAD-binding domain. The presence of the KtrAB system was demonstrated by detection of the C-terminal Strep-tag of the KtrA protein in cell extracts of *Cg_pktrBA*. Upon addition of IPTG to cell cultures and protein extraction a protein band of approx. 27 kDa was detected, corresponding to the Strep-tagged version of KtrA (Fig. S1).

Functionality of the KtrAB transport system was addressed by expression of *ktrBA* genes in *C. glutamicum* wild type cells and cells of the mutant lacking both potassium transporters (CglK and Kup). Under standard laboratory conditions *C. glutamicum* does not require addition of potassium and mutants lacking potassium transporters grow indistinguishable from wild type cells at alkaline pH [2]. However, potassium accumulation was shown to be crucial for survival under acidic conditions. Cells were inoculated at pH 7.5 on plates containing different potassium concentrations. While in the presence of 50 mM potassium all strains were able to grow to a comparable extent, at 5 mM potassium growth of the strain lacking all potassium uptake systems was impaired. This strain did not grow at all if no potassium was added (Fig. 5A). Growth of wild type cells was slightly decreased in presence of low potassium concentrations, but mutants harboring the *ktrBA* genes were not affected upon addition of 5 mM or if potassium was lacking in comparison to plates containing 50 mM potassium (Fig. 5A). Moreover, at very low potassium concentrations the presence of *ktrBA* genes resulted in slightly better growth in comparison to wild type cells (Fig. 5A). From these observations it was concluded that loss of potassium uptake systems in *C. glutamicum* can be complemented by the expression of the *ktrBA* operon from *C. jeikeium* indicating that the KtrAB transporter is functional in *C. glutamicum*.

3.3. Impact of ktrBA expression on potassium uptake and accumulation

As a basis for further investigations, potassium uptake and the cytoplasmic potassium concentration in *C. glutamicum* cells were analyzed by flame photometry [2]. Under optimal growth conditions (pH 8.5), external potassium was taken up by wild type cells lacking or harboring the *ktrBA* expression plasmid. Comparable rates of potassium accumulation and comparable internal potassium levels were observed in both strains within 8 h (data not shown, Fig. 2). Obviously, the contribution of the KtrAB transport system to potassium uptake in *C. glutamicum* is not significant in comparison to potassium uptake via the channel CglK under these conditions. Consequently, we performed experiments under conditions of an increased demand for potassium, namely at neutral and acidic medium pH. In comparison to the results obtained at pH 8.5 the internal potassium concentration in both *C. glutamicum* strains was maintained at higher levels at pH 7.5 and 6.5 after 8 h of incubation. Whereas at pH 8.5 the internal potassium concentration was comparable in both cell types, at pH 7.5 and 6.5 the internal potassium concentration in the recombinant strain was 170 mM and 120 mM higher than in wild type cells, respectively (Fig. 2). These findings underline the increased potassium requirement of *C. glutamicum* under neutral and acidic conditions and demonstrate that potassium accumulation in *C. glutamicum* is increased by the heterologous uptake system KtrAB of *C. jeikeium* under acidic stress conditions.

The known KtrAB transport systems require sodium ions for activity but it is unknown whether sodium activates the transporter

