Identification and functional characterization of cation/proton antiport systems in *Corynebacterium glutamicum*

Inaugural-Dissertation

zur

Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zu Köln

vorgelegt von

Anna Margarida Bartsch

aus Stuttgart

Köln

2015

Berichterstatter:	Prof. Dr. Reinhard Krämer
	Prof. Dr. Ulf-Ingo Flügge
Tag der mündlichen Prüfung:	26.11.2014

"Zwei Dinge sind zu unserer Arbeit nötig:
Unermüdliche Ausdauer und die Bereitschaft,
etwas, in das man viel Zeit und Arbeit
gesteckt hat, wieder wegzuwerfen."
Albert Einstein

Summary

Corynebacterium glutamicum is a Gram-positive soil bacterium of the actinomycetes sub-order *Corynebacterineae* and was first described in 1958 as a glutamic acid secreting bacterium. *C. glutamicum* is one of the major industrial production organisms for amino acids and related compounds. In addition, *C. glutamicum* serves as a model organism for closely related, dangerous human pathogens like *Mycobacterium tuberculosis* and *Corynebacterium diphtheriae*.

This work aimes to get a better insight into Na^+- and K^+-homeostasis in *C. glutamicum* whereas the main focus was placed on the export of the cations. Good candidates for this function are cation/proton antiporters which exchange internal Na^+ and/or K^+ with external protons. Four putative cation/proton antiporter candidates were found in the genome of *C. glutamicum*: Mrp1, Mrp2, ChaA and NhaP. Their function as Na^+/H^+- and/or K^+/H^+-antiporters was proven on a physiological and on a biochemical level.

The growth phenotype of *C.glutamicum* antiporter deficient mutants in presence of high NaCl or KCl concentrations revealed that Mrp1 and Mrp2 are the cation/proton antiporters with highest relevance during salt stress. The sole absence of Mrp1 lead to a Na^+ sensitive phenotype whereas the lack of Mrp2 caused K^+ sensitivity. ChaA and NhaP were of minor physiological importance. Antiporter deficient mutants also showed increased internal sodium concentrations ($[Na^+]_i$). Interestingly, the increase of $[Na^+]_i$ caused the reduction of the internal potassium concentration ($[K^+]_i$). It could be shown that this fact is not caused by an altered membrane potential so that the cells probably actively downregulate $[K^+]_i$. Complementation studies with a *C. glutamicum* antiporter quadruple mutant (AQM) revealed that Mrp1 and ChaA are involved in Na^+ ion export over a wide pH range of pH 6.5 to pH 9.0. The expression of *mrp2* and *nhaP* at pH 9.0 also improved growth of the mutant. In addition, Mrp1, ChaA and NhaP were able to reduce the internal Na^+ concentration in the *C. glutamicum* AQM background.

The acridine orange fluorescence assay was used for the biochemical characterization of the putative antiporters. The corresponding *C. glutamicum* genes were expressed in the antiporter-deficient *Escherichia coli* strain KNabc and everted membrane vesicles were used to determine cation/proton antiport activities. All four antiporters were able to mediate Na^+/H^+- and K^+/H^+-antiport whereas Mrp1 showed a preference for sodium and NhaP seemed to prefer potassium.

Zusammenfassung

Corynebacterium glutamicum ist ein Gram-positives Bodenbakterium aus der Actinobakterienunterordnung Corynebacterineae und wurde zum ersten Mal 1958 als ein Glutamat sezernierendes Bakterium beschrieben. In der Biotechnologie wird *C. glutamicum* zur Produktion von Aminosäuren und verwandten Produkten verwendet. Außerdem dient *C. glutamicum* als wichtiger Modellorganismus für seine human-pathogenen Verwandten wie zum Beispiel *M. tuberculosis* und *C. diphtheriae*.

Das Ziel dieser Arbeit ist die Untersuchung der Na^+- und K^+-Homöostase in *C. glutamicum*, wobei das Hauptaugenmerk auf dem Export dieser Kationen lag. Hervoragende Kandidaten für diese Funktion sind Kationen/Protonen-Antiporter, die intrazelluläre Na^+- und/oder K^+-Ionen gegen externe Protonen tauschen. Im Genom von *C. glutamicum* wurden vier putative Kationen/Protonen-Antiporter gefunden: Mrp1, Mrp2, ChaA and NhaP. Ihre Funktion als Na^+/H^+- und/oder K^+/H^+-Antiporter wurde physiologisch und biochemisch untersucht.

Wachstumsexperimente mit Antiportermutanten in Gegenwart von hohen NaCl oder KCl Konzentrationen zeigten, dass Mrp1 und Mrp2 die wichtigsten Kationen/Protonen-Antiporter unter Salzstress sind. Die alleinige Abwesenheit von Mrp1 bzw. Mrp2 führte bereits zu Na^+- bzw. K^+-Sensitivität. ChaA und NhaP spielten eine untergeordnete Rolle. Außerdem zeigten die Antiportermutanten eine erhöhte interne Na^+-Konzentration. Ein Anstieg dieser führte zu einer Abnahme des internen Kaliumgehaltes. Dies konnte nicht auf ein verändertes Membranpotential zurückgeführt werden, sodass die Zellen die interne Kaliumkonzentration aktiv runter regulieren. Komplementationsstudien mit einer *C. glutamicum* Mutante, der alle Antiporter fehlen, konnten zeigen, dass Mrp1 und ChaA am Export von Na^+-Ionen in einem pH-Bereich von 6.5-9.0 beteiligt sind. Bei pH 9.0 konnte auch die Expression von *mrp2* und *nhaP* das Wachstum der Mutante verbessern. Außerdem konnten Mrp1, ChaA und NhaP die interne Na^+-Konzentration der Mutante verringern.

Die biochemische Charakterisierung der Antiporter erfolgte in invertierten Membranvesikeln aus *E. coli* KNabc. Die entsprechenden Gene aus *C. glutamicum* wurden in diesem Stamm exprimiert und anschließend die Aktivität der Kationen/Protonen-Antiporter mit Hilfe eines Acridinorangefluoreszenz-basierten Tests bestimmt. Alle vier Antiporter waren in der Lage Na^+/H^+- und K^+/H^+-Austausch zu vermitteln, wobei Mrp1 eine Präferenz für Na^+-Ionen und NhaP für K^+-Ionen zeigte.

Table of contents

Abbreviations

$[K^+]_i$ ..... intracellular K^+ concentration

$[Na^+]_i$ ..... intracellular Na^+ concentration

AO ..... acridine orange, N,N,N',N'-Tetramethylacridine-3,6-diamine

AQM ..... antiporter quadruple mutant, *C. glutamicum* Δ*mrp1*Δ*mrp2*Δ*chaA*Δ*nhaP*

BLAST ..... basic local alignment search tool

BTP ..... bis-tris-propane, buffer

CHES ..... N-cyclohexyl-2-aminoethanesulfonic acid, buffer

DNA ..... deoxyribonucleic acid

HCl ..... hydrochloric acid

HEPPS ..... 3-[4-(2-hydroxyethyl)-1-piperazinyl]propanesulfonic acid, buffer

KOH ..... potassium hydroxide

MES ..... 2-(N-morpholino)ethanesulfonic acid, buffer

MOPS ..... 3-(N-morpholino)propanesulfonic acid, buffer

NaOH ..... sodium hydroxide

o/n ..... over night

OD_{600} ..... optical density at 600 nm

ORF ..... open reading frame

PCR ..... polyermase chain reaction

pmf ..... proton motive force

rpm ..... revolutions per minute

smf ..... sodium motive force

TAE ..... tris-acetate-EDTA

Tris ..... tris(hydroxymethyl)aminomethane

WT ..... wild type

1 Introduction

1.1 *Corynebacterium glutamicum*

Corynebacterium glutamicum is a Gram-positive soil bacterium which was first described in 1958 as a glutamic acid secreting bacterium [68]. As a member of the actinomycetes sub-order *Corynebacterineae* this bacterium has an outer membrane-like cell wall structure with mycolic acid esters [32]. Together with *Escherichia coli*, *C. glutamicum* is one of the major industrial production organisms for amino acids and related compounds [7]. With an annual market of 2.5 and 1.5 million tons of L-glutamate and L-lysine, respectively, are the most important amino acids that are produced fermentatively with *C. glutamicum* [7, 32]. L-glutamate serves as a flavor enhancer whereas L-lysine is widely used in animal nutrition. In addition, the fermentation of nucleotides became of great commerical interest because the purine ribonucleoside 5'-monophosphates guanylic acid (GMP), inosinic acid (IMP) and xanthylic acid (XMP) were found to act as strong flavor enhancers [21, 121]. Again *C. glutamicum* is the organism of choice to produce these compounds [22, 23].
Besides its importance in biotechnology, *C. glutamicum* also serves as an important model organism. Dangerous human pathogens like *Mycobacterium tuberculosis, Mycobacterium leprae* and *Corynebacterium diphtheriae* also belong to the *Corynebacterineae* and thus they are close relatives [32, 115].
C. glutamicum fulfills some important requirements. First of all this bacterium is non-pathogenic and thus easy to handle. Furthermore, it grows fast to high optical densities and is able to use a variety of different carbon sources in parallel [39, 51, 87]. In addition, a big toolbox for genetic engineering is available [69]. All this is helpful for the improvement of productions strains as well as for the identification of putative drug targets, which are, for example, transporters involved in ion homeostasis.

1.2 The need for ion homeostasis

All living cells show an unequal distribution of ions across their cell surface membrane. Ion homeostasis describes the maintenance of these ion gradients which are crucial for survial. Almost all enzymes have a very narrow pH optimum for optimal function and many of them

also need ion cofactors. Furthermore, the structure folding of proteins and also protein-protein interactions are only stable within a narrow range of ionic strength and pH. For these reasons it is important for the cell to regulate the intracellular pH, osmotic strength of the cytoplasm and the overall charge balance by means of ion homeostasis. The cell's challenge is to discriminate between the ions that are present in the cytoplasm. Those with essential roles have to be accumulated to an appropriate concentration whereas useless or toxic ions need to be excluded from the cell. This process also has to function under varying extracellular conditions, for example pH- or salt stress. In addition, the selective ion transport processes also lead to the formation of charge differences between the two sides of the membrane with an excess of negatively charged ions on the inside, known as membrane potential. The energy that is stored in the electrochemical potential of the ion gradients can be used to facilitate important processes. For example, using the electrochemical proton potential, ATP is synthesized by the ATP synthase. Furthermore, the existing ion gradients and electrochemical potentials are used for secondary transport, such as the import of nutrients or the export of toxic metabolites, against their concentration gradient, either by a symport or antiport mechanism [58, 78, 138]. Besides the maintenance of electrochemical ion potentials, pH homeostasis is of major importance. Microbes are able to colonize many different environments whose pH values range from below pH 0 (acid mine waters) up to pH 13 (soda lakes) [63, 111, 120]. In all cases, the intracellular pH has to be kept in a much narrower range and for this reason efficient pH homeostasis mechanism are required.

1.3 Importance of cations for bacterial cells

H^+, Na^+ and K^+ are the major and most important monovalent cations for bacterial cells. Like other living cells, bacteria tend to create gradients of these ions between the cytoplasm and the environment. Normally the intracellular sodium concentration is much lower than that of the surrounding medium and vice versa for potassium [25].

As mentioned earlier, a electrochemical proton potential is the driving force for several important cellular processes such as ATP synthesis and transport of toxic ions or metabolites. Furthermore, the intracellular H^+ concentration is the pH of the cytoplasm and thus is equally important to provide an appropriate intracellular environment.

Sodium ions play a very important physiological role because a number of endergonic and exergonic processes are connected to the cycling of Na^+ ions across the membrane [58]. Especially for cells living in extreme environments, e.g. marine, halophilic and alkalophilic bacteria, sodium cycling displays an essential physiological function [25, 65]. Sodium ions can not only be used for sodium-coupled energy conservation and energy transduction but also for pH homeostasis and activation of enzymes [129]. Furthermore, alkalophilic *Bacillus* spp. as well as *Vibrio alginolyticus* strains can use a sodium gradient to drive flagellar movement [25]. Often the essential role of sodium ions for growth of bacterial cells is ascribed to the function in solute uptake by Na^+/solute-cotransport. For example, *E. coli* uses Na^+ to transport proline, panthothenate and melobiose [25, 65]. The electrochemical sodium potential needed for all these processes is established by the action of Na^+/H^+ antiporters which use the electrochemical H^+ potential or by primary Na^+ pumps such as decarboxylases, ATPases or Na^+-dependent electron transport complexes [25, 65].

Although, or perhaps in particular because, sodium is the most abundant cation in natural environments, potassium represents the most abundant cation in the prokaryotic cytosol [20, 89]. K^+ ions are involved in a large variety of cellular processes. Besides its role in the control of the plasma membrane potential, potassium is required for the regulation of the internal pH value and the activation of intracellular enzymes [11, 20, 36, 44]. In addition, for *C. glutamicum* and *E. coli* it was shown that potassium is essential for growth at low external pH values [44]. Moreover, potassium can act as an osmotic solute and upon hyperosmotic stress many bacteria rapidly accumulate K^+ ions to restore their turgor pressure [26, 36, 106, 124].

1.4 Cation transporting systems

The described fundamental physiological functions of cations are largely dependent on the presence of specific uptake- and extrusion systems. *C. glutamicum* harbours several transport systems mediating efflux and influx of the cations H^+, K^+ and Na^+. The transport systems that are relevant for cation homeostasis in *C. glutamicum* are shown in figure 1.

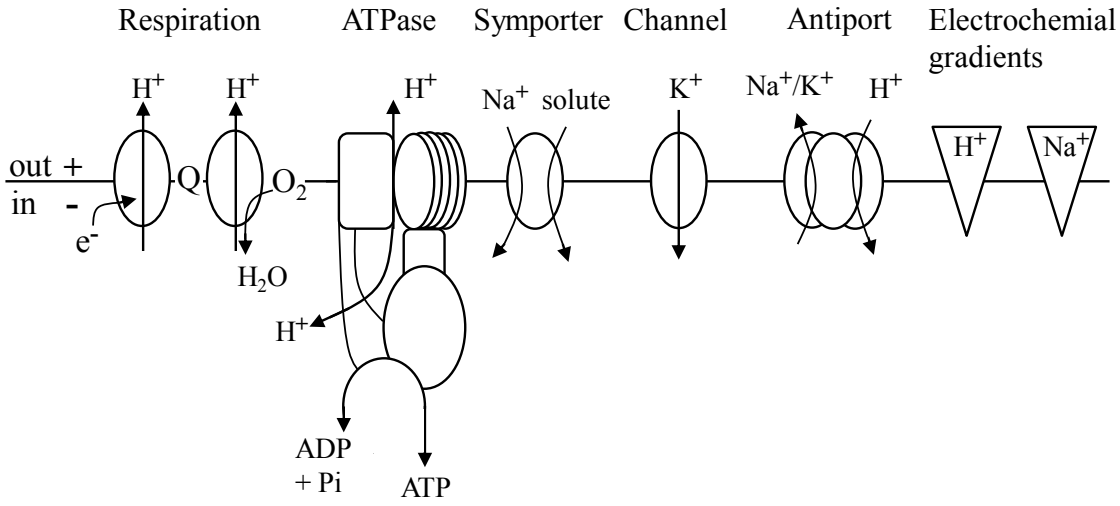

Figure 1: The major transporters that are relevant for cation homeostasis in *C. glutamicum*
The respiratory chain establishes an electrochemical proton potential at the plasma membrane. This in turn can be used to energize the synthesis of ATP by the F_1F_O-ATPase or the export of Na^+ and K^+ ions by antiporters. The main entry route for sodium is the sodium-solute symport whereas the only known way for potassium to enter *C. glutamicum* cells is a channel. In addition, the direction of the electrochemical potentials of H^+ and Na^+ ions is shown. Figure modified from [125].

1.4.1 Respiratory chain and ATP synthase

The respiratory chain plays a crucial role for bacteria. It translocates protons across the membrane and thereby establishes the primary electrochemical potential which is the driving force for many important transport-associated processes. *C. glutamicum* contains a so-called branched electron transport chain. The reducing equivalents are first transferred to menaquinone and then either to a supercomplex comprising the cytochrome bc1 complex and the cytochrome aa3 oxidase or to the cytochrome bd oxidase [12]. When using oxygen as terminal electron acceptor these three enzymes couple electron transfer to the export of protons and thus to the generation of the electrochemical proton potential. In detail, the cytochrome bc1-aa3 supercomplex exports six protons per two electrons while the bd oxidase only translocates two H^+ per electron pair.

As many other aerobic respiring bacteria, *C. glutamicum* not only gains ATP by substrate level phoysphorylation but also by oxidative phosphorylation [73]. The F_1F_O-ATPase is essential for oxidative phosphorylation and uses the electrochemical proton potential, which was established by the respiratory chain, for the generation of ATP. By fulfilling its function the ATPase transports protons along their electrochemical potential into the cell. It is thought that three to four protons are needed to form one ATP [95]. The ATPase can also work in reverse and hydrolyse ATP to export protons, however this was not shown yet for *C. glutamicum*.

1.4.2 The Na^+ cycle

As mentioned earlier, the essential role of sodium ions for bacterial cells is often ascribed to its function in solute uptake. Na^+-substrate cotransport is a common way which is used for substrate uptake in all living cells [138]. The prerequisite for this type of transport is the presence of an electrochemical sodium potential that provides energy for the transport of substrates against their concentration gradient. Na^+ ions that enter the cell are exported by Na^+/H^+ antiporters in exchange with extracellular protons. This exchange helps to maintain the sodium gradient but it also driven by the electrochemical proton potential which again has to be maintained by the H^+ ATPase as described above (Fig. 1).

1.4.3 Potassium transport systems

Bacteria are able to accumulate potassium by a number of different uptake systems. With the help of these uptake systems the intracellular potassium concentration of most bacteria can reach values of several hundred millimolar, even if the external K^+ concentration is very low [36, 84]. The adaptation to different extracellular potassium availabilities is reflected in differences in kinetics, regulation and energetic coupling [36, 123].

Potassium channels are the evolutionary oldest form of potassium uptake systems from which active carrier systems developed [28]. The transport mediated by channels is passive, which means that K^+ ions rapidly diffuse across the membrane down their electrochemical potential [27, 55, 62]. In addition to passive transport systems, there are also active potassium uptake systems present in most bacteria. One example for a primary active system is the *E. coli* Kdp system. This transporter is an inducible P-type ATPase which is only expressed when the cell needs K^+ and the uptake by other systems is not sufficient [36]. However, the stimulus for this transporter is largely unknown. Secondary active transporters are, for example, the Trk and Kup systems which are K^+/H^+ symporters [36, 124, 128]. Some bacteria also have an additional type of potassium uptake system, the secondary, Na^+-dependent Ktr [94].