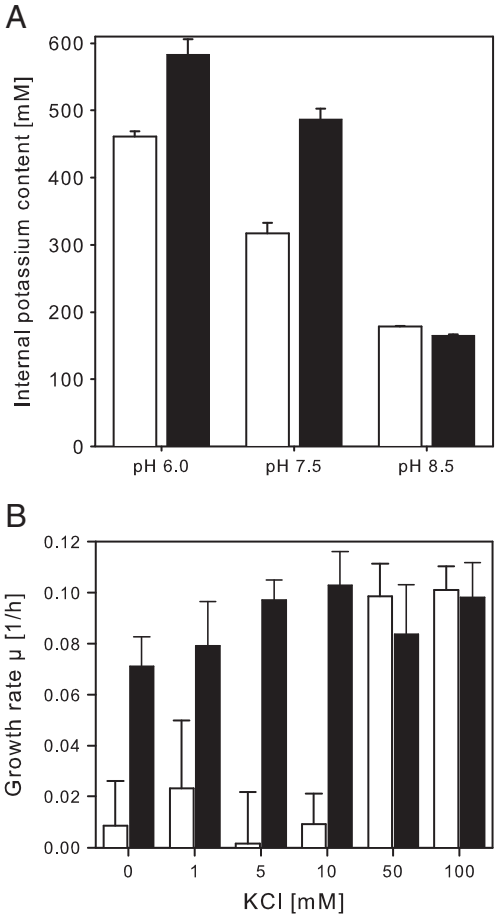

Fig. 2. Impact of *ktrBA* expression on potassium uptake and accumulation in *C. glutamicum* and sodium dependence of KtrAB function. (A) Cells of the wild type harboring a control plasmid (white bars) or the *ktrBA* expression vector (strain *Cg_pktrBA*, black bars) were inoculated in minimal medium of pH 8.5, 7.5 and 6.5 in presence of 1 mM potassium. The internal potassium content was measured by flame photometry and the values after 8 h of incubation are shown. (B) Cells of the strains Δ*cglK*Δ*kup* and Δ*cglK*Δ*kup*_p*ktrBA* were grown in liquid media at a pH level of 6.5 at the indicated potassium concentrations in absence (white bars) or presence (black bars) of sodium (no addition or addition of 100 mM NaCl), respectively, and growth rates were estimated.

or KtrAB functions as a K^+/Na^+ symporter [15]. To test the dependence of the KtrAB transporter of *C. jeikeium* on sodium, growth experiments in absence or presence of NaCl under potassium limiting conditions using the Δ*cglK*Δ*kup* mutant at low pH were performed. Whereas cells of the Δ*cglK*Δ*kup* mutant were not affected by addition of NaCl (data not shown), expression of *ktrBA* caused significantly increased growth rates of the mutant at low potassium concentrations in the presence of NaCl only (Fig. 2B). At high potassium concentrations no difference of Δ*cglK*Δ*kup* cells harboring or lacking KtrAB was found (Fig. 2B). The data indicate that the demand for potassium of *C. glutamicum* can be accomplished by KtrAB in a sodium-dependent manner pointing to the important role of sodium ions for the KtrAB function.

3.4. Impact of ktrBA expression on bioenergetic parameters

To investigate the impact of the KtrAB-dependent potassium transport on the maintenance of bioenergetic homeostasis, membrane potential ($\Delta\Psi$), internal pH and proton motive force (pmf) were measured in cells of the wild type and the Δ*cglK*Δ*kup* mutant as well as the corresponding strains harboring the KtrAB transporter under acidic stress conditions. Cells were grown in the absence of potassium in minimal medium at pH 7.5 for 3 h in order to deplete the internal potassium concentration, and subsequently the external pH was shifted to 6.0. Simultaneously, bioenergetic parameters were measured in absence of potassium or immediately after addition of 50 mM potassium. In *C. glutamicum* cells lacking the potassium transport systems CglK and Kup, a collapse of the pH gradient was observed indicated by low internal pH values of 6.3 and 6.1 irrespective of the potassium concentration (Fig. 3). Consequently, high values for the membrane potential were determined in both strains (184 and 183 mV). In contrast, wild type cells maintained a more neutral internal pH of 6.7 and a lower membrane potential (143 mV) in the presence of potassium. In its absence, however, the internal pH dropped to 6.2 and the membrane potential was found to be increased to 183 mV (Fig. 3). Expression of *ktrBA* enabled the Δ*cglK*Δ*kup* mutant and, interestingly, even wild type cells to maintain more neutral internal pH values (6.8 and 6.9) and lower values of the membrane potential (143 and 126 mV), respectively, than the parental strains (Fig. 3). The improved pH homeostasis was even more pronounced at low potassium concentrations resulting in 0.7 units higher internal pH values in *ktrBA* expression strains compared to the parental strains. For all strains comparable pmf values were observed (Fig. 3).