In most species two or more independent saturable K^+-transport systems are present [36]. However, so far it is unknown why some strains harbour a variety of potassium uptake systems while others only have simple channels. One explanation for this difference might be the adaptation to different environments and pathogenicity [123].

Although there were some studies on potassium export, it is still only rudimentarily understood. The most completely described potassium efflux system is the *E. coli* Kef system. This system is a glutathione-gated potassium/proton-antiporter that exchanges cytoplasmic potassium with extracellular protons and protects the cell against the toxicity of electrophilic compounds [91, 113]. K^+/H^+ antiporters are ideal candidates to mediate the efflux of K^+ ions [36]. Indeed, some cation/proton-antiporters already have been found with this function, for example the *Vibrio cholerae* Mrp systems or the antiporter ChaA from *E. coli* [29, 41].
Besides *Helicobacter pylori*, *C. glutamicum* also belongs to the apparently exceptional bacteria that harbour a potassium channel as the only functional uptake system [44, 123]. The function of CglK mediated K^+ uptake is the internal accumulation of high potassium concentrations which is particular important under conditions of acidic and osmotic stress. This also includes the adjustment of the membrane potential and the internal pH [44, 99]. The genome of *C. glutamicum* also contains an ORF (*cg0187*) putatively encoding a protein which is similar to the *E. coli* Kup systems. Under all conditions tested so far, no potassium uptake function could be ascribed to this transporter [44]. There have not yet been any investigations on the export of potassium in *C. glutamicum*.

1.4.4 Cation/proton antiporters

Cation/proton antiporters play crucial roles in bacteria and their need was first postulated by Mitchell in 1961 [92]. Na^+/H^+- and K^+/H^+-antiporters are described to be involved in cytoplasmic pH homeostasis, alkali tolerance, and resistance to elevated temperature and osmolality fluctuations [101, 120]. Concomitantly they mediate resistance to toxic levels of the transported cation. As secondary active transporters, antiporters use the electrochemical proton potential across the membrane as energy source. Thus, in the case of electrogenic Na^+/H^+ antiporters that transport more protons than sodium ions, the primary electrochemical proton potential is converted into an electrochemical sodium potential which in turn can be used for sodium-substrate costransport. The genomes of most non-marine bacteria contain five to nine genes that are predicted to encode putative Na^+/H^+- or K^+/H^+-antiporters [83]. Generally it is thought that bacteria exceedingly exposed to many different types of stress harbour a higher number of antiporters than pathogenic bacteria that live inside host cells.
Based on sequence similarity, monovalent cation/proton antiporters were classified into at least eight different protein families. The two main clusters are the cation:proton antiporter (CPA)

family, sub-divided into CPA1, CPA2 and CPA3, and the Nha family, containing NhaA, NhaB, NhaC and NhaD proteins. Another family contains calcium/proton (CaCA) antiporters of which some are also able to transport Na^+ and K^+ ions. The majority of the antiporters are single gene products which in some cases form homooligomers to function [83].

On the structural level the CPA3 antiporters, also called Mrp (multiple resistance and pH-related), are the most complex type of antiporters. They are composed of six to seven different proteins that are thought to form hetero-oligomeric complexes in the membrane in which all members are required for full functionality. The according genes are organized in operons. *mrp* operons from different organisms were also named according to their function, for example pha (pH adaptation), sha (sodiumhydrogen antiporter) and mnh (multi-subunit Na^+/H^+ antiporter) [125]. The *mrp* operons can be divided into two groups: group 1 and group 2 operons (Fig. 2). Most experimental data published so far were obtained with group 1 Mrp systems. These antiporters are encoded by seven genes ordered from *mrpA* to *mrpG*, and are found in Gram-positive as well as Gram-negative bacteria. For group 2 operons there is no separate *mrpB* gene but a larger so-called *mrpA'* gene. This type of operon is dominantly found in Gram-negative bacteria. However, there are some groups of bacteria and archaea, such as enteric bacteria, lactobacilli and streptococci, that do not possess any Mrp antiporter systems [125].

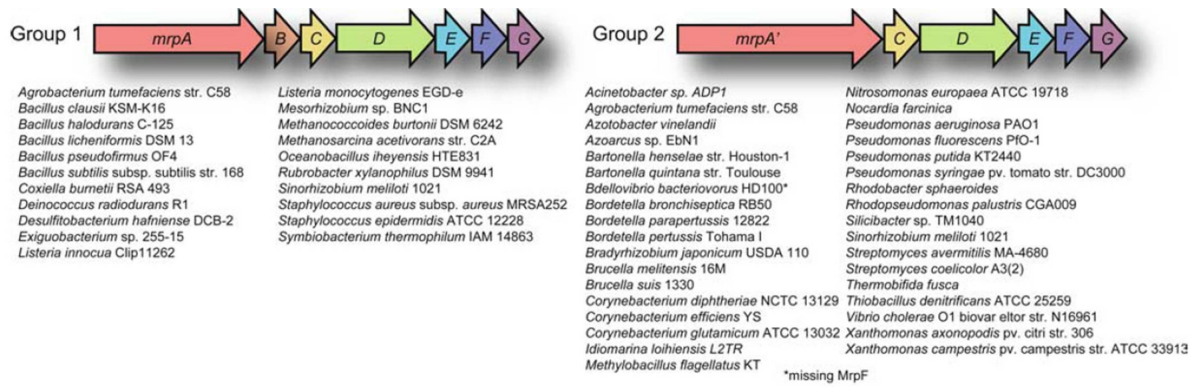

Figure 2: Gene arrangements of group 1 and group 2 *mrp* operons.
Group 1 operons include seven genes named *mrpA-G* and are found in Gram-positive and Gram-negative bacteria. Group 2 operons are dominantly found in Gram-negative bacteria and consist of six genes: the larger *mrpA'* and *mrpC-G*. Figure taken from [125].

Cytoplasmic pH homeostasis at alkaline external pH of alkaliphilic *Bacillus halodurans* was the first role described for a Na^+/H^+ antiporter from the Mrp family [54]. The import of protons supports the cytoplasmic pH homeostasis at external alkaline pH values. A *Bacillus halodurans* Mrp mutant did not grow at alkaline pH because the intracellular pH could not be retained lower than that of the medium. In addition, the Mrp system contributed to resistance to cytotoxic Na^+ [71]. Meanwhile important physiologic roles in alkali-, Na^+- and K^+-resistance have been ascribed to other Mrp systems which were found to mediate $Na^+(Li^+)/H^+$- and/or K^+/H^+-antiport [10, 54, 60, 76, 77, 105, 141].

The genome of *C. glutamicum* contains genes for two CPA3 antiporters (Mrp1, Mrp2), one CPA1 antiporter (NhaP) and one CaCA antiporter (ChaA). Interestingly, genes for antiporters of the Nha family are missing [103]. Both *mrp* antiporters belong to the Mrp group 2, which means that both are encoded by an operon of six genes (Fig. 3)[125]. The precise function of these systems in *C. glutamicum* is not understood. The assumption that they might be Na^+/H^+ antiporters is based on the observation that insertion mutants of both show a sodium-sensitive phenotype [43]. Interestingly, the *Vibrio cholerae* Mrp system, which also belongs to group 2, is able to transport potassium ions in addition to sodium and lithium ions [29]. Whether this is true for Mrp1 and/or Mrp2 from *C. glutamicum* has to be investigated. A *nhaP* insertion mutant was only briefly investigated and did not show a pH- or Na^+-sensitive phenotype [43]. A *chaA* insertion mutant was only investigated in regard to its involvement in potassium transport which could not be confirmed under the conditions tested [8]. Thus, for all four putative cation/proton antiporters from *C. glutamicum* there is not any proof for their function as cation/proton antiport activity so far.

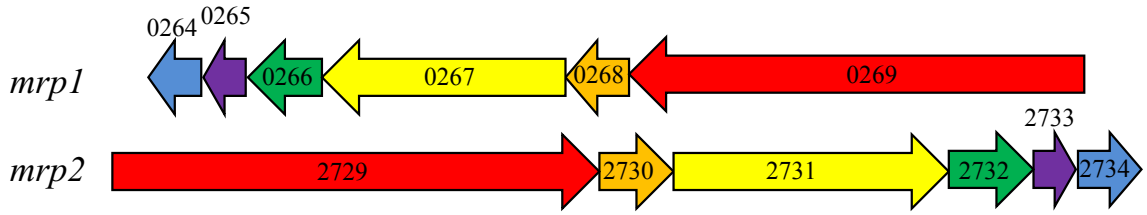

Figure 3: The *mrp*-like operons *mrp1* and *mrp2* from *C. glutamicum*
Mrp1 is encoded by the genes *cgl0269-cgl0264* and Mrp2 includes *cgl2729-cgl2734* whereas *cgl0269* and *cgl2729* correspond to *mrpA'*.

2 Thesis objectives

This work aims to get a better insight into cation homeostasis in *Corynebacterium glutamicum*. Particular attention is directed on sodium and potassium ions. To do so it is necessary to understand the two ion fluxes that are involved in this process: import and export.

The main entry route for sodium ions into bacterial cells is represented by Na^+-solute symporters which were not investigated in this work. The only way for potassium to enter *C.glutamicum* is the potassium channel CglK. A basic characterization of CglK was already done before [44, 99]. As the entry routes for Na^+ and K^+ ions were already known, the main focus was directed on the export of these ions. Good candidates for this function are cation/proton antiporters which exchange internal Na^+ and/or K^+ with external protons.

There were already four putative candidates known: the two complex cation/proton-antiporters Mrp1 and Mrp2 and two simple antiporters ChaA and NhaP. Whether theses candidates function as Na^+/H^+ and/or K^+/H^+ antiporters should be proven on a physiological and on a biochemical level.

The physiological characterization should be performed with the native host *C.glutamicum*. The investigation of single and multiple deletion mutants as well as a completely cation/proton antiporter deficient strain represents the main part of this project. Their growth behavior in presence of elevated Na^+ or K^+ concentrations should give information about the transported cation. As mutants probably accumulate one or the other cation, the measurement of the intracellular cation concentrations is required to support the physiological information. In addition, the influence of the external pH value has to be considered. At high external pH values less protons are available and cells lacking one or more antiporters might have problems with pH homeostasis and/or cation export.

As physiological experiments can only give hints on the transported cation, a biochemical approach has to be used to prove Na^+/H^+ and/or K^+/H^+ antiport function. A system for this has previously been established and uses everted *E.coli* membrane vesicles containing the antiporter to be investigated. Addition of acridine orange to the vesicles and establishment of a pH gradient (acid inside) leads to quenching of fluorescence. The addition of a transported cation leads to dequenching of fluorescence and reflects cation-dependent proton movement out of the everted membrane vesicles [126]. This approach allows the determination of the substrate specificities as well as transport kinetics.

3 Materials & Methods

3.1 Bacterial strains, oligonucleotides and plasmids

All strains, oligonucleotides and plasmids used in this work are listed in table 1, table 2 and table 3, respectively.

Table 1: Strains used in this work
Cells were stored at -80°C using Roti®-Store cryo vials as recommended by the manufacturer (Carl Roth GmbH & Co. KG, Karlsruhe, Germany).

E. coli	**Description**	**Reference**
DH5αMCR	F$^-$ *endA1 supE44 thi-1* λ^- *recA1 gyrA96 relA1 deoR* Δ(*lacZYA-argF*) U169 80Φd*lacZ*ΔM15 *mcrA* Δ(*mrr hsdRMS mcrBC*)	[50]
KNabc	TG1 (Δ*nhaA* Δ*nhaB* Δ*chaA*)	[97]
KNabc_pGEM3Zf(+)	KNabc carrying pGEM3Zf(+)empty plasmid	This study
KNabc_pGEM3Zf(+)_Bpmrp	KNabc carrying pGEM3Zf(+)_Bpmrp	This study
KNabc_pGM36	KNabc carrying pGM36	This study
KNabc_pGEM3Zf(+)_Pro$_{nhaA}$*mrp1*	KNabc carrying pGEM3Zf(+)_Pro$_{nhaA}$*mrp1*	This study
KNabc_pGEM3Zf(+)_*mrp2*	KNabc carrying pGEM3Zf(+)_*mrp2*	This study
KNabc_pGEM3Zf(+)_*chaA*	KNabc carrying pGEM3Zf(+)_*chaA*	This study
KNabc_pGEM3Zf(+)_*nhaP*	KNabc carrying pGEM3Zf(+)_*nhaP*	This study

C. glutamicum	**Description**	**Reference**
ATCC13032	wild type	[1]
ATCC13032_pEKEx2	ATCC13032 carrying pEKEx2 empty plasmid	This study
Δ*mrp1*	ATCC13032 with deletion of the *mrp1* operon (*cgl0269-cgl0264*)	This study
Δ*mrp2*	ATCC13032 with deletion of the *mrp2* operon (*cgl2729-cgl2734*)	This study
Δ*chaA*	ATCC13032 with deletion of the gene coding for ChaA (*cgl1082*)	This study
I:*nhaP*	ATCC13032 with insertion of pDRIVE in the gene coding for NhaP (*cgl1436*)	[64]
Δ*mrp1*Δ*mrp2*Δ*chaA*	ATCC13032 with deletion of *mrp1*, *mrp2* and *cgl1082*	This study
Δ*mrp1*Δ*mrp2*Δ*nhaP*	ATCC13032 with deletion of *mrp1*, *mrp2* and *cgl1436*	This study
AQM	ATCC13032 with deletion of *mrp1*, *mrp2*, *cgl1082* and *cgl1436*	This study
AQM_pEKEx2	AQM carrying pEKEx2 empty plasmid	This study
AQM_pEKEx2_*mrp1*-His	AQM carrying pEKEx2_*mrp1*-His	This study
AQM_pEKEx2_*mrp2*-His	AQM carrying pEKEx2_*mrp2*-His	This study
AQM_pEKEx2_*chaA*-His	AQM carrying pEKEx2_*chaA*-His	This study
AQM_pEKEx2_*nhaP*-His	AQM carrying pEKEx2_*nhaP*-His	This study
Δ*mrp1*Δ*mrp2*Δ*chaA*Δ*nhaP*Δ*cg3038*	ATCC13032 with deletion of *mrp1*, *mrp2*, *cgl1082*, *cgl1436* and *cg3038*	This study

Table 2: Oligonucleotides used in this work.
Ribosome binding sites are in italic and the sequence for the 6xHis-tag is underlined. Restriction sites are in bold and the corresponding restriction endonucleases are named in brackets. All primers were purchased from Operon (Cologne, Germany) and solved in water to obtain a concentration of 100 nmol/ml.

Name	**Sequence 5'→3'**	**Application**
Del_cpaA_1_F	GTATA**GTCGAC**GTCTTGCATTGCCGCTGC TGAT (SalI)	Deletion of the *mrp1* operon (*cgl0269-cgl0264*)
Del_cpaA_1_R	GTACTA**TCTAGA**GCTTTTAGAAAAAAGG GGCGTGCC (XbaI)	Deletion of the *mrp1* operon (*cgl0269-cgl0264*)

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Table 2 - continued from previous page

Name	Sequence 5'→3'	Application
Del_cpaA_2_F	GTTAC**TCTAGA**TATCCCTAATCGCCCATA GAACCCTC (XbaI)	Deletion of gene *cgl0269* or the *mrp1* operon (*cgl0269-cgl0264*)
Del_cpaA_2_R	GTATA**GGATCC**GTCCTCGGACTTCGGTGA TTTG (BamHI)	Deletion of gene *cgl0269* or the *mrp1* operon (*cgl0269-cgl0264*)
Del_cpaB_1_F	GTATT**GTCGAC**TGGAATGGCGCACCTTCA (SalI)	Deletion of gene *cgl2729* or the *mrp2* operon (*cgl2729-cgl2734*)
Del_cpaB_1_R	GCGC**TCTAGA**GTGTTCTCCAGTTCCCCTT TGC (XbaI)	Deletion of gene *cgl2729* or the *mrp2* operon (*cgl2729-cgl2734*)
Del_cpaB_2_F	GTACTA**TCTAGA**CTGAAGGCTTTCTGGAG GTTTTAGAG (XbaI)	Deletion of the *mrp2* operon (*cgl2729-cgl2734*)
Del_cpaB_2_R	GTATA**GGATCC**GGCATGAGCACCGCACT GAA (BamHI)	Deletion of the *mrp2* operon (*cgl2729-cgl2734*)
Del_Cgl0269_F	GTACTA**GTCGAC**CCGTGATCAGCATCAAT GTGAG (SalI)	Deletion of gene *cgl0269*
Del_Cgl0269_R	GTTAC**TCTAGA**TTCTCGCACTGACAGTCG CGATA (XbaI)	Deletion of gene *cgl0269*
Del_Cgl2729_F	GCACA**TCTAGA**CACATGGTAGCCAACCTT TTC (XbaI)	Deletion of gene *cgl2729*
Del_Cgl2729_R	GCTAT**GGATCC**TCGCGTACCACATGACA GAA (BamHI)	Deletion of gene *cgl2729*
Del_NhaP_1_F	GCAT**GTCGAC**CGCTGTTATCGGCTTCT TCG (SalI)	Deletion of the gene coding for NhaP (*cgl1436*)
Del_NhaP_1_R	GCGC**TCTAGA**ATAAACAGCAGCAGGCTG ATC (XbaI)	Deletion of the gene coding for NhaP (*cgl1436*)
Del_NhaP_2_F	GATA**TCTAGA**GAATAAGGTCTCTTCGGGT CAGG (XbaI)	Deletion of the gene coding for NhaP (*cgl1436*)
Del_NhaP_2_R	GCAT**GGATCC**TATTGGGCAGGTGCGCT ATC (BamHI)	Deletion of the gene coding for NhaP (*cgl1436*)
Del_ChaA_1_F	TATA**GTCGAC**ACCTGGGCAAGGGAATC CAC (SalI)	Deletion of the gene coding for ChaA (*cgl1082*)
Del_ChaA_1_R	GCGC**TCTAGA**GTTCTCTAGTCAAACTTTC TTAA (XbaI)	Deletion of the gene coding for ChaA (*cgl1082*)
Del_ChaA_2_F	GCGC**TCTAGA**TTTAGGTAGCCTGGTGGG AAT (XbaI)	Deletion of the gene coding for ChaA (*cgl1082*)
Del_ChaA_2_R	GCGC**GGATCC**AGCTTGAAGGGATGCAA CTC (BamHI)	Deletion of the gene coding for ChaA (*cgl1082*)
Del_cg3038_F1	GCAT**GTCGAC**ACGCCCAATCCTCCTAC TAC (SalI)	Deletion of gene *cg3038*
Del_cg3038_R1	GCGC**TCTAGA**CTCTAGGTGGCGTTTTAAG (XbaI)	Deletion of gene *cg3038*
Del_cg3038_F2	GCGC**TCTAGA**CACTGTCTCGTGTATCTAT ATCG (XbaI)	Deletion of gene *cg3038*
Del_cg3038_R2	GCAT**GGATCC**GTGGTGGTGGTTGATGAT TG (BamHI)	Deletion of gene *cg3038*
ContrDel_cpaA_F	CGCTGATTGGCTTGCTATACAC	Control of deletion of the *mrp1* operon (*cgl0269-cgl0264*)
ContrDel_cpaA_R	TTGAGGGTGGCCATGGAGTC	Control of deletion of the *mrp1* operon (*cgl0269-cgl0264*)

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Table 2 - continued from previous page

Name	Sequence 5'→3'	Application
ContrDel_cpaB_F	CGCAAAGGACCGTGTCAATG	Control of deletion of the *mrp2* operon (*cgl2729-cgl2734*)
ContrDel_cpaB_R	TTCACCGTTGGTGACCTTTG	Control of deletion of the *mrp2* operon (*cgl2729-cgl2734*)
Contr_Del_chaA_F	TGATCAGGGCAAGATCAAGC	Control of deletion of the gene coding for ChaA (*cgl1082*)
Contr_Del_chaA_R	AGAAACGCCGGAATGAACTC	Control of deletion of the gene coding for ChaA (*cgl1082*)
Contr_Del_nhaP_F	GCAGGATCACTCGGCTTCTC	Control of deletion of the gene coding for NhaP (*cgl1436*)
Contr_Del_nhaP_R	CGACAATTAGCCTAGGTGAG	Control of deletion of the gene coding for NhaP (*cgl1436*)
Contr_Del_3038_F	CGTCGAACTATTCGTCTCTG	Control of deletion of gene *cg3038*
Contr_Del_3038_R	ATGATCGGAATCGCCACAGG	Control of deletion of gene *cg3038*
Kompl_Cgl0269_F	GATAC**GGATCC**TCATCGGTTTGCCTCCTC TTT (BamHI)	Cloning of gene *cgl0269* into pEKEx2
Kompl_Cgl0269_R	TATACG**CCTGCAGG***AGGAGAGTATCT*ATG AGTTTGCTATTTGTTGTG (SbfI)	Cloning of gene *cgl0269* into pEKEx2
Kompl_Cgl2729_F	GTACTA**CCTGCAGG***AGGAGACTATCT*GTG CTCATTCTTTTTCTCGCGCTCAC(SbfI)	Cloning of gene *cgl2729* into pEKEx2
Kompl_Cgl2729_R	GCACT**GGATCC**TTATTTTCCTTCCTGCTT TAAAC (BamHI)	Cloning of gene *cgl2729* into pEKEx2
Kompl_cpaA_F	GTACT**CCTGCAGG**TTATTTGGATCGTCTG TTAT (SbfI)	Cloning of the *mrp1* operon (*cgl0269-cgl0264*) into pEKEx2
Kompl_cpaA_F2	GCGC**TCTAGA**TTATTTGGATCGTCTGTTA TC (XbaI)	Cloning of the *mrp1* operon (*cgl0269-cgl0264*) into pEKEx2
Kompl_cpaA_FHis	GCGT**CCTGCAGG**TTAGTGGTGATGATGGT GATGTTTGGATCGTCTGTTAT (SbfI)	Cloning of the *mrp1* operon (*cgl0269-cgl0264*) into pEKEx2
Kompl_cpaB_R	GTACT**CCTGCAGG**TCAAGACTTCTTCGGC TTCAA (SbfI)	Cloning of the *mrp2* operon (*cgl2729-cgl2734*) into pEKEx2
Kompl_cpaB_R2	GCGC**TCTAGA**TCAAGACTTCTTCGGCTTC AAC (XbaI)	Cloning of the *mrp2* operon (*cgl2729-cgl2734*) into pEKEx2
Kompl_cpaB_RHis	GCGT**CCTGCAGG**TTAGTGGTGATGATGGT GATGAGACTTCTTCGGCTTCAA (SbfI)	Cloning of the *mrp2* operon (*cgl2729-cgl2734*) into pEKEx2
Kompl_chaA_F	TATA**CCTGCAGG***AGGAGAGTATCT*ATGCC GTTTTCTTGGCTAAAAC (SbfI)	Cloning of the gene coding for ChaA (*cgl1082*) into pEKEx2
Kompl_chaA_R	GCGC**GAATTC**TTAGTGGTGATGATGGTGA TGGGCGAACATGCTCATCAT (EcoRI)	Cloning of the gene coding for ChaA (*cgl1082*) into pEKEx2
Kompl_nhaP_F	GCTA**GGTACC**TTAGTGGTGATGATGGTGA TGGCGTTCAGCCGCAACAAG (KpnI)	Cloning of the gene coding for NhaP (*cgl1436*) into pEKEx2
Kompl_nhaP_R	GCGC**CCTGCAGG***AGGAGAGTATCT*ATGAC GATATTGTTCATGCTC (SbfI)	Cloning of the gene coding for NhaP (*cgl1436*) into pEKEx2
Ves_cpaA_R	GCGC**TCTAGA**ATGAGTTTGCTATTTGTT GTG (XbaI)	Cloning of the *mrp1* operon (*cgl0269-cgl0264*) into pGEM3Zf(+)
Ves_cpaB_F	GCGC**TCTAGA**GTGCTCATTCTTTTTCTCG CGCTCAC (XbaI)	Cloning of the *mrp2* operon (*cgl2729-cgl2734*) into pGEM3Zf(+)
Vesicle_cpaA_R	GATA**TCTAGA**CTGCTGTACAACGACAA GAC (XbaI)	Cloning of the *mrp1* operon (*cgl0269-cgl0264*) and its promotor region into pGEM3Zf(+)
Vesicle_cpaB_F	GATA**TCTAGA**TCAACGCAACCCTGATC AAC (XbaI)	Cloning of the *mrp2* operon (*cgl2729-cgl2734*) and its promotor region into pGEM3Zf(+)
Vesicle_chaA_F	GCGC**GAATTC**CGCTCTAACCGTGTCC ATTC (EcoRI)	Cloning of the gene coding for ChaA (*cgl1082*) and its promotor region into pGEM3Zf(+)

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Table 2 - continued from previous page

Name	Sequence 5'→3'	Application
Vesicle_chaA_R	GCGC**CCTGCAGG**TCAGGCGAACATGCTCA TCAT (SbfI)	Cloning of the gene coding for ChaA (*cgl1082*) and its promotor region into pGEM3Zf(+)
Vesicle_nhaP_F	GCTA**CCTGCAGG**CTAGCGTTCAGCCGCAA CAAG (SbfI)	Cloning of the gene coding for NhaP (*cgl1436*) and its promotor region into pGEM3Zf(+)
Vesicle_nhaP_R	GCTA**GGTACC**ATTGGGCAGGTGCGCTA TCC (KpnI)	Cloning of the gene coding for NhaP (*cgl1436*) and its promotor region into pGEM3Zf(+)
mrp1_R	ATGAGTTTGCTATTTGTTGTG	Cloning of *mrp1* (*cgl0269-cgl0264*) and the $nhaA_{E.coli}$ or $mrp_{B.\ pseudofirmus\ OF4}$ promotor into pGEM3Zf(+)
mrp2_F	ATGCTCATTCTTTTTCTCGCGCTCAC	Cloning of *mrp2* (*cgl2729-cgl2734*) and the $nhaA_{E.coli}$ or $mrp_{B.\ pseudofirmus\ OF4}$ promotor into pGEM3Zf(+)
Pro_Bpmrp_F	GCGC**TCTAGA**CGAACTTGACCTAAGCC (XbaI)	Cloning of the *mrp1/mrp2* operon and the $nhaA_{E.coli}$ or $mrp_{B.\ pseudofirmus\ OF4}$ promotor into pGEM3Zf(+)
Pro_nhaA_F	GCGC**TCTAGA**GAAAAGAAGAACTAACTCG (XbaI)	Cloning of the *mrp1/mrp2* operon and the $nhaA_{E.coli}$ promotor into pGEM3Zf(+)
Pro_Bpmrp_R_mrp1	AACAAATAGCAAACTCATATCACATACCT CCTTAAATGG	Cloning of the *mrp1* operon (*cgl0269-cgl0264*) and the $mrp_{B\ .pseudofirmus\ OF4}$ promotor into pGEM3Zf(+)
Pro_nhaA_R_mrp1	AACAAATAGCAAACTCATTTTTTATTTCT CTTTCAGGTGAATAG	Cloning of the *mrp1* operon (*cgl0269-cgl0264*) and the $nhaA_{E.\ coli}$ promotor into pGEM3Zf(+)
Pro_Bpmrp_R_mrp2	CGAGAAAAAGAATGAGCATATCACATACC TCCTTAAATGG	Cloning of the *mrp2* operon (*cgl2729-cgl2734*) and the $mrp_{B.\ pseudofirmus\ OF4}$ promotor into pGEM3Zf(+)
Pro_nhaA_R_mrp2	CGAGAAAAAGAATGAGCATTTTTTATTTC TCTTTCAGGTGAATAG	Cloning of the *mrp2* operon (*cgl2729-cgl2734*) and the $nhaA_{E.\ coli}$ promotor into pGEM3Zf(+)
Seq_0269_2_R	GTGAACGGCATCTTCTTGAC	Control PCRs
Seq_0269_2_F	GCATTTGGGTTGGATCGTAG	Control PCRs
Seq_0269_R	AGAGCTCGCCCAACGTATCC	Control PCRs
Seq_0269_F	CGCATCAAGCCCAGCAGAAG	Control PCRs
Seq_0267_R	GCCACGCCACCGACATAAAG	Control PCRs
Seq_0267_F	GTGTGGCGTGAAGTCTTCTG	Control PCRs
Seq_2731_F	CCACTGCCCTGTTGGTTTCC	Control PCRs
Seq_2731_2_F	CCATGCTGACGGTGAGTTCC	Control PCRs
Seq_2731_R	CAAGGTGAGCAGCACGTATG	Control PCRs
Seq_2729_F	CGGCCGTATCTTGGGAACTG	Control PCRs
Seq_2729_R	AACCGATGACGATCAGGTAG	Control PCRs
Seq_2729_2_F	GCTGGATACCGTGTTGAATG	Control PCRs
Seq_2729_2_R	GTGCGATCCACAGCTTAAAC	Control PCRs

Table 3: Plasmids used in this study

Plasmid	Relevant characteristic	Reference
pJET	Cloning vector. Amp^R	Thermo Scientific ,USA
pGEM3Zf(+)	Cloning vector. Amp^R	Promega
pGEM3Zf(+)_Bpmrp	pGEM3Zf(+) derivative; contains the full *mrp* operon from *B. pseudofirmus* OF4	[126]
pGM36	pBR322 derivative; contains the full *nhaA* gene from *E.coli*	[48]
pGEM3Zf(+)_*mrp1*	pGEM3Zf(+) derivative; contains the full *mrp1* operon (*cgl0269-cgl0264*) from *C. glutamicum*	This study
pGEM3Zf(+)_*mrp2*	pGEM3Zf(+) derivative; contains the full *mrp2* operon (*cgl2729-cgl2734*) from *C. glutamicum*	This study
pGEM3Zf(+)_*chaA*	pGEM3Zf(+) derivative; contains the full gene coding for ChaA (*cgl1082*) from *C. glutamicum*	This study
pGEM3Zf(+)_*nhaP*	pGEM3Zf(+) derivative; contains the full gene coding for NhaP (*cgl1436*) from *C. glutamicum*	This study
pGEM3Zf(+)_Pro_{nhaA}*mrp1*	pGEM3Zf(+) derivative; contains the *mrp1* operon (*cgl0269-cgl0264*) from *C. glutamicum* under the control of the $nhaA_{E.\ coli}$ promotor	This study
pEKEx2	Expression vector; Km^R, $lacI^Q$, *tacP*, *ori* of pBL1, pUC18 mcs	[33]
pEKEx2_*mrp1*-His	pEKEx2 derivative; contains the *mrp1* operon (*cgl0269-cgl0264*) from *C. glutamicum* followed by the sequence for a 6xHis-tag	This study
pEKEx2_*mrp2*-His	pEKEx2 derivative; contains the *mrp2* operon (*cgl2729-cgl2734*) from *C. glutamicum* followed by the sequence for a 6xHis-tag	This study
pEKEx2_*chaA*-His	pEKEx2 derivative; contains the gene coding for ChaA (*cgl1082*) from *C. glutamicum* followed by the sequence for a 6xHis-tag	This study
pEKEx2_*nhaP*-His	pEKEx2 derivative; contains the gene coding for NhaP (*cgl1436*) from *C. glutamicum* followed by the sequence for a 6xHis-tag	This study
pK19msB	Gene deletion plasmid for *C. glutamicum*. Kan^R, $oriV_{E.coli}$, oriT, mob, sacB	[117]
pK19msB_0269	pK19msB derivative; contains the flanking regions of gene *cgl0269* from *C. glutamicum*	This study
pK19msB_2729	pK19msB derivative; contains the flanking regions of gene *cgl2729* from *C. glutamicum*	This study
pK19msB_cpaA	pK19msB derivative; contains the flanking regions of the *mrp1* operon (*cgl0269-cgl0264*) from *C. glutamicum*	This study
pK19msB_cpaB	pK19msB derivative; contains the flanking regions of the *mrp2* operon (*cgl2729-cgl2734*) from *C. glutamicum*	This study
pK19msB_chaA	pK19msB derivative; contains the flanking regions of the gene coding for ChaA (*cgl1082*) from *C. glutamicum*	This study
pK19msb_nhaP	pK19msB derivative; contains the flanking regions of the gene coding for NhaP (*cgl1436*) from *C. glutamicum*	This study
pK19msB_3038	pK19msB derivative; contains the flanking regions of gene *cg3038* from *C. glutamicum*	This study

3.2 Media and cultivation of *E. coli* and *C. glutamicum*

3.2.1 Media preparation

All media and solutions were sterilized by autoklaving or passing through a membrane syringe filter (pore size 0.22 μm). For plates, agar (15 g/l) was added to the liquid medium before sterilization.

3.2.2 Media and cultivation of *E. coli*

E. coli was grown in LB, LBK or L_0 (see below). If appropriate, 25 μg/ml kanamycin and/or 100 μg/ml carbenicillin were added. Incubation occured at 37°C and 125 rpm.

	LB	LBK	L_0
Tryptone	10 g/l	10 g/l	10 g/l
Yeast extract	5 g/l	5 g/l	5 g/l
NaCl	10 g/l	-	-
KCl	-	6 g/l	-
pH	7.0 by NaOH	7.0 by KOH	7.0 by KOH

Precultivation was performed in a volume of 5 ml in glass test tubes. For growth experiments, a volume of 10 ml was used in 100 ml baffled shaking flasks. For membrane vesicle preparation, cultivation was carried out with 500 ml medium in 2 l baffled shaking flasks.

3.2.3 Media and cultivation of *C. glutamicum*

As complex medium for *C. glutamicum* LB, L_0 and L_0S (L_0 with 400 mM sorbitol, 7 mM KCl, 100 mM BTP, pH 7.5 by HCl) were used. If appropriate, 25 μg/ml kanamycin and/or 1% glucose were added.
Minimal media used were MMI and CgXII (see below).

	MMI	CgXII
$(NH_4)_2SO_4$	5 g/l	20 g/l
urea	5 g/l	5 g/l
KH_2PO_4	7.3 mM	1 g/l
K_2HPO_4	5.7 mM	1 g/l
NaH_2PO_4	7.3 mM	-
Na_2HPO_4	5.7 mM	-

CgXII was supplemented with 250 mg/l $MgSO_4$ * 7 H_2O, 10 mg/l $CaCl_2$, 1 mg/l $FeSO_4$ * 7 H_2O, 1.375 mg/l $MnSO_4$ * 4 H_2O, 0.1 mg $ZnSO_4$ * 7 H_2O, 0.031 mg $CuSO_4$ * 5 H_2O, 2 μg $NiCl_2$ * 6 H_2O, 30 μg/ml protocatechuic acid and 200 μg/l biotin. MOPS (0.2 M), HEPPS (0.2 M), MES (0.2 M), CHES (0.2 M) or BTP (0.1 M) were used to buffer CgXII. The pH was adjusted with NaOH, KOH or HCl. If appropriate, 25 μg/ml kanamycin was added. Incubation occured at 30°C and 125 rpm.

The first preculture was grown in a volume of 5 ml in glass test tubes, followed by a second preculture in 10 ml medium in 100 ml baffled shaking flasks. Growth experiments were carried out in 10 ml medium in 100 ml baffled shaking flasks or in 200 μl medium in 96-well microtiter plates.

3.2.4 Cultivation of *C. glutamicum* in microtiter plates

C. glutamicum cells from an overnight preculture were washed once in CgXII minimal medium (w/o glucose) and resuspended to an OD_{600} of 20. A volume of 5 μl was added to 195 μl medium in a microtiter plate well. Plates were sealed with a Breathe-Easy® sealing membrane (Diversified Biotech, Dedham, USA). Incubation occured at 30°C and 1200 rpm on a special microtiter plate shaker. The optical density was measured at 595 nm in a plate reader (Tecan infinite® F200 Pro, Tecan Group Ltd., Männedorf, Germany). For the determination of growth rates the measured values were converted into OD_{600} using equation 1. The corresponding curve is shown in figure 4 (p. 26).

$$OD_{600} = 3.634(OD_{595})^4 - 8.45339(OD_{595})^3 + 9.86447(OD_{595})^2 + 0.33856(OD_{595}) + 0.00144 \quad (1)$$

3.3 Molecular biology methods

3.3.1 DNA purification, restriction digest and ligation

Plasmid DNA from *E. coli* was isolated with the High Pure Plasmid Isolation Kit (Roche, Switzerland). DNA concentration was determined with the Ultrospec 2100 Pro spectrophotometer (GE Healthcare, Freiburg, Germany). FastDigest® restriction enzymes were used for restriction digest, ligation was performed with T4 DNA Ligase (Thermo Scientific, USA).

3.3.2 Polymyerase chain reaction

Polymerase chain reaction (PCR [93]) was performed in an analytikjena FlexCycler (Analytik Jena, Jena, Germany). For preparative PCR Phusion® High-Fidelity DNA Polymerase (Thermo Scientific, USA) was used with chromosomal or plasmid DNA as a template. PCR fragments were either digested and directly ligated into the destination plasmid or sub-cloned into pJET using the CloneJet PCR Cloning Kit (Thermo Scientific, USA). For analytic purposes colony PCRs were performed with EconoTaq® PLUS GREEN 2X Master Mix (Lucigen, USA). An *E. coli* or *C. glutamicum* colony was resuspended in 50 μl water and 1 μl was used as template.