In order to investigate further consequences of the KtrAB-dependent improvement of pH homeostasis the activity of the respiratory chain was examined in the same experimental set up. Respiratory activity in wild type cells without addition of potassium was 38 nmol O_2 min^{-1} ml^{-1} OD_{600}^{-1} and increased by 50% in presence of 5 or 50 mM potassium (Fig. 4). For the Δ*cglK*Δ*kup* mutant the respiratory activity was significantly lower than in wild type cells (25 nmol O_2 min^{-1} ml^{-1} OD_{600}^{-1}) and did not increase upon addition of potassium (Fig. 4). In contrast, expression of *ktrBA* in the Δ*cglK*Δ*kup* mutant resulted in higher rates of oxygen consumption in comparison to the parental strain. In the wild type background, however, expression of the *ktrBA* genes did not increase oxygen consumption irrespective of the potassium concentration.

3.5. Impact of ktrBA expression on survival and growth of C. glutamicum under stress conditions

After proving the functionality of *C. jeikeium* KtrAB in *C. glutamicum* and quantifying the beneficial impact on bioenergetic homeostasis, we investigated whether growth and survival of *C.*

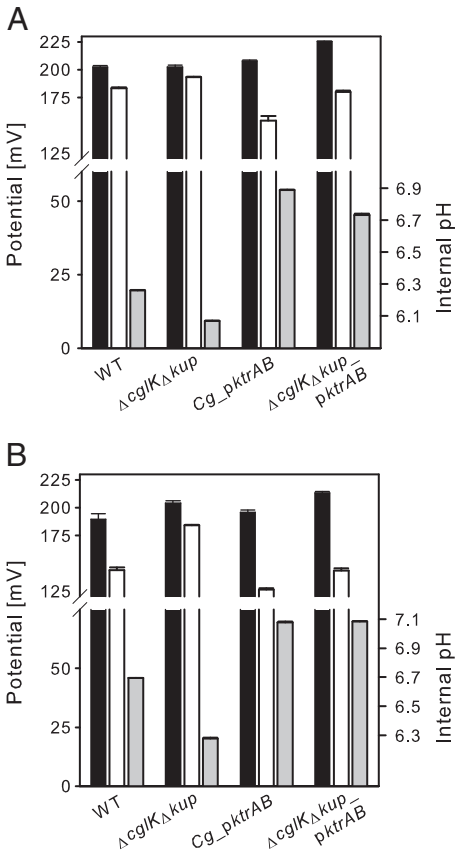

Fig. 3. Impact of *ktrBA* expression on bioenergetic parameters in *C. glutamicum*. Cells of the wild type (WT) and the mutant lacking the *cglK* and *kup* gene (Δ*cglK*Δ*kup*) as well as both strains harboring the *ktrBA* expression vector (*Cg*_p*ktrBA*, Δ*cglK*Δ*kup*_p*ktrBA*) were exposed to a pH shift in minimal medium from pH 7.5 to pH 6 without addition of potassium (A) or in presence of 50 mM potassium (B). Membrane potential (white bars) and pH gradient (grey bars) were measured and the corresponding pmf (black bars) was calculated. All values are presented as potential and are given in mV. On the right site the corresponding internal pH is indicated.