3.3.3 Agarose gel electrophoresis and isolation of DNA from agarose gels

PCR products and digested plasmid DNA were seperated by gel electrophoresis on 0.8% agarose gels (Agarose NEEO Ultra-Quality, Roth, Karlsruhe, Germany) in 1x TAE buffer at 85 V [116]. Afterwards DNA was stained by incubating the gel in 1 μg/ml ethidium bromide solution. Visualization was performed with the UVP BioDoc-It™ Imaging system (Analytik Jena, Jena, Germany). The High Pure PCR Product Purification Kit (Roche, Switzerland) was used to isolate DNA from agarose gels.

3.3.4 Competent *E. coli* cells and transformation

Competent *E. coli* DH5α were prepared as described previouly and stored at -80° [116]. Chemical competent *E. coli* KNabc were prepared using the $CaCl_2$ method. 100 μl of a L_0 over-night culture were used to inoculate 10 ml of the same medium in a Corning® 50 ml

centrifuge tube (Sigma Aldrich, USA). Cells were grown until an OD_{600} of about 0.4-0.6 was reached. 1 ml per reaction was transferred to a reaction tube and centrifuged for one minute at 11,000 rpm. Cells were resuspended in 500 μl ice-cold $CaCl_2$ (0.05 M) by vortexing and incubated on ice for 10 minutes. Subsequently cells were centrifuged as before, resuspended in 300 μl ice-cold $CaCl_2$ (0.05 M) by pipetting and incubated on ice for 30 minutes.
The transformation procedure was the same for both *E. coli* strains. 10 μl of ligation mixture or 0.25 μl of purified plasmid was added to the competent cells. After an incubation on ice for 30 minutes, a heat shock was performed (42°C, 2 minutes). 600 μl of LB or L_0 was added to the cells, followed by incubation at 37°C and 125 rpm for one hour. Afterwards cells were centrifuged (11,000 rpm; 1 min) and resuspended in 100 μl of fresh medium. An appropriate amount was plated on agar plates which were incubated at 37°C over night.

3.3.5 Competent *C. glutamicum* cells and transformation

Preparation of competent *C. glutamicum* cells and transformation were performed as described previously with following exceptions [131]. Cells were washed only once with 50 ml ice-cold 10% (v/v) glycerol, frozen in 55 μl aliquots and stored at -80°C.

3.3.6 Gene deletion in *C. glutamicum*

Gene deletion in *C. glutamicum* was performed as described before [117, 32]. DNA sequences of about 500 bp homologous to the up- and downstream regions of the target gene were amplified by PCR and directly ligated into pK19mobsacB. PCR with primers flanking the target sequence was applied to verify successful target gene deletion.

3.4 Biochemical methods

3.4.1 Preparation of everted membrane vesicles from *E. coli*

A single colony was used to inoculate 5 ml LBK medium. After 8 hours 2.5 ml of the preculture was used to inoculate 500 ml LBK medium, this culture was incubated for 16 hours. If appropriate 50-100 mM NaCl were added to both cultures. Cells were harvested (5,000 rpm, 15 min), washed with 50 ml TCDG buffer (10 mM Tris (pH 8.0 by HCl), 140 mM

choline-Cl, 5 mM $MgCl_2$, 10% (v/v) glycerol, 1 mM DTT (fresh)) and resuspended in 20 ml of the same buffer supplemented with DNase and protein inhibitor cocktail. Vesicles were made by single passage through a French®Pressure Cell Press at 1,100 psi. Cell debris was removed by centrifugation (4,000 rpm, 20 min). Supernatants were centrifuged twice at 12,00 rpm for 10 minutes. Vesicles were collected by ultracentrifugation at 40,000 rpm for 90 minutes and resuspended in 1 ml of fresh TCDG buffer. All steps were performed at 4°C. Samples were stored at -20°C or -80°C.

3.4.2 Determination of protein concentration

Protein concentrations of everted membrane vesicle preparations were determined with a modified Lowry method [104]. BSA was used as standard and the assay volume was down-scaled to 1/10.

3.4.3 Acridine orange fluorescence assay

To measure ΔpH-dependent antiport activity aliquots of vesicles (66 μg) were added to 2 ml of an assay buffer (50 mM Bis-Tris-Propane, adjusted to the indicated pH, 140 mM choline-Cl, 2.5 mM $MgCl_2$ and 1 μM acridine orange (AO)) and incubated for 30 minutes at room temperature to equilibrate. The measurement occured in a thermostated cuvette (23°C). Respiration-dependent formation of the ΔpH was initiated by the addition of 2.5 mM Tris-succinate (pH 8.0), and the resulting quenching of AO fluorescence was monitored with an Aminco-Bowman spectrofluorometer (excitation at 493 nm and emission at 528 nm). Antiport activity was assessed as the percent of dequenching. NaCl or KCl were added at 2.5 mM in the pH-profile determinations and at 0.2-5 mM for the determinations of halfmaximal effective concentrations (apparent K_M). Finally, 10 mM NH_4Cl was added to the assay buffer to abolish any ΔpH.

3.4.4 Determination of intracellular cation concentrations

A volume of 1.8 ml cell suspension was centrifuged for 2 minutes at 11,000 rpm. The supernatant was removed and stored at -20°C. The pellet was resuspended in 1.8 ml of distilled water and cells were broken by incubation in an ultrasonic bath at 85°C for 45 minutes. Cell debris was removed by centrifugation at 20,000 rpm for 30 minutes. The supernatant was

removed and used to determine cation concentrations with a flame photometer (ELEX 6361, Eppendorf, Germany). Calibration was performed with solutions of 20-150 μM NaCl or 50-300 μM KCl. For calculations of the intracellular cation concentrations a cytoplasmic volume of 1.6 μl per mg of cell dry matter (CDM) was assumed and equation 2 was used to calculate the CDM. The contamination by external medium in the pellet was assumed to be 1.5 times the cytoplasmic volume.

$$CDM[mg] = 0.36 * OD_{600} * cellvolume[ml] \tag{2}$$

3.4.5 Determination of osmolality

The osmolality of media was measured with a cryoscopic osmometer (Osmomat® 030, Gonotec GmbH, Berlin, Germany). Calibration was performed with solutions of 0.1-2.5 osmol/kg and samples were analysed as recommended by the manufacturer.

3.4.6 Radioactive measurement of membrane potential

The membrane potential was essentially determined from the distribution of the membrane permeable, lipophilic cation [^{14}C]-Tetraphenylphosphoniumbromid (TPP^+) as described before [114, 66]. The accumulation of TPP^+ inside the cell depends on the electrical potential across the membrane. In equilibrium the electrical membrane potential is equal to the chemical potential of TPP^+. Therewith it is possible to calculate the electrical potential when knowing the intra- and extracellular concentration of TPP^+ using equation 3.

$$\Delta\Psi = (-2.303R * T/F) * log([TPP^+]_{\text{in}}/[TPP^+]_{\text{ex}}) \tag{3}$$

with F = faraday constant, R = universal gas constant, T = asolute temperature.

A volume of 2 μl/(ml cell suspension) TPP^+ working solution (final concentration 10 μM, specific activity 2,21 x 10^9 D/min mmol) were added to the cells. After 30 minutes 200 μl cell suspension were subjected to silicone oil centrifugation (70 μl 1.09 g/cm^3 silicone oil, 30 μl 20% perchloric acid) for the determination of the extracellular TPP^+ concentration.

The radioactivity of 150 μl of the supernatant was measured by liquid scintillation counting. To determine the intracellular TPP^+ concentration 150 μl of the cell suspension was directly measured and the concentrations were calculated with equation 4.

$$[TPP^+]_{\text{in}} = [TPP^+]_{\text{total}} - [TPP^+]_{\text{ex}} \tag{4}$$

4 Results

4.1 *C. glutamicum* harbours several putative cation/proton antiporters

C. glutamicum needs Na^+ ions to perform pH homeostasis at alkaline pH [43]. In many bacteria Na^+/H^+ antiporters are not only used for the extrusion of sodium ions but also for pH homeostasis [101]. For this reason it has to be assumed that *C.glutamicum* also possesses at least one sodium/proton antiporter.

The genome of *C. glutamicum* comprises genes for five transporters that have similarity to known cation/proton antiporters (Tab. 4). There are two large gene clusters of six genes each, *cgl0269-cgl0264* and *cgl2729-cgl2734*. They were named *mrp1* and *mrp2*, respectively, according to their classification into the cation:proton antiporter-3 (CPA3) family. In both cases the proteins encoded by the first genes of the operon, Cgl0269 and Cgl2729, have the highest number of predicted transmembrane helices (Tab. 4). Both proteins have similarity to components of the Mrp systems of *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus pseudofirmus* OF4 and *Vibrio cholerae* which were described to function as Na^+/H^+- and/or K^+/H^+-antiporters (Tab. 5, [29, 126]).

Table 4: *C. glutamicum* genes encoding putative cation/proton antiporters

Gene(s)	Transporter classification	# encoded aminoacids	# predicted helices
mrp1			
cgl0269		964	23
cgl0268		127	2
cgl0267	CPA3	510	14
cgl0266		151	1
cgl0265		90	3
cgl0264		106	3
mrp2			
cgl2729		1019	24
cgl2730		163	3
cgl2731	CPA3	556	14
cgl2732		169	1
cgl2733		91	2
cgl2734		126	3
chaA	CaCA		
cgl1082		350	10
nhaP	CPA1		
cgl1436		516	11
MFS transporter	MFS		
cgl2743		398	12

Table 5: Percentage of identical amion acids of *C. glutamicum* antiporters or antiporter components with those from other bacteria.

	Cgl0269	Cgl2729	$ChaA_{Cg}$	$NhaP_{Cg}$	Cgl2743
$MnhA1_{Sa}$	31 %	36 %	-	-	-
$MrpA_{Bs}$	32 %	36 %	-	-	-
$MrpA_{Bp}$	33 %	33 %	-	-	-
$MrpA'_{Vc}$	32 %	34 %	-	-	-
$ChaA_{Ec}$	-	-	33 %	-	-
$NhaP_{Pa}$	-	-	-	24 %	-
$NhaP_{Mj}$	-	-	-	23 %	-
$MdfA_{Ec}$	-	-	-	-	27 %

Cg = *C. glutamicum*, Sa = *S. aureus*, Bs = *B. subtilis*, Bp = *B. pseudofirmus* OF4, Vc = *V. cholerae*, Ec = *E. coli*, Pa = *P. aeruginosa*, Mj = *M. jannaschii*

The gene product of *cgl1082* is named ChaA and belongs to the Ca^{2+}:cation antiporter (CaCA) family, members of which are ubiquitously found in animals, plants, yeast, archaea and widely divergent bacteria [40]. ChaA is similar to the $Na^+/H^+(Ca^{2+}/H^+)$ antiporter ChaA from *Escherichia coli* (Tab. 5, [61, 100]). *C. glutamicum* also has a NhaP-type antiporter from the cation:proton antiporter-1 (CPA1) family, members of which are found in Gram-positive and Gram-negative bacteria as well as in cyanobacteria, yeast, fungi, plants and animals [40]. It is encoded by gene *cgl1436* which is annotated as Na^+/H^+ and K^+/H^+ antiporter. A BLAST analysis revealed similarity to NhaP antiporters of *Pseudomonas aeruginosa* and *Methanococcus jannaschii* which both exchange sodium ions with protons (Tab. 5, [56, 130]). Gene *cgl2743* encodes a major facilitator superfamily permease that is similar to the *E. coli* MdfA which was shown to mediate Na^+/K^+-dependent alkali tolerance (Tab. 5, [86]).

4.2 Construction of cation/proton antiporter deficient *C. glutamicum*

Cation/proton antiporters transport cations out of the cell and concomitantly carry protons into the cell. Two important functions for (bacterial) cells can be derived from these transport directions. On the one hand, they might be involved in cation homeostasis. In the case of sodium the export function is crucial to maintain the inwardly directed sodium gradient which is used, for example, for solute uptake. On the other hand, the antiporters might be important players in pH homeostasis at alkaline pH. Due to their proton importing function they could help preventing the alkalinization of the cytoplasm.

In order to prove the function of the putative cation/proton antiporters in *C. glutamicum* several cation/proton antiporter mutant strains were constructed. First of all, *C. glutamicum* single deletion mutants lacking the *mrp1* operon, the *mrp2* operon or the *chaA* gene were made. A *C. glutamicum nhaP* insertion mutant was already existing [43]. Based on the *C. glutamicum mrp2* mutant a Δ*mrp1*Δ*mrp2* double deletion mutant was constructed. This double mutant was the basis for two triple deletion combinations: Δ*mrp1*Δ*mrp2*Δ*chaA* and Δ*mrp1*Δ*mrp2*Δ*nhaP*. Based on *C. glutamicum* Δ*mrp1*Δ*mrp2*Δ*chaA*, a mutant strain lacking four antiporters (Mrp1, Mrp2, ChaA, NhaP), the so-called *C. glutamicum* AntiporterQuadrupleMutant (AQM), was constructed. *C. glutamicum* AQM served as the basis for a mutant lacking all five putative cation/proton antiporters. In all cases, the successful deletion of the gene(s) was proven by PCR. The primers used for the verification as well as the sizes of the corresponding PCR fragements are given in table 6.

Table 6: Primers used for the verification of gene deletion and sizes of the resulting PCR fragments

Gene	Primers	size of the WT product	size of the mutant product
mrp1	ContrDel_cpaA_F ContrDel_cpaA_R	7671 bp	1808 bp
mrp2	ContrDel_cpaB_F ContrDel_cpaB_R	8138 bp	1724 bp
chaA	Contr_Del_chaA_F Contr_Del_chaA_R	2527 bp	1474 bp
nhaP	Contr_Del_nhaP_F Contr_Del_nhaP_R	2941 bp	1390 bp
cgl2743	Contr_Del_3038_F Contr_Del_3038_R	2595 bp	1398 bp

The constructed mutant strains were used to test the dependence of *C. glutamicum* on cation/proton antiporters under different stress conditions. The expression of the antiporter encoding genes in the antiporter deficient strains represents a second approach that was used to investigate their function.

4.3 Conversion of OD_{595} measured in a plate reader to OD_{600} measured in a photometer

Growth experiments in 96-well microtiter plates are often used to test many different conditions at the same time. This method was also used in the present study to investigate the growth phenotype of antiporter deficient mutants. An important characteristic to describe the growth of bacteria is the growth rate. The major problem is that the values for the optical density that are obtained from the plate reader can not be directly compared to those from shaking flask experiments where the optical density is measured with a photometer. Concluding, also the growth rates can not be compared. For this reason, one goal was to find out whether it is possible to convert the optical density measured in a plate reader to the optical density measured in a photometer.

Several dilutions of an over night culture of the *C. glutamicum* wild type were measured in both a plate reader (TECAN infinite F200 Pro) and a photometer (Ultrospec 2100 Pro, GE Healthcare).

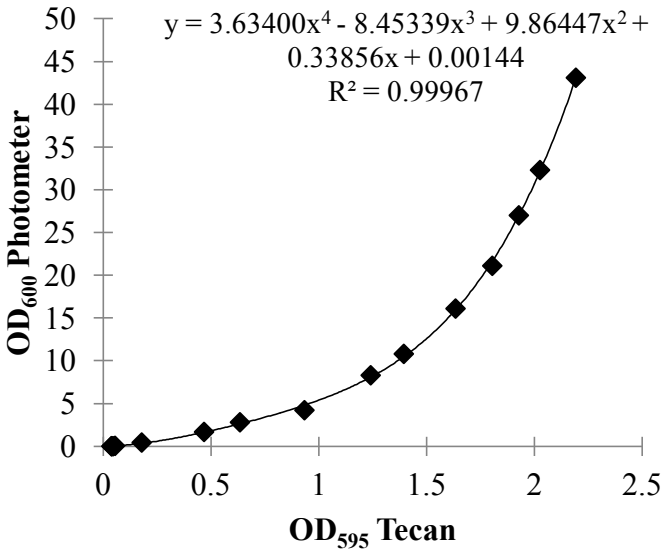

Figure 4: Correlation of OD_{595} measured in the Tecan plate reader to OD_{600} measured in the photometer
Several dilutions of an over night culture of the *C. glutamicum* wild type were measured in both a plate reader (TECAN infinite F200 Pro) and a photometer (Ultrospec 2100 Pro, GE Healthcare). Microsoft Excel was used to fit a fourth-degree polynomial.

Figure 4 shows the correlation between the OD_{595} (Tecan) and the OD_{600} (photometer). The measured values fit into a fourth-degree polynomial. The equation is given in the figure (see also p. 17) and was used in all microtiter plate growth experiments in this study to convert data from the Tecan plate reader. With the new values, growth rates were determined which may be compared to those from shaking flask experiments.

4.4 Investigation of *C. glutamicum* cation/proton antiporter single mutants

4.4.1 The absence of Mrp1 leads to Na^+-sensitivity, a lack of Mrp2 causes K^+-sensitivity

In order to investigate the physiological function of the putative cation/proton antiporters, the growth of single deletion mutants was investigated in minimal medium with high extracellular sodium or potassium concentrations. If a growth deficit was observed the intracellular cation concentrations were determined as well to check for altered values due to the lack of antiporters. Determination of internal cation concentrations is not possible at the high external concentrations used in the growth experiments. Thus a different approach was used in which the cells were incubated for one hour in MMI minimal medium, containing 20 mM Na^+ and 20 mM K^+, at indicated pH values.

The deletion of the complete *mrp1* operon slightly reduced the growth rate of *C. glutamicum* under control conditions at neutral and alkaline pH (Fig. 5A, grey bars). In presence of 1 M NaCl growth of the mutant was clearly impaired at pH 7.5 and was completely abolished at pH 8.5 (yellow bars). A high extracellular potassium concentration led to a small decrease of the growth rate (light green bars). In regard to the intracellular cation content, an increase of sodium was observed for *C. glutamicum* Δ*mrp1* compared to the *C. glutamicum* wild type while the potassium concentration was reduced (Fig. 5B).

At pH 7.5 a *C. glutamicum* mutant lacking the *mrp2* operon grows like the wild type independent of the applied cation concentration (Fig. 6A). For pH 8.5 the same is true under control conditions and at a high NaCl concentration. However, at a high KCl concentration the growth rate of the mutant was reduced compared to the wild type. Intracellular sodium concentrations were similar to wild type levels, the potassium concentration was reduced (Fig. 6B).

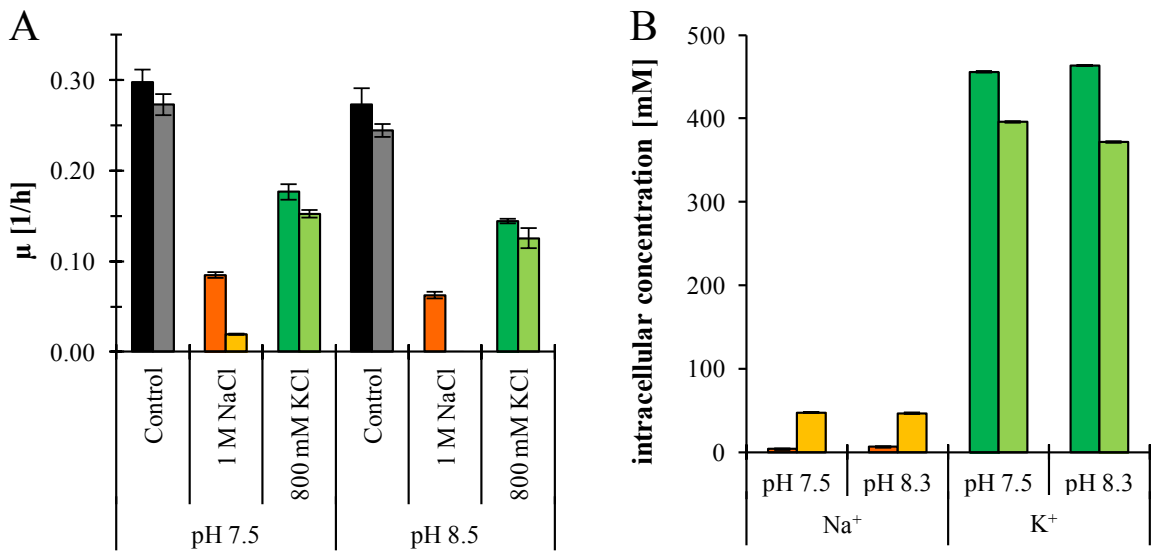

Figure 5: Growth rates (A) and intracellular cation concentrations (B) of *C. glutamicum* wild type and *C. glutamicum* Δ*mrp1*. Cells were grown in microtiter plates in CgXII minimal medium at indicated pH values. Growth rates were determined after conversion of the plate reader data according to equation 1 (p. 17). The error bars indicate standard deviations for growth rates of three microtiter plate wells. In all three wells *C. glutamicum* Δ*mrp1* did not grow with 1 M NaCl at pH 8.5. To determine intracellular cation concentrations cells were incubated for one hour in MMI minimal medium (20 mM Na^+, 20 mM K^+) at indicated pH values and washed twice in 20 mM BTP (+10 mM KCl, +5 mM NaCl) at same pH and osmolality. Intracellular concentrations are the means from two independent experiments. ■■■ wild type, ■■■ Δ*mrp1*.

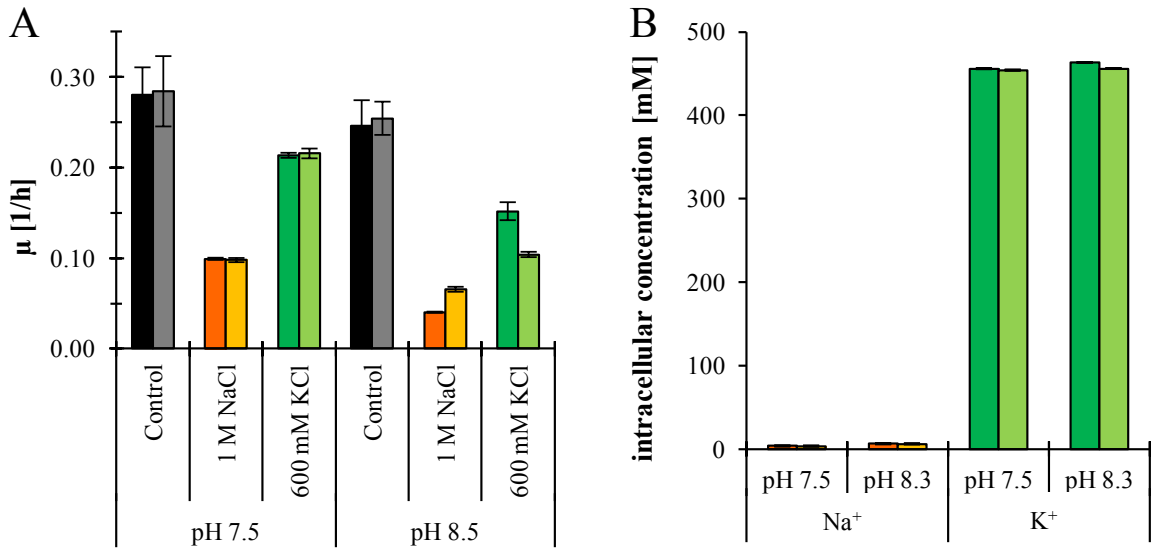

Figure 6: Growth rates (A) and intracellular cation concentrations (B) of *C. glutamicum* wild type and *C. glutamicum* Δ*mrp2*. Cells were grown in microtiter plates in CgXII minimal medium at indicated pH values. Growth rates were determined after conversion of the plate reader data according to equation 1 (p. 17). The error bars indicate standard deviations for growth rates of three microtiter plate wells. To determine intracellular cation concentrations cells were incubated for one hour in MMI minimal medium (20 mM Na^+, 20 mM K^+) at indicated pH values and washed twice in 20 mM BTP (+10 mM KCl, +5 mM NaCl) at same pH and osmolality. Intracellular concentrations are the means from two independent experiments. ■■■ wild type, ■■■ Δ*mrp2*.

Summarizing, the observed Na^+-sensitivity of the *C. glutamicum* Δ*mrp1* mutant points at a function of this transporter as in Na^+ export at neutral and alkaline pH. In contrast, Mrp2 is likely to be working as a K^+ exporter although a K^+-sensitive phenotype was only observed at pH 8.5.

4.4.2 The presence of Mrp1 and Mrp2 masks ChaA and NhaP activity

In contrast to the clear phenotypes of the *C. glutamicum mrp1* and *mrp2* deletion mutants, the phenotype of *C. glutamicum* cells lacking ChaA or NhaP did not differ from the wild type (Fig. 7). Independent of the applied pH value neither a high NaCl concentration nor a high KCl concentration had an effect on the growth rate.

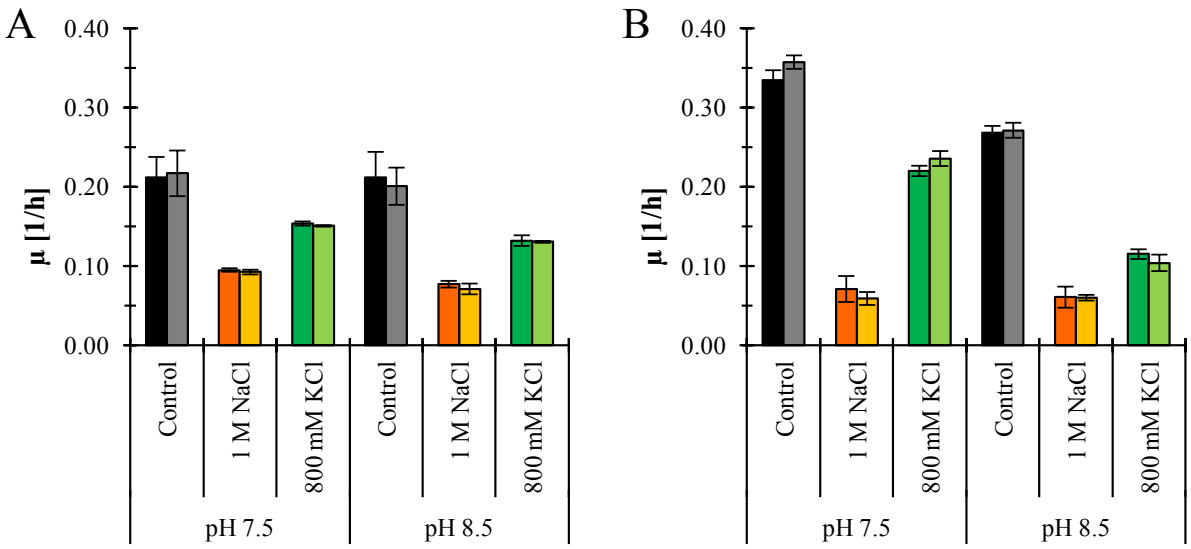

Figure 7: Growth rates of *C. glutamicum* Δ*chaA* and *C. glutamicum* IM*nhaP*.
Cells were grown in microtiter plates in CgXII minimal medium at indicated pH values. Growth rates were determined after conversion of the plate reader data according to equation 1 (p. 17). The error bars indicate standard deviations for growth rates of three microtiter plate wells. **A** *C. glutamicum* Δ*chaA*, **B** *C. glutamicum* Δ*nhaP*, ■■■ wild type, ■■■ Δ*chaA*/IMnhaP.

These results indicate that the Mrp antiporters are able to compensate for the lack of ChaA and NhaP. The fact that the deletion of one *mrp* operon already resulted in a cation-sensitive phenotype, although four other putative cation/proton antiporters are present, suggests that the Mrp antiporters play the major role in cation extrusion in *C. glutamicum*. For this reason *chaA* and *nhaP* were deleted in the *C. glutamicum* Δ*mrp1*Δ*mrp2* double deletion mutant background to find out more about the function and the impact of ChaA and NhaP.

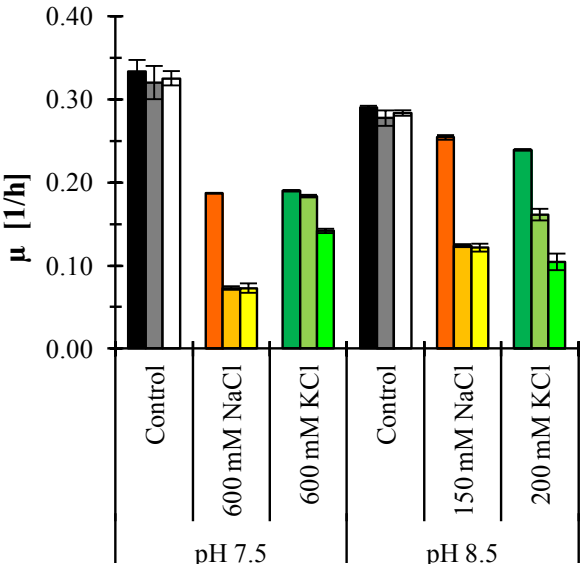

Figure 8: Growth rates of *C. glutamicum* wild type, Δ*mrp1*Δ*mrp2*Δ*chaA* and Δ*mrp1*Δ*mrp2*Δ*nhaP*.
Cells were grown in microtiter plates in CgXII minimal medium at indicated pH values and cation concentrations. Growth rates were determined after conversion of the plate reader data according to equation 1 (p. 17). The error bars indicate standard deviations for growth rates of three microtiter plate wells. ■■■ wild type, ■■■ Δ*mrp1*Δ*mrp2*Δ*chaA*, □■■ Δ*mrp1*Δ*mrp2*Δ*nhaP*.

At both applied pH values, *C. glutamicum* Δ*mrp1*Δ*mrp2*Δ*chaA* and *C. glutamicum* Δ*mrp1*Δ*mrp2*Δ*nhaP* did not differ in growth from the wild type under control conditions (Fig. 8). Both triple deletion mutants were comparably sodium sensitive. Potassium sensitivity at neutral pH was only observed for *C. glutamicum* Δ*mrp1*Δ*mrp2*Δ*nhaP*. At alkaline pH this mutant was even more K^+-sensitive than the *C. glutamicum* Δ*mrp1*Δ*mrp2*Δ*chaA* mutant. Due to the fact that no comparison was made to the *C. glutamicum* Δ*mrp1*Δ*mrp2* double mutant, it is difficult to designate a function for these two antiporters, especially in regard to sodium. At least, the fact that a *C. glutamicum mrp* double mutant additionally lacking NhaP is more potassium sensitive than the same mutant additionally lacking ChaA indicates a K^+ export function of NhaP.

4.4.3 A completely antiporter deficient *C. glutamicum* is still able to grow

The above results show that *C. glutamicum* mutants lacking three of five putative cation/proton antiporters are still able to grow. Thus the next step was to investigate a mutant strain devoid of the genes for four antiporters: *mrp1*, *mrp2*, *chaA* and *nhaP*. This mutant was called *C. glutamicum* AntiporterQuadrupleMutant (AQM).

Growth in minimal medium with high extracellular sodium or potassium concentrations was investigated. In addition, intracellular cation concentrations were determined. Under control conditions, *C. glutamicum* AQM grew like the *C. glutamicum* wild type at pH 7.5 but showed a slightly reduced growth rate at pH 8.5 (Fig. 9A). Compared to the *C. glutamicum* wild type, *C. glutamicum* AQM was sensitive to both sodium and potassium. In contrast to the *C. glutamicum* wild type, growth rates were more reduced at alkaline pH resulting in nearly abolished growth in presence of 400 mM KCl at pH 8.5.

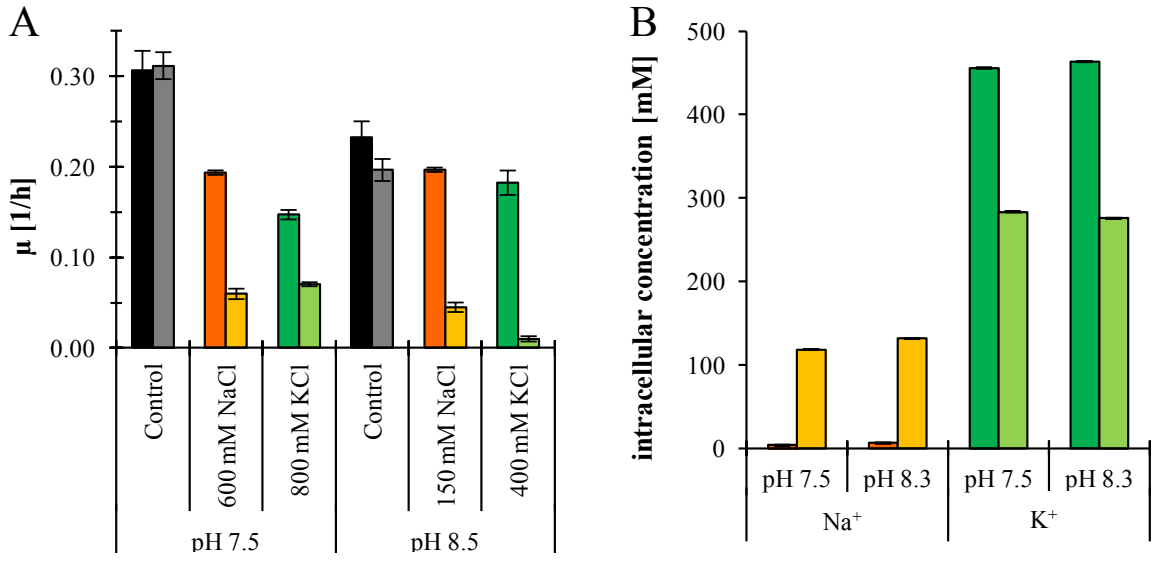

Figure 9: Growth rates (A) and intracellular cation concentrations (B) of *C. glutamicum* wild type and *C. glutamicum* AQM. Cells were grown in microtiter plates in CgXII minimal medium at indicated pH values. Growth rates were determined after conversion of the plate reader data according to equation 1 (p. 17). The error bars indicate standard deviations for growth rates of three microtiter plate wells. To determine intracellular cation concentrations cells were incubated for one hour in MMI minimal medium (20 mM Na^+, 20 mM K^+) at indicated pH values and washed twice in 20 mM BTP (+10 mM KCl, +5 mM NaCl) at same pH and osmolality. Intracellular concentrations are the means from two independent experiments. ■■■ wild type, ■■■ AQM.

As observed before, the *C. glutamicum* wild type contained low amounts of Na^+ ions and high amounts of K^+ ions (Fig. 9B). For *C. glutamicum* AQM a dramatic increase in intracellular sodium was observed. At the same time the intracellular potassium concentration was considerably reduced. The above results show that *C. glutamicum* is still able to grow even if four of its five (putative) cation/proton antiporters are missing. However, it was ruled out that the fifth putative cation extrusion system, Cgl2743, is responsible for this observation. The deletion of the corresponding gene *cgl2743* in the *C. glutamicum* AQM background had no effect under all conditions tested (data not shown). For this reason the quintuple mutant is not further mentioned in this work.

4.5 Comparative investigation of different cation/proton antiporter deficient *C. glutamicum* strains

The above experiments approaching the cation sensitivity of antiporter mutant strains clearly showed that the cation/proton antiporters seem to have redundant functions in *C. glutamicum*. In addition, the salt concentrations tested were not the same for the different mutants. Thus it is difficult to assess the physiological function of the antiporters by comparing the results of these single experiments. A direct comparison of all different mutant strains makes it easier to assign functions to the putative antiporters and investigate their role in cation as well as pH homeostasis.

4.5.1 Mrp1 and Mrp2 are the most important cation/proton antiporters in *C. glutamicum*

Cation/proton antiporters need cations as well as protons to fulfill their funtion. For this reason the external avaiblability of protons, namely the extracellular pH value, plays an important role. Cation sensitivity might be higher at alkaline pH due to a lower external proton availability. But also the capability of the different antiporters to take over the function of a missing one, namely redundancy, could be pH dependent.

In order to check the above mentioned assumptions, different *C. glutamicum* cation/proton antiporter deficient strains were grown in CgXII-BTP minimal medium at different pH values and cation concentrations. Bis-Tris-Propane (BTP) was used as buffer to allow the adjustment

of the pH by HCl, thereby avoiding the addition of varying concentrations of NaOH or KOH. Hence the standard CgXII-BTP medium only contained ≈1.6 mM Na^+ and ≈19 mM K^+ as determined by flame photometry.

In standard CgXII-BTP medium (control conditions) the growth rates of all mutant strains were similar to the wild type at all applied pH values (Fig. 10A). In presence of additional 300 mM NaCl all mutants lacking Mrp1 showed a reduced growth rate compared to the wild type (Fig. 10B). At an acidic pH of 6.5 it made no difference if other antiporters than Mrp1 were missing. At higher pH values of 7.0 to 8.75 the additional lack of Mrp2 (*C. glutamicum* Δ*mrp1*Δ*mrp2*) further reduced the growth rate. Deletion of more antiporter genes in *C. glutamicum* Δ*mrp1*Δ*mrp2* resulted in mutant strains largely behaving similar to the Mrp double mutant. Only at pH 8.0 mutants lacking ChaA (*C. glutamicum* Δ*mrp1*Δ*mrp2*Δ*chaA* and *C. glutamicum* AQM) had a lower growth rate than the double mutant or the *C. glutamicum* Δ*mrp1*Δ*mrp2*Δ*nhaP* triple mutant. In presence of a high potassium concentration between pH 6.5 and 7.5 all mutants showed similar or higher growth rates than the wild type when taking the standard deviations into account (Fig. 10C). However, the mean growth rates at pH 7.5 were lower for cells lacking both Mrps compared to cells only lacking Mrp2. At pH 8.0, mutants lacking more antiporters than Mrp2 were more K^+ sensitive than the *C. glutamicum* Δ*mrp2* single mutant. However, at pH 8.75 all strains lacking *mrp2* were similarly impaired with exception of *C. glutamicum* Δ*mrp1*Δ*mrp2*Δ*nhaP* which had a more reduced growth rate.