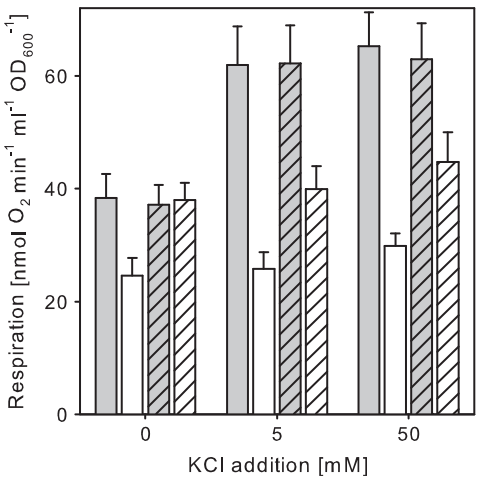

Fig. 4. Impact of *ktrBA* expression on the activity of the respiratory chain in *C. glutamicum*. Cells of the wild type (grey bars) and the mutant lacking the *cglK* and *kup* gene (white bars) as well as both strains harboring the *ktrBA* expression vector (hatched bars) were inoculated in minimal medium pH 6 in presence of the indicated potassium concentration. Activity of the respiratory chain was measured by determining the oxygen consumption rate.

glutamicum are affected in a KtrAB-dependent manner under stress conditions. *C. jeikeium* is part of the human skin flora and exposed to low pH values (around pH 5.5) and desiccation in combination with low potassium concentrations (approx. 10 mM) during colonization of this habitat. Therefore, the KtrAB-dependent pH and desiccation tolerance of *C. glutamicum* were analyzed as an example. The increased tolerance of *C. glutamicum* cells expressing the *ktrBA* genes towards neutral pH was already described and interestingly even the pH tolerance of wild type cells was increased in a KtrAB-dependent manner (Fig. 5A). At pH 6.5 even at high potassium concentrations growth of wild type cells harboring the control vector without potassium transporter genes was strongly impaired (Fig. 5C). In contrast, cells harboring the KtrAB transporter grew even at low potassium concentrations down to 1 mM. At pH 6 growth of both strains was strongly impaired (Fig. 5C). In comparison, upon ectopic overexpression of the channel encoding gene *cglK* growth of *C. glutamicum* was not improved (Fig. S2). The results demonstrate that the KtrAB transport system of *C. jeikeium* confers a higher resistance towards potassium limitation under acidic stress conditions to *C. glutamicum*.

To test the correlation between potassium transport and survival of dehydration, wild type and the Δ*cglK*Δ*kup* mutant harboring the control vector pEKEX2 or the *ktrBA* expression plasmid were exposed to desiccation in the presence of 5 mM potassium. After 24 and 48 h survival of cells was determined by re-suspending the cells and transferring 5 μl of this suspension onto BHI agar plates. The mutant Δ*cglK*Δ*kup*, lacking all potassium transport systems, was strongly impaired in survival in comparison to wild type cells (Fig. 5B). Expression of the *ktrBA* genes did complement the sensitivity of the Δ*cglK*Δ*kup* strain and improved survival of wild type cells, respectively (Fig. 5B). In conclusion, under these conditions the presence of potassium and appropriate potassium uptake systems are crucial for long-term survival and resistance of *C. glutamicum* towards desiccation stress.

4. Discussion

4.1. Potassium channels are sufficient for stress-dependent potassium uptake

The requirement for potassium under stress conditions was observed for bacteria as well as eukaryotic organisms [3]. Potassium accumulation as an immediate response upon an osmotic upshift is observed in many bacteria like *E. coli*, *B. subtilis* or cyanobacteria and was shown to be essential for survival [1,7,16]. During desiccation bacterial cells are exposed to physiological challenges comparable to osmotic stress situations [17]. In this study, we showed that a CglK mutant of *C. glutamicum* was more sensitive towards acidic conditions and desiccation than wild type cells indicating that potassium uptake is required under these conditions. Also in *Helicobacter pylori* the potassium channel HpKchA acts as sole potassium uptake system and it was demonstrated that the presence of this channel is required for the persistence of *H. pylori* in the gastric environment [6]. Consequently, supply of cells with potassium seems to be required during various stress conditions and a potassium channel is sufficient to meet the demand.