Summarizing, the comparative investigation of different cation/proton antiporter mutants in minimal medium showed that Mrp1 is the main player in sodium ion extrusion. Deletion of the complete *mrp1* operon leads to a clear Na^+-sensitive phenotype. Although the *C. glutamicum* Δ*mrp2* single mutant is not sodium sensitive, the *C. glutamicum* Δ*mrp1*Δ*mrp2* double mutant is more impaired than the *C. glutamicum* Δ*mrp1* mutant. From this observation it can be concluded that Mrp2 is also involved in Na^+ export, but to a much smaller extent than Mrp1. However, this is not true for pH 6.5 where the *C. glutamicum* Δ*mrp1* mutant resembled the double mutant, meaning that Mrp2 is not able to transport Na^+ ions at acidic pH. Furthermore, the experiment hints at a probable function of ChaA in sodium export, at least at pH 8.0, although higher extracellular NaCl concentrations might be needed for a more distinct effect. In regard to potassium, it was shown that Mrp2 is the main player in K^+ export although this only becomes evident at pH 8.0 or higher. The additional deletion of the other antiporter

genes only showed a slight effect at pH 8.0 and thus indicates that the other antiporters are of minor physiologic importance in presence of high extracellular KCl concentrations.

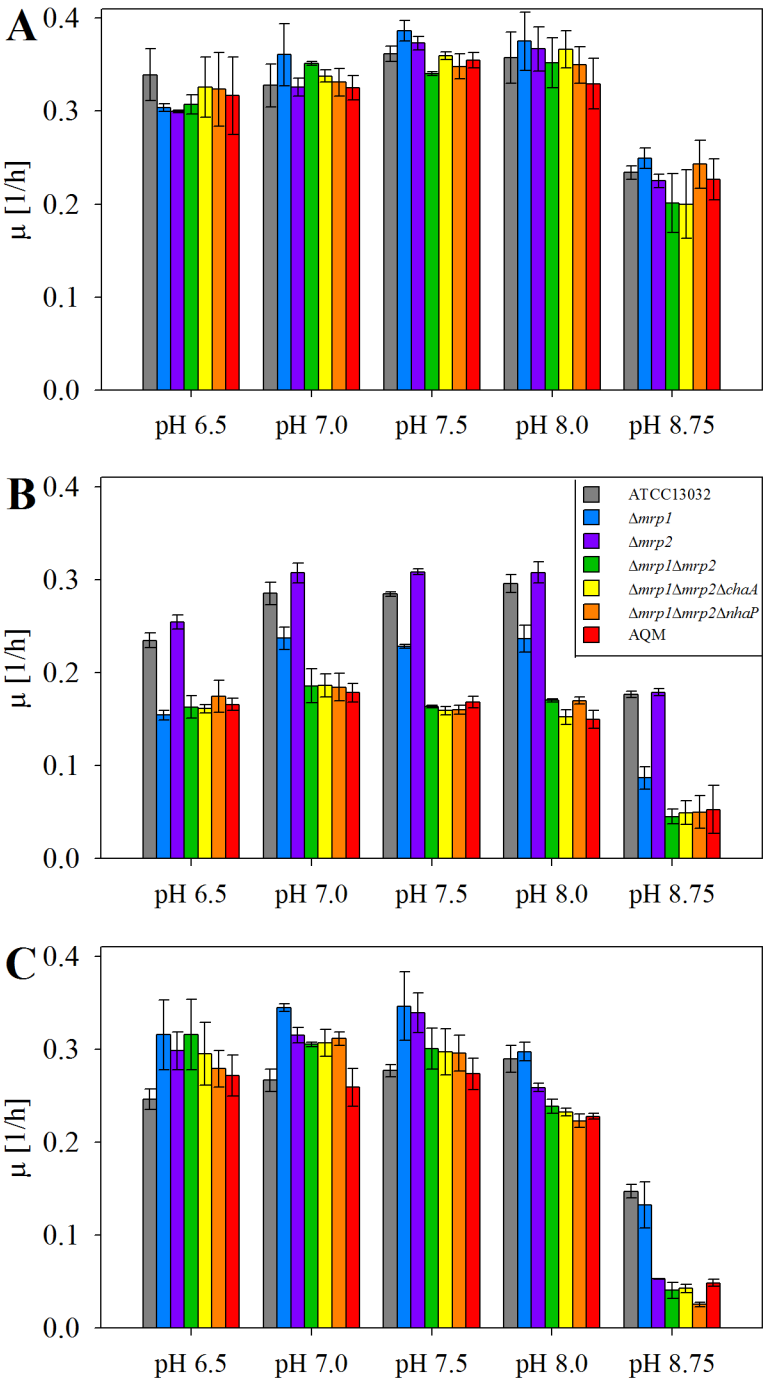

Figure 10: Growth rates of *C. glutamicum* wild type and cation/proton antiporter deficient strains in minimal medium. Cells were grown in microtiter plates in CgXII-BTP minimal medium at indicated pH values. Growth rates were determined after conversion of the plate reader data according to equation 1 (p. 17). The error bars indicate standard deviations for growth rates of three microtiter plate wells. **A** control, **B** +300 mM NaCl, **C** +600 mM KCl

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4.5.2 The presence of Mrp1 is crucial for good growth in complex medium

The comparative investigation of cation/proton antiporter mutants showed that it is difficult to assess K^+/H^+ antiport function on the physiological level. One reason is that K^+ ions are not cytotoxic, the other reason is the fact that *C. glutamicum* by its own accumulates potassium up to very high intracellular concentrations. In contrast, the functional attribution of Na^+ export is very easy.

Except the addition of high amounts of sodium to the medium there is another possibility to interfere with ion homeostasis closer to physiologic conditions. Feeding complex medium forces the uptake of sodium ions via Na^+/solute symporters. In *C. glutamicum*, for example, the uptake of branched-chain amino acids as well as methionine, alanine, glutamine, threonine and glutamate is mediated by sodium-coupled symport [127, 38, 15, 119, 31]. To maintain the gradient for this transport process, the Na^+ ions have to be exported again which is mediated by Na^+/H^+ antiporters. Thus growth of cation/proton antiporter mutants might be impaired in complex medium.

First of all a suitable complex medium had to be found which allows relatively good growth of *C. glutamicum* AQM and permits the determination of the intracellular sodium concentration. In a first attempt the *C. glutamicum* wild type and *C. glutamicum* AQM were grown in standard LB medium and in L_0 medium, which is LB without NaCl.

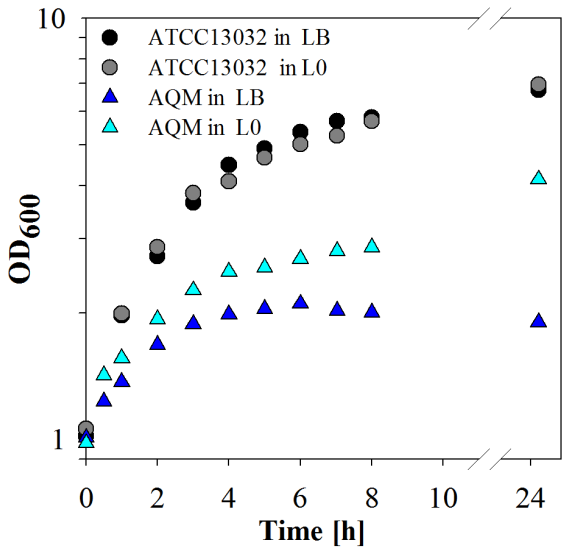

Figure 11: Growth of *C. glutamicum* wild type and *C. glutamicum* AQM in LB and L_0 complex medium
Cells were grown in LB and L_0 complex medium after precultivation in CgXII-BTP pH 7.5.

The *C. glutamicum* wild type grew nearly identical in both media (Fig. 11). For *C. glutamicum* AQM growth was much better in L_0 medium. In addition, the intracellular sodium concentrations could not be determined for samples from LB medium due to the high Na^+ concentration of this medium. Based on these results, L_0 complex medium was chosen for further experiments.

The planned growth experiments in complex medium should include a preculture in CgXII-BTP minimal medium to allow good growth for all mutants. When looking at internal ion concentrations, it has to be considered that bacteria respond to osmotic stress by increasing or decreasing the cytoplasmic ion strength. Thus a change in osmolality has to be avoided when the medium is changed. The CgXII-BTP minimal medium, which should be used for the preculture, has an osmolality of about 0.7 osmol/kg. In contrast, L_0 complex medium only has about 0.1 osmol/kg. Concluding, the osmolality of the complex medium had to be increased. For this purpose, sorbitol was used. In addition, bis-tris-propane (BTP) was added as a buffer substance to ensure a constant pH during cultivation. The modified medium contained 400 mM sorbitol and 100 mM BTP. The pH was adjusted to 7.5 with KOH to keep the sodium concentration low. The resulting medium was called L_0S and had an osmolality of about 0.72 osmol/kg. The newly developed L_0S complex medium was used to test whether growth of cation/proton antiporter mutants is impaired when sodium uptake is forced due to use of Na^+/solute symporters.

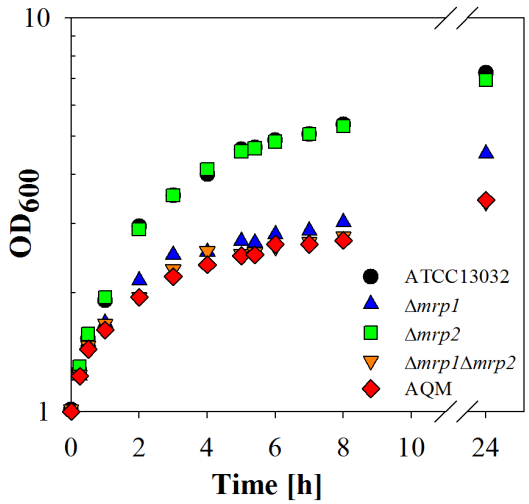

Figure 12: Growth of *C. glutamicum* wild type and cation/proton antiporter mutants in L_0S medium. Cells were grown in L_0S complex medium after precultivation in CgXII-BTP pH 7.5.

Growth of the *C. glutamicum* wild type and the *C. glutamicum* Δ*mrp2* mutant were identical (Fig. 12). In contrast, growth of the *C. glutamicum* Δ*mrp1* mutant was impaired dramatically. The *C. glutamicum* Δ*mrp1*Δ*mrp2* double mutant grew even worse than the *C. glutamicum* Δ*mrp1* mutant and also reached a lower final optical density. The phenotype of the mutant lacking all antiporters (*C. glutamicum* AQM) was identical to that of the double mutant. Again it was shown that the two Mrp antiporters play a crucial role in sodium extrusion in *C. glutamicum* and that the other two antiporters, ChaA and NhaP, seem to be of minor importance under this condition.

4.5.3 An increase of internal [Na^+] causes the reduction of internal [K^+]

The impaired growth of antiporter mutants in complex medium might be the result of missing Na^+/H^+ antiport function. The cells might have a reduced or absent capability to export Na^+ ions that enter the cell via Na^+/solute symporters leading to an increased intracellular sodium concentrations. Concluding, the measurement of the intracellular cation concentrations during growth in L_0S complex medium can be used to assign Na^+/H^+ antiport function to the putative cation/proton antiporters of *C. glutamicum*. Three different time points were chosen to take samples for the determination of intracellular cation concentrations. The fastest reliable time point was after 30 minutes. The 4 hours time point represents the status of growing cells while the 8 hours time point reflects the status of cells starting to enter the stationary phase.
In general, the intracellular Na^+ concentrations after precultivation were very low. For the *C. glutamicum* wild type and the *C. glutamicum* Δ*mrp2* mutant a value of about 1.2 mM was determined after 16 hours growth in CgXII-BTP minimal medium (Fig. 13A, o/n). The other mutant strains contained about 12-17 mM. During growth in complex medium, the intracellular Na^+ concentrations were clearly increased in all strains. After 30 minutes the *C. glutamicum* wild type and the *C. glutamicum* Δ*mrp2* mutant showed identical values of about 150 mM Na^+. After eight hours a decrease to about 40 mM was observed. The lack of Mrp1 lead to a drastically increased intracellular sodium concentration of about 340 mM, which stayed constant during cultivation. The *C. glutamicum* Δ*mrp1*Δ*mrp2* and *C. glutamicum* AQM strains showed even higher Na^+ concentrations (about 440 mM) at the beginning of the cultivation, which decreased over time to values of about 340 mM and 365 mM, respectively.

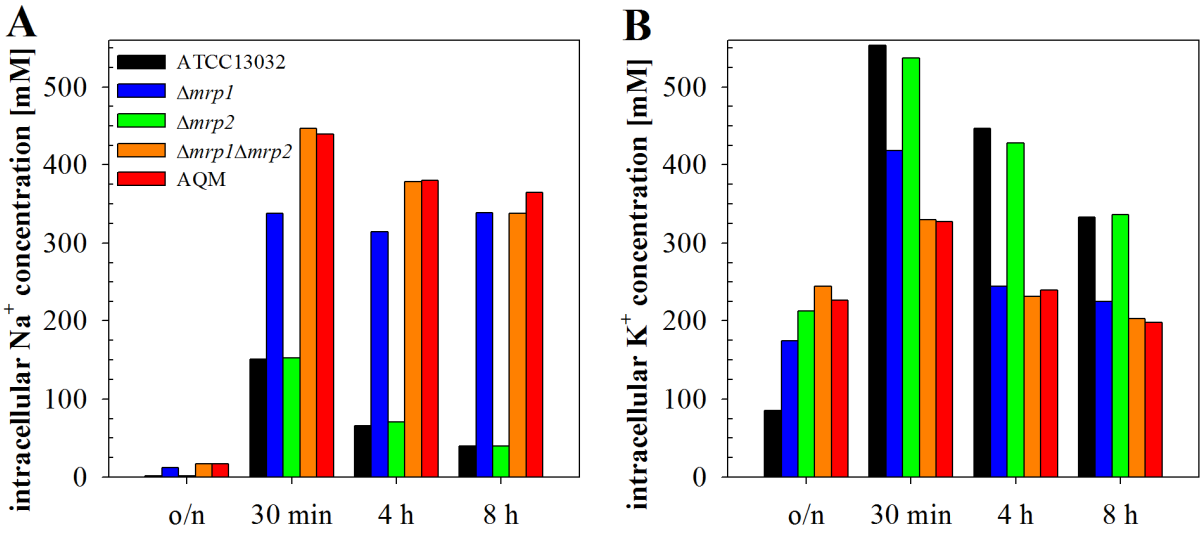

Figure 13: Intracellular cation concentrations of *C. glutamicum* wild type and cation/proton antiporter deficient strains during growth in L_0S complex medium. Cells were grown in L_0S complex medium after precultivation in CgXII-BTP pH 7.5. **A** intracellular Na^+, **B** intracellular K^+

The intracellular K^+ concentrations showed an inversed pattern (Fig. 13B). Again, the phenotypes of the *C. glutamicum* wild type and the *C. glutamicum* Δ*mrp2* mutant were very similar. Both strains showed very high potassium concentrations of about 540 mM at the beginning of the cultivation. Over time these values decreased to about 330 mM. Deletion of the *mrp1* operon lead to lower K^+ values of initially about 420 mM and about 225 mM after eight hours. The *C. glutamicum* Δ*mrp1*Δ*mrp2* and *C. glutamicum* AQM strains showed even lower intracellular K^+ concentrations at the beginning (about 330 mM) which then decreased to a level slightly lower than that of the *C. glutamicum* Δ*mrp1* mutant.

The above results confirm the function of Mrp1 in Na^+ export. In addition, the fact that the deletion of the *mrp2* operon in *C. glutamicum* Δ*mrp1* further increases the intracellular sodium concentration shows that Mrp2 is able to export Na^+ ions as well. The further deletion of genes encoding the ChaA and/or NhaP antiporter had no effect on the intracellular sodium concentration. Hence, it is not possible to specify the function of ChaA and NhaP with this type of experiment.

Interestingly, in the case of an increased intracellular sodium concentration, a reduced intracellular potassium concentration was observed. There are at least two possible explanantions. First, the cell might need to regulate the intracellular ion strength. In the mutant cells, the export of Na^+ ions is restricted or abolished and therefore the intracellular amount of K^+ ions is adjusted. The second explanation might be an altered membrane

potential. In *C. glutamicum* potassium uptake is mediated by the potassium channel CglK, which is driven by the membrane potential [44, 99]. If the membrane potential is not high enough, potassium uptake might be influenced. To check this assumption the membrane potential was measured for the *C. glutamicum* wiltype and for *C. glutamicum* AQM, both growing in L_0S complex medium.

After growing four hours in L_0S complex medium, cells of the *C. glutamicum* wild type and the *C. glutamicum* AQM strain showed similar membrane potentials (Fig. 14A). In addition, the intracellular cation cocentrations were determined. Again, an increase of the sodium concentration and a reduction of the potassium concentration was observed for the mutant (Fig. 14B). This result supports the hypothesis that *C. glutamicum* is able to adjust the intracellular K^+ concentration in response to a change of the internal Na^+ concentration and thereby regulates the ion strength of the cytoplasm.