4.2. Potassium uptake represents a bottleneck for bioenergetic homeostasis

The sole presence of a potassium channel seems not to be sufficient, however, under potassium limitation as indicated by the significantly improved survival of *C. glutamicum* strains expressing *ktrBA*. A plausible reason for this limitation is the sole driving force for the CglK-dependent potassium accumulation, the membrane potential, which was determined in *C. glutamicum* wild type cells as 143 mV minimum at low pH. This facilitates a 400-fold internal accumulation of potassium according to the Nernst equation. Since the internal potassium concentration in *C. glutamicum* at acidic pH was around

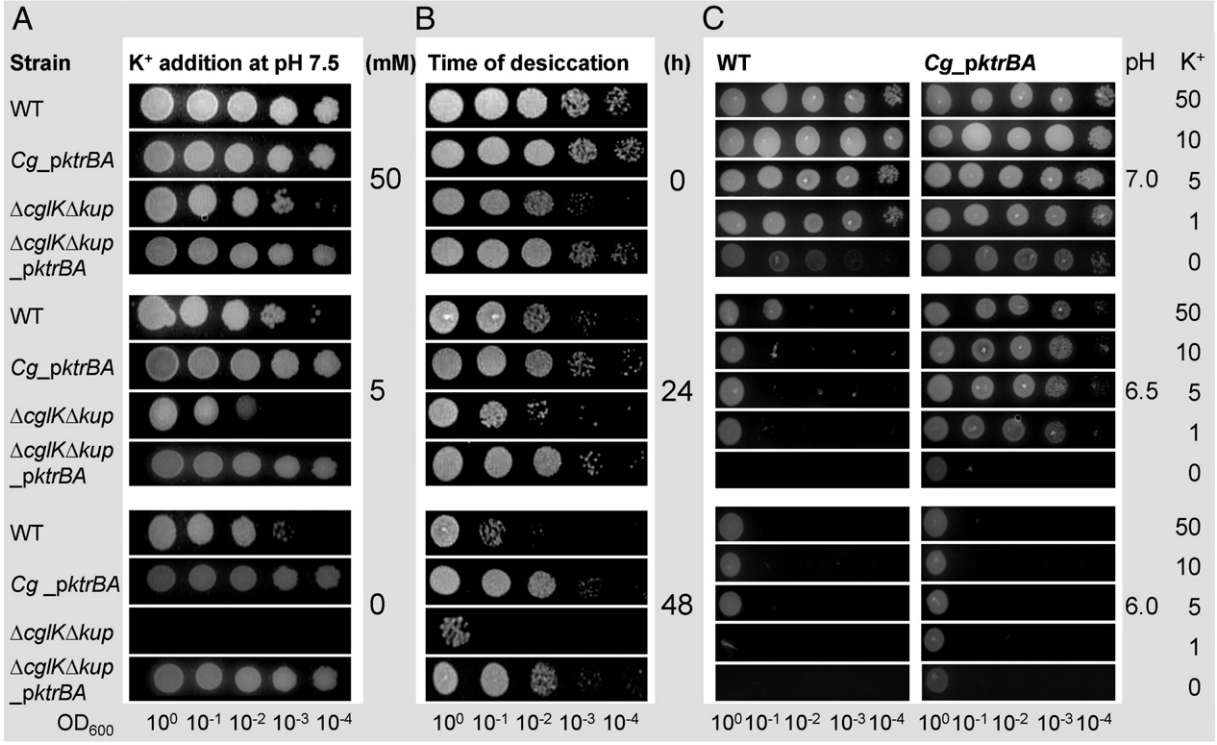

Fig. 5. Impact of *C. jeikeium* KtrAB on growth and survival of *C. glutamicum* during potassium limitation and/or acidic stress as well as desiccation. Cells of the wild type (WT), the Δ*cglK*Δ*kup* mutant and the strains *Cg_pktrBA* and Δ*cglK*Δ*kup_pktrBA*, harboring the plasmid pEKEX2_*ktrBA*, were grown on agar plates of pH 7.5 (A) supplemented with 50, 5 mM or no potassium or exposed to desiccation (B) for 0, 24 and 48 h and survival was determined by the drop assay. The potassium-dependent acidic stress resistance of wild type and *Cg_ktrBA* cells was addressed (C) by growth on agar plates of pH 7.0, 6.5 and 6.0 supplemented with 50, 10, 5, 1 mM or no potassium. The OD600 values indicate the cell density of cultures applied for the assay by transfer of 4 μl onto the plate (A, C) or exposed to desiccation (B). Upon incubation for 24 h documentation by photography was performed.