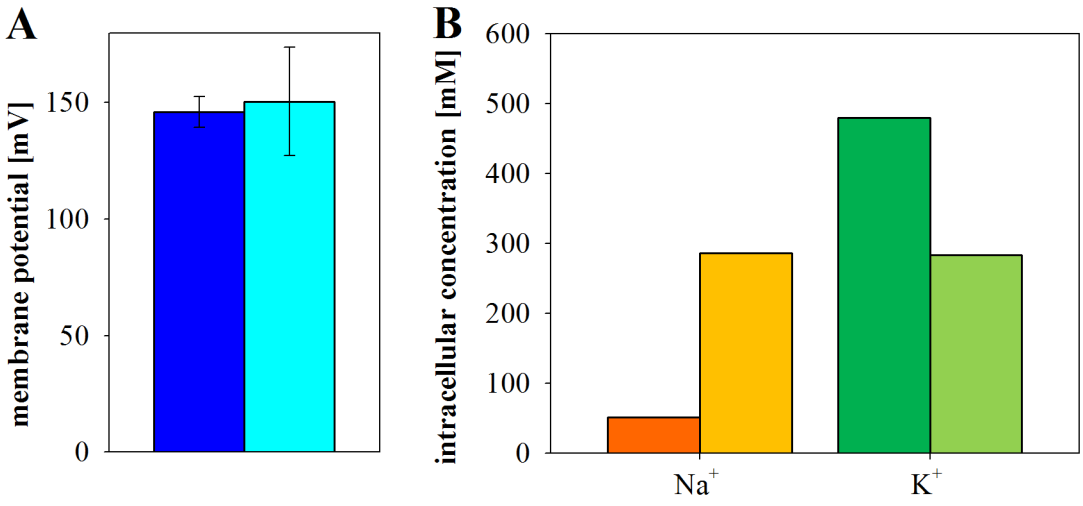

Figure 14: Membrane potential (A) and intracellular cation concentrations (B) of *C. glutamicum* wild type and *C. glutamicum* AQM during growth in complex medium. Cells were grown in L_0S complex medium after precultivation in CgXII-BTP (pH 7.5). Membrane potential and intracellular cation concentrations were determined after 4 hours. Error bars indicate the standard deviations for three independent measurements of one culture. ■■■ *C. glutamicum* wild type, ■■■ *C. glutamicum* AQM.

4.5.4 Glucose as carbon source reduces the sodium uptake via Na^+/solute symporters

The growth experiments in complex medium were performed to force the cells to use Na^+/solute symporters to take up the carbon sources in the medium and thereby force the uptake of sodium. The results shown above indicate that this approach was successful. The fact that the high intracellular sodium concentrations of the mutants are really connected to the Na^+-driven uptake of the carbon sources can also be proven by the addition of a carbon source whose uptake is not sodium-driven, for example glucose. In most bacteria, glucose is prefered to other carbon sources which is reflected in a down-regulation of the activity of other carbon source uptake systems. Thus the addition of glucose to complex medium should result in a decreased use of Na^+/solute symporters which in turn should lower the amount of sodium ions that enter the cell. To prove this assumption, the *C. glutamicum* wild type as well as *C. glutamicum* AQM were grown in L_0S complex medium ±1% glucose.

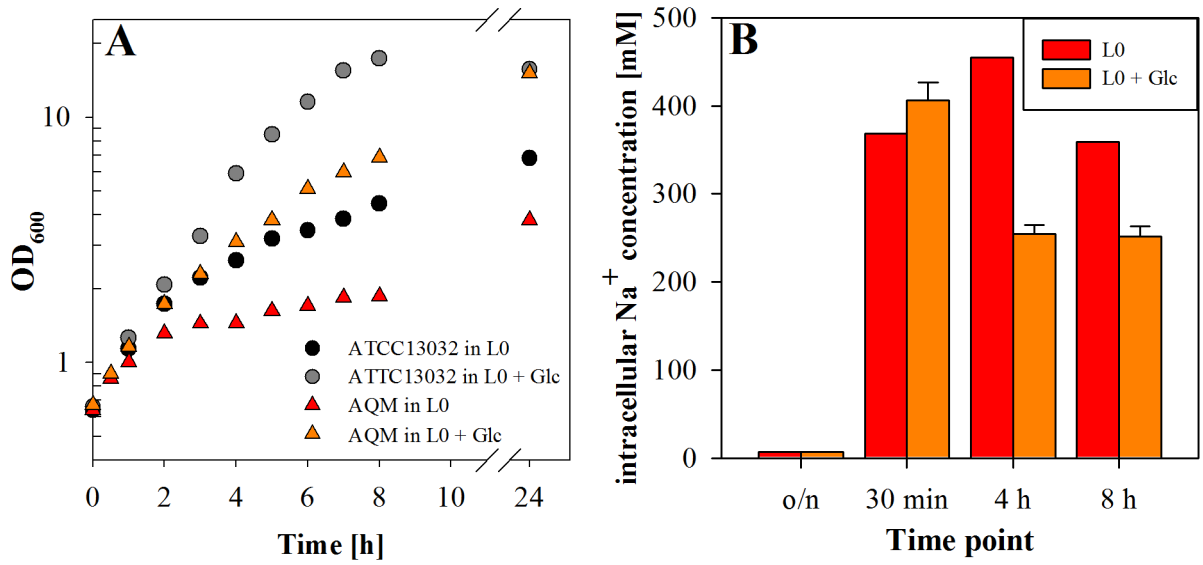

Figure 15: Growth (A) and intracellular Na^+ concentrations (B) of *C. glutamicum* AQM during growth in complex medium supplemented with glucose. After precultivation in CgXII-BTP pH 7.5, cells were grown in L_0S supplemented with 1% glucose. Intracellular cation concentrations were determined for *C. glutamicum* AQM at indicated time points. Error bars indicate the standard deviation for the internal concentration of three independent cultures.

In complex medium without added glucose the growth of *C. glutamicum* AQM was clearly impaired as observed in the previous experiment (Fig. 15A). The addition of glucose to the complex medium dramatically improved growth. The *C. glutamicum* wild type and the mutant reached a similar final optical density of about 14-15 which is consistent with the sole

use of 1% glucose for growth. For *C. glutamicum* AQM also intracellular sodium concentrations were determined. As before, the mutant cells showed very low cytosolic Na^+ concentrations after precultivation in minimal medium (Fig. 15B, o/n). After 30 minutes in complex medium, the intracellular sodium concentration increased drastically but similar for both media. Though, after four hours, cells using glucose as carbon source showed a much lower intracellular sodium concentration (455 mM $\leftrightarrow$ 255 mM). After eight hours, the difference of intracellular Na^+ concentration between both media was 110 mM. Nevertheless, the values were still very high.

This experiment shows that *C. glutamicum* cells prefer glucose as carbon source, also in complex medium. Thus the cells do not use Na^+/solute symporters and take up less sodium ions. In addition, it was shown that the carbon source uptake via Na^+/solute symporters is, at least to a significant extent, responsible for the high intracellular sodium concentration in *C. glutamicum* cation/proton antiporter deficient strains. With glucose as carbon source in addition to the complex medium ingredients, *C. glutamicum* AQM cells could grow rather well although still having a very high intracellular Na^+ concentration.

4.6 Complementation of *C. glutamicum* AQM

The investigation of antiporter deletion mutants indicated that Mrp1 functions as Na^+ exporter and Mrp2 is involved in both K^+- and Na^+-export. For ChaA and NhaP no clear results were obtained so far. For this reason complementation studies were performed using *C. glutamicum* AQM as a test strain to seperately express the different antiporter genes. For this purpose the corresponding antiporter encoding genes were cloned into the pEKEx2 expression vector. The *C. glutamicum* wild type and *C. glutamicum* AQM carrying the empty plamid were used as control strains.

4.6.1 Mrp1 and ChaA function as Na^+/H^+ antiporters within a wide pH range

First, the capability of the (putative) cation/proton antiporters to improve growth of *C. glutamicum* AQM in CgXII-BTP minimal medium with 300 mM NaCl was investigated by the seperate expression of the corresponding genes. Several pH values were tested because the function of the antiporters might be pH-dependent.

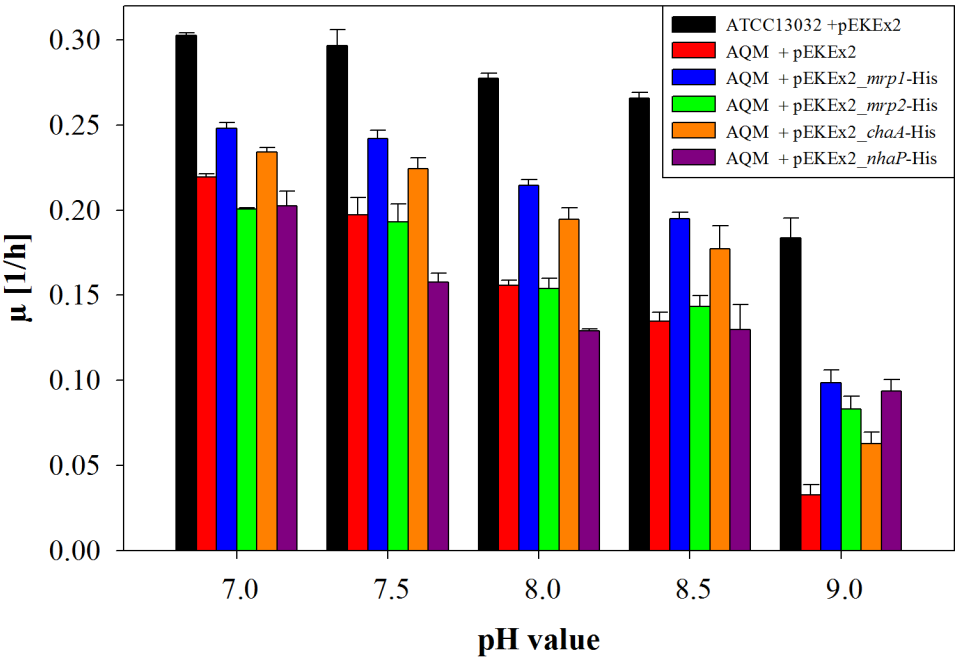

Figure 16: Growth rates of *C. glutamcium* wild type and complemented *C. glutamcium* AQM in minimal medium. Cells were grown in microtiter plates in CgXII-BTP at indicated pH values and in presence of 300 mM NaCl. For gene expression 10 μM IPTG were added. Growth rates were determined after conversion of the plate reader data according to equation 1 (p. 17). The error bars indicate standard deviations for growth rates of three microtiter plate wells.

In the range of pH 7.0 to pH 8.5 only the expression of the *mrp1* operon and the *chaA* gene improved growth of *C. glutamicum* AQM. The growth rates were a little bit higher for *C. glutamicum* AQM pEKEx2_*mrp1*-His compared to *C. glutamicum* AQM pEKEx2_*chaA*-His (Fig. 16). At pH 9.0 all four antiporters, including Mrp2 and NhaP, were beneficial for the mutant. Surprisingly, the expression of *nhaP* lead to the same growth rate as obtained with the expression of the *mrp1* operon. The expression of the *mrp2* operon did not improve the growth deficiency of *C. glutamicum* AQM which is inconsistent with the results of previous experiments. In previous experiments the *C. glutamicum* Δ*mrp1*Δ*mrp2* mutant was significantly more sodium sensitive than the *C. glutamicum* Δ*mrp1* single mutant. Thus, a function in Na^+ export was assumed which could not be fully confirmed here. However, Mrp2 was functional at pH 9 and thus it might be that the expression level was just too low to see an effect under the other conditions . Apart from that, it was shown that Mrp1 and also ChaA seem to function as Na^+ exporter within a wide pH range.

4.6.2 Mrp1, ChaA and NhaP are able to reduce the intracellular Na^+ concentration of *C. glutamicum* AQM

Another approach to prove Na^+export function was a growth experiment in complex medium. The expression of a functional Na^+/H^+ antiporter should improve growth of the antiporter-deficient mutant due to a decrease of the intracellular sodium concentration.
The expression of the *mrp1* operon completely restored the growth of *C. glutamicum* AQM back to wild type level (Fig. 17). The expression of the other antiporter genes also improved growth but to a suboptimal extent. Mutant cells expressing *chaA* reached a higher final optical density than cells expressing *mrp2* or *nhaP*.

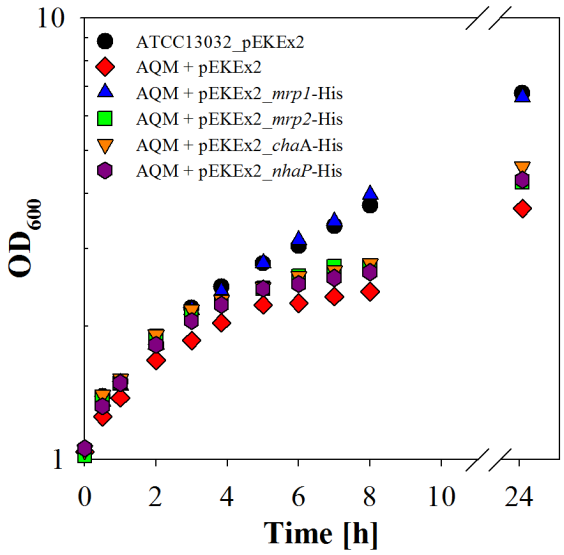

Figure 17: Growth of *C. glutamcium* wild type and complemented *C. glutamcium* AQM in complex medium. Cells were grown in L_0S after a preculture in CgXII-BTP (pH 7.5). For gene expression 10 μM IPTG were added.

As expected from the restored growth phenotype, a significant decrease of the intracellular Na^+ concentration was measured in *C. glutamicum* AQM pEKEx2_*mrp1*-His (Fig. 18A) which was accompanied by an increase of the intracellular K^+ concentration (Fig. 18B). However, in both cases wild type-like values were not reached. After 30 minutes of growth in complex medium *C. glutamicum* AQM pEKEx2_*mrp2*-His also showed a decreased intracellular sodium concentration. However, over time an increase back to a mutant-like level was observed. In this strain, the intracellular potassium concentrations were even lower than in *C. glutamicum* AQM. Again the result is contrary to the results of previous experiments. Most probably the expression level was not high enough to see a clear effect of Mrp2 function. ChaA

and NhaP were also able to reduce the amount of cytoplasmic Na^+ but to a lower extent than Mrp1. Intracellular potassium concentrations were unaltered in cells of *C. glutamicum* AQM expressing *chaA* or *nhaP*.

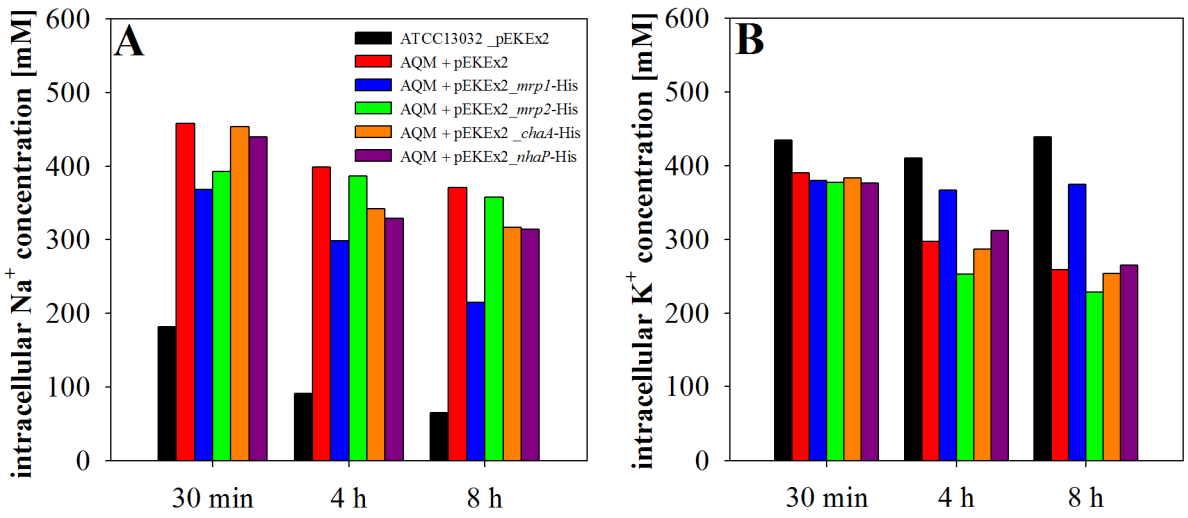

Figure 18: Intracellular cation concentrations of *C. glutamicum* wild type and complemented *C. glutamicum* AQM during growth in complex medium. Cells were grown in L_0S after precultivation in CgXII-BTP (pH 7.5). For gene expression 10 μM IPTG were added. Intracellular cation concentrations were determined at indicated time points.

Summarizing, this experiment proved that Mrp1 and ChaA are involved in sodium export. In addition, also NhaP was shown to reduce the amount of cytoplasmic Na^+ ions which hints at a function as Na^+/H^+ antiporter. The results for Mrp2 are not matching with the results obtained before. Most probably the *mrp2* operon was not expressed properly or the assembly of the protein complex in the membrane was inaccurate.

4.7 Biochemical characterization of cation/proton antiporters from *C. glutamicum*

4.7.1 Establishment of the acridine orange fluorescence measurement

The physiological characterization only gave hints on the cation exporting function of the putative cation/proton antiporters of *C. glutamicum*. However, a biochemical approach is needed to prove that these transport systems exchange cations for protons. A widely used method to do so is the acridine orange fluorescence quenching/dequenching assay. Inside-out membrane vesicles from the antiporter-deficicent *E. coli* KNabc are energized by addition of an electron donor, for example succinate. Due to respiration, protons are pumped into the vesicles resulting in the development of a proton gradient (ΔpH, acid in). At the same time also acridine orange molecules accumulate inside the vesicles which leads to fluorescence quenching.

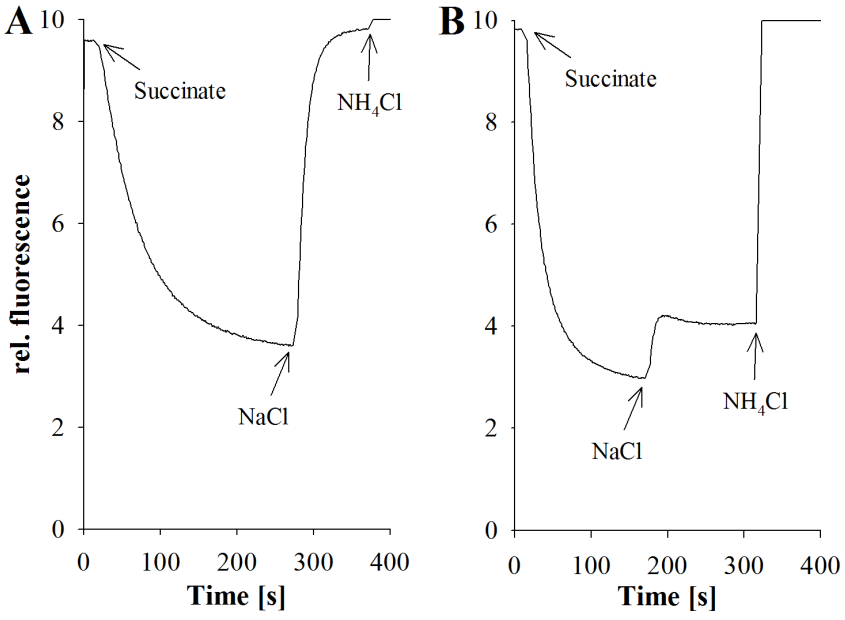

Figure 19: Na^+/H^+ antiport activities of *E. coli* NhaA (A) and *B. pseudofirmus*OF4 Mrp (B) at pH 8.0. Fluorescence-based assays of the Na^+/H^+ antiport activities in *E. coli* KNabc vesicles were conducted at pH 8.0 and 2.5 mM NaCl. The complete fluorescence measurement is shown. The assay protocol is described in *Material&Methods*.