600 mM an external potassium concentration of at least 1.5 mM is required. If the potassium availability is lower, lower levels of internal potassium can be maintained causing the collapse of internal pH homeostasis under acidic stress conditions. Overexpression of the homologous potassium channel encoding gene *cglK* did not lead to improved survival under acidic conditions and did not cause an improved maintenance of bioenergetic parameters [2]. The potassium potential at the cytoplasmic membrane might be adjusted faster upon overexpression of *cglK* because more channels mediate a higher potassium flux, but at thermodynamical equilibrium the same internal potassium concentration will be achieved. Consequently, survival of cells depends on the availability of potassium and efficient transport systems are required for growth and survival [6]. This conclusion is in agreement with the exclusive occurrence of Ktr- and Trk-type potassium transport systems in representatives of the *Actinobacteria* that are supposed to be challenged by such stress conditions.

The presence of the *C. jeikeium* potassium transporter KtrAB could complement the loss of homologous potassium transport systems in *C. glutamicum* and, moreover, was found to be beneficial even for *C. glutamicum* wild type cells. Cells harboring the KtrAB transporter can accumulate higher levels of internal potassium which causes an improved homeostasis of the internal pH and the membrane potential in particular under conditions of potassium limitation. The internal pH in *ktrBA* expressing cells was kept 0.7 units higher upon application of acidic stress. A decrease of the internal pH by 0.7 units under the same conditions caused the reduction of growth rate by 50% in wild type cells [11]. This fact seems to be the basic advantage of *ktrBA* expressing cells over wild type cells. In agreement with the more neutral internal pH during acidic stress the membrane potential was found to be not increased in *C. glutamicum* cells harboring the KtrAB system and the respiratory activity can be increased in a KtrAB-dependent manner.

4.4. The presence of active potassium transport systems improves survival under stress conditions

We showed that the maintenance of bioenergetic parameters in *C. glutamicum* upon expression of the *ktrBA* genes of *C. jeikeium* was improved and this fact was correlated to the significantly enhanced growth at acidic pH and survival during desiccation stress. The increased survival of *C. glutamicum* cells harboring KtrAB was more pronounced under potassium limitation and in the presence of sodium, which is in agreement with the properties of KtrAB transporters. Interestingly, *C. jeikeium* is exposed to both types of stress conditions during colonization of the human skin. The pH on dry skin is approx. 5.5 or can drop to 5.0 in sweat and low potassium (5 mM) and high sodium concentrations (80 mM) on skin or in sweat can be found [18]. This is in agreement with the hypothesis that the KtrAB system is of relevance for acclimatization of *Actinobacteria* towards low pH and desiccation stress. In agreement, desiccation tolerance was concluded for *C. diphtheriae* which was isolated after 9 weeks of desiccation in silica gel-dried swabs. It was also shown for *M. tuberculosis* that can remain viable in dust for up to 120 days and upon storage under vegetable oils for approx. 2 years [19,20]. The comparison of potassium transport systems in these strains revealed that in contrast to *C. glutamicum* mycobacteria harbor genes encoding the high-affinity potassium ATPase Kdp and *C. diphtheriae* carries a KtrAB transport system in addition to a CglK-type channel. The correlation between desiccation tolerance and effective potassium uptake is underlined by the presence of genes encoding the KtrAB and Kdp carrier in the genome of the highly desiccation-tolerant *Deinococcus radiodurans* R1 [21].

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