After the steady state is reached, a monovalent cation is added as substrate for antiport. The antiporter-mediated transport of the cations into the vesicles leads to a concomitant release of protons. This in turn results in the release of acridine orange molecules and thereby in an increase of fluorescence. The activities of the tested antiporters are assessed by the percent of dequenching after cation addition.

To establish this assay, vesicles containing the *E. coli* NhaA and the *B. pseudofirmus* OF4 Mrp antiporters were prepared. Both antiporters are described as Na^+/H^+ antiporters and should show a high activity in the assay [48, 126]. At the beginning a high fluorescence was recorded which dropped immidiately upon the addition of succinate, displaying the establishment of a ΔpH (Fig. 19). The addition of 2.5 mM NaCl resulted in an increase of fluorescence and showed that Na^+ ions were transported into the vesicles. To completely abolish the ΔpH, 10 mM NH_4Cl were added which further increased fluorescence. Both *E. coli* NhaA and *B. pseudofirmus* OF4 Mrp showed high activity, the latter one to a lesser extent than NhaA (Fig. 19).

This experiment showed that the acridine orange fluorescence assay indeed functions in our lab as described in the literature and can be used to investigate the cation/proton antiporters of *C. glutamicum*.

4.7.2 *C. glutamicum* antiporters accept Na^+ and K^+ ions with different preferences

First of all, the four cation/proton antiporters of *C. glutamicum* were subjected to the experiment described in the previous section to check whether they are capable of Na^+/H^+- and/or K^+/H^+-antiport. Expression of the antiporters was performed in *E. coli* KNabc which is devoid of the cation/proton antiporters NhaA, NhaB and ChaA. Antiporter genes were under the control of their own promotors except for *mrp1* which was cloned behind the $nhaA_{E.\ coli}$ promotor to improve the expression. The activities are shown as the percent of dequenching after the addition of the cation whereas background activities were subtracted.

Mrp1 was shown to transport Na^+ ions as well as K^+ ion whereas in both cases the activities were higher at pH 8.5 compared to pH 8.0 (Fig. 20, top left). In addition, sodium/proton antiport activity was much higher than potassium/proton antiport activity. Mrp2 showed a higher activity with Na^+ ions at pH 8.5 compared to pH 8.0 (Fig. 20, top right). For potassium the measured activities were similar for both pH values. The same was observed for the Na^+/H^+ antiport activity of ChaA (Fig. 20, bottom left). With K^+ ions as substrate

the activity of ChaA was higher at pH 8.0 than at pH 8.5. NhaP also transported both cations (Fig. 20, bottom right). In the case of sodium the activity increased with increasing pH. Activities for potassium were higher than for sodium but similar for both pH values.

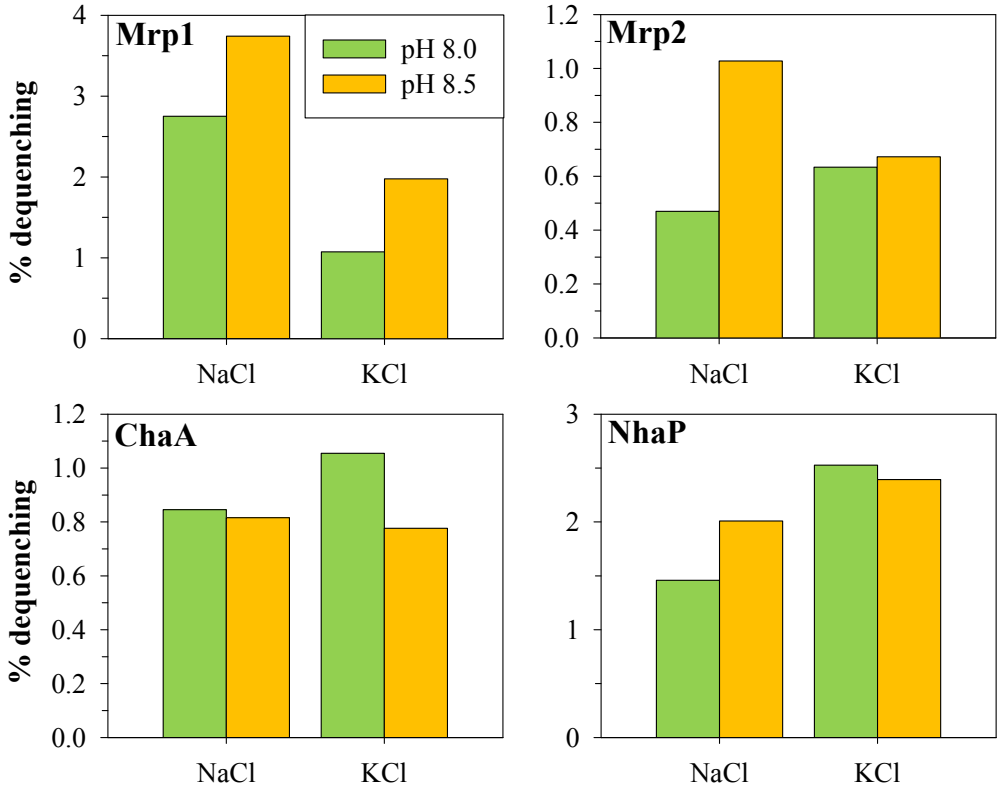

Figure 20: Na^+/H^+ and K^+/H^+ antiport activities of *C. glutamicum* cation/proton antiporters.
Fluorescence-based assays of the Na^+/H^+ and K^+/H^+ antiport activities in *E. coli* KNabc vesicles were conducted at indicated pH values and 2.5 mM NaCl or KCl. Vesicles are energized by addition of succinate. Due to respiration, protons are pumped into the vesicles resulting in the development of a proton gradient (ΔpH, acid in). At the same time acridine orange molecules accumulate inside the vesicles which leads to fluorescence quenching. After the steady state is reached, the cation is added. The antiporter-mediated transport of the cations into the vesicles leads to a concomitant release of protons. This in turn results in the release of acridine orange molecules and thereby in an increase of fluorescence. The activities are assessed by the percent of dequenching after cation addition.

The above measurements showed that all antiporters are able to transport Na^+ ions and K^+ ions. Mrp1 showed a preference for sodium whereas NhaP preferred potassium. These results are in agreement with the results of the physiological characterization. In the cases of ChaA and Mrp2 no significant preference was observed. At least for Mrp2 a tendency towards potassium as substrate rather than sodium was observed which supports the physiological data obtained with deletion mutants.

4.7.3 Mrp1 is a $Na^+(K^+)/H^+$ antiporter with low K_m values

Vesicles containing Mrp1 showed high cation/proton antiport activities and were therefore subjected to a more detailed investigation. First, the ability of Mrp1 to complement the growth deficiency of the *E. coli* test strain was tested. *E. coli* KNabc lacks the main cation/proton antiporters NhaA, NhaB and ChaA. For this reason cells are not able to grow in presence of high extracellular NaCl concentrations (Fig. 21, black circles). In contrast, cells expressing the *mrp1* operon from *C. glutamicum* under control of the *E. coli nhaA* promotor grew well after a lag phase (Fig. 21, white circles). The fact that Mrp1 is able to complement the sodium-sensitive phenotype of the *E. coli* test strain proves that the antiporter was functionally expressed and fulfilled the same function, i.e. Na^+ export, as in the native host.

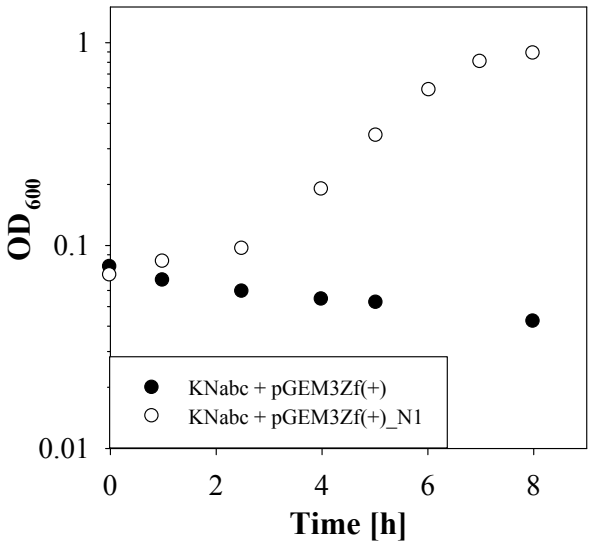

Figure 21: Growth of *E. coli* KNabc+pGEM3Zf(+) and *E. coli* KNabc+pGEM3Zf(+)_N1 with 100 mM NaCl. Cells were grown in LBK complex medium (pH 7.5) supplemented with 100 mM NaCl.

To characterize the cation/proton antiport function of the *C. glutamicum* Mrp1 in more detail, the apparent K_m values were determined with the acridine orange fluoerscence assay. The determination and comparison of v_{max} makes no sense because it mainly depends on the expression level which in turn differs between different antiporters and also between different vesicle preparations of the same antiporter.

For Na^+/H^+ antiport a pH of 8.5 was chosen, K^+/H^+ antiport was investigated at pH 8.95. The apparent K_m values were determined twice for two independent vesicle preparations. Apparent K_m values of 0.65 mM and 0.46 mM were determined for Na^+ as a substrate (Fig. 22A). Using K^+, values of 0.94 mM and 0.82 mM were obtained (Fig. 22B). In both cases the K_m values were low but within the range of published data for other cation/proton antiporters [29, 46, 109, 126]. Interestingly, there is only a small difference in the apparent K_m for Na^+ ions and K^+ ions.

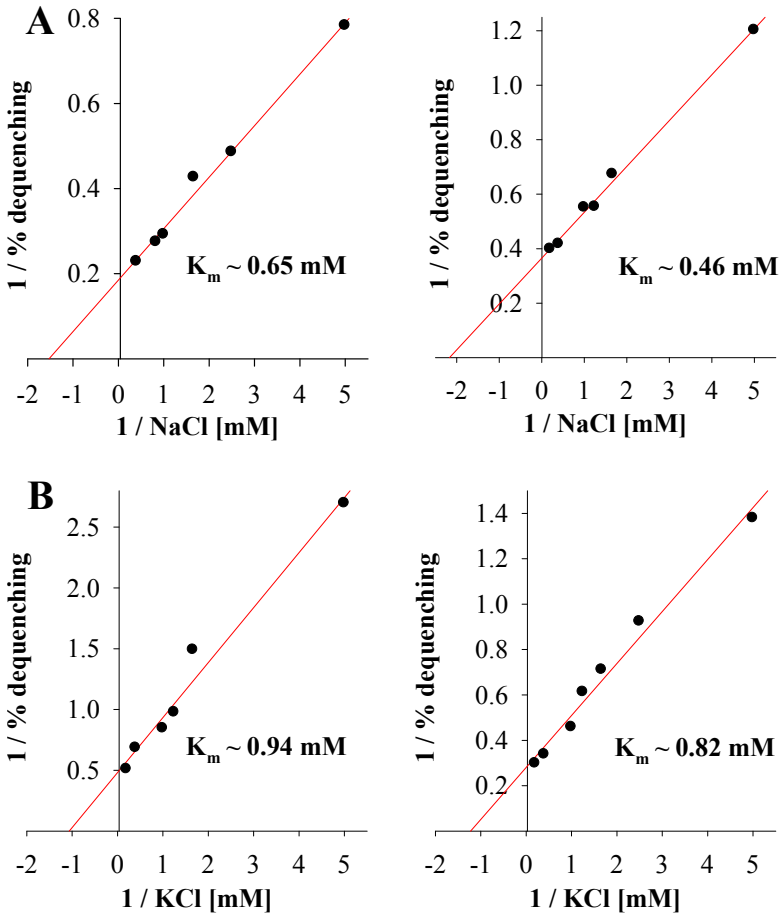

Figure 22: Determination of kinetic parameters of *C. glutamicum* Mrp1 in everted membrane vesicles. Fluorescence-based assays of the Na^+/H^+ (A) and K^+/H^+ (B) antiport activities in *E. coli* KNabc vesicles were conducted at pH 8.5 and pH 8.95, respectively, over a range of concentrations of added NaCl and KCl; the apparent K_m was determined for two independent vesicle preparations. Vesicles are energized by addition of succinate. Due to respiration, protons are pumped into the vesicles resulting in the development of a proton gradient (ΔpH, acid in). At the same time acridine orange molecules accumulate inside the vesicles which leads to fluorescence quenching. After the steady state is reached, the cation is added. The antiporter-mediated transport of the cations into the vesicles leads to a concomitant release of protons. This in turn results in the release of acridine orange molecules and thereby in an increase of fluorescence. The activities are assessed by the percent of dequenching after cation addition. Double reciprocal plots are shown.

4.7.4 NhaP is a $K^+(Na^+)/H^+$ antiporter with clear preference for potassium

The general biochemical characterization of the cation/proton antiporters from *C. glutamicum* (see section 4.7.2) also showed high activities for NhaP and more interestingly, a clear preference for potassium. For this reason, everted membrane vesicles containing NhaP were subjected to a determination of kinetic parameters whereas pH 8.5 was chosen for both Na^+/H^+- and K^+/H^+-antiport activities.

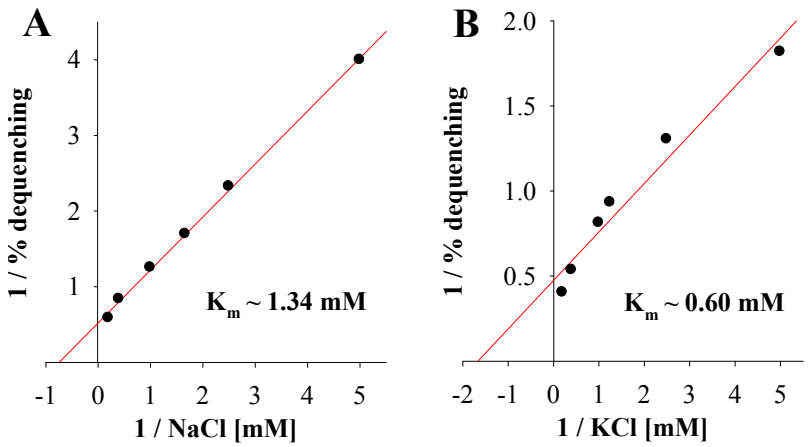

Figure 23: Determination of kinetic parameters of *C. glutamicum* NhaP in everted membrane vesicles. Fluorescence-based assays of the Na^+/H^+ and K^+/H^+ antiport activities in *E. coli* KNabc vesicles were conducted at pH 8.5 over a range of concentrations of added NaCl and KCl. Vesicles are energized by addition of succinate. Due to respiration, protons are pumped into the vesicles resulting in the development of a proton gradient (ΔpH, acid in). At the same time acridine orange molecules accumulate inside the vesicles which leads to fluorescence quenching. After the steady state is reached, the cation is added. The antiporter-mediated transport of the cations into the vesicles leads to a concomitant release of protons. This in turn results in the release of acridine orange molecules and thereby in an increase of fluorescence. The activities are assessed by the percent of dequenching after cation addition. Double reciprocal plots are shown.

For Na^+ as a substrate an apparent K_m of 1.34 mM was determined (Fig. 23A). For potassium a K_m of 0.6 mM was obtained (Fig. 23B). These results show that NhaP prefers K^+ ions to Na^+ ions with the K_m for sodium being more than twice as high. Compared to Mrp1, NhaP has a lower affinity for Na^+ ions. However, the the affinity for K^+ ions is higher and the K_m of 0.6 mM is probably one of the lowest reported so far for bacterial K^+/H^+ antiporters.

5 Discussion

5.1 Ion homeostasis is crucial for all living cells

5.1.1 Importance of ion homeostasis for bacterial stress response

C. glutamicum is the major work horse for the biotechnological large-scale production of amino acids, especially L-glutamate und L-lysine which have an annual market of 2.5 and 1.5 million tons [7, 32]. In addition, *C. glutamicum* is the organism of choice for the production of the purine ribonucleoside 5'-monophosphates guanylic acid (GMP), inosinic acid (IMP) and xanthylic acid (XMP) which act as strong flavor enhancers [21, 22, 23, 121].
During fermentation cells are subjected to a variety of different stress factors. Changes in temperature and elevated CO_2 concentrations mainly occur due to the high cell densities used in large bioreactors and insufficient stirring leads to local pH shifts in the medium [67, 72]. In addition, high concentrations of both substrates and products cause osmotic stress which in turn affects important production parameters. It could be shown that the biomass formation rate is decreased in medium with high osmolarity [110]. Furthermore, the uptake of PTS sugars is inhibited by increasing osmolarity [49]. But also production rate and product yield are affected [132].
Nonionic stress leads to water efflux out of the cell which is compensated by the accumulation of compatible solutes, ionic stress leads to ion influx which need to be exported again [14, 107, 139]. It is obvious that the transport of ions plays an important role for *C. glutamicum* in order to cope with these stress conditions. Cation/proton antiporters are involved in both pH- and cation homeostasis in bacteria and thus are of fundamental importance for cellular fitness and performance of production strains. The identification of cation/proton antiporters as well as their detailed characterization could help to further improve the stress resistance for biotechnological large-scale productions.

5.1.2 Cation/proton antiport is an important pathogenicity factor

Besides its importance in biotechnology, *C. glutamicum* also serves as an important model organism for dangerous human pathogens like *Mycobacterium tuberculosis, Mycobacterium leprae* and *Corynebacterium diphtheriae*, all belonging to the actinomycetes suborder *Corynebacterineae*. As a close relative *C. glutamicum* can be used to identify putative drug targets and investigate them in detail. One group of drug targets are transporters that are involved in ion homeostasis, such as cation/proton antiporters. The knowledge about the function of the antiporters is the first step to develop new drugs and therapeutical strategies that have minimal side effects but an acceptable antimicrobial potency.

Cation/proton antiporters play a crucial role in ion homeostasis of all living cells. In many studies it could be shown that Na^+/H^+- and K^+/H^+-antiporters are involved in cytoplasmic pH homeostasis, resistance to elevated temperature and tolerance to alkali and to osmolality fluctuations [101, 120]. These properties are of high impact for pathogenic species because they have to deal with different and changing environments, both inside and outside their host organism. Thus an extensive homeostatic capacity is required to adapt to changing pH and ionic conditions. Cation/proton antiporters are able to confer resistance to sodium and/or potassium and are able to support pH homeostasis. For this reason their presence displays a great advantage for a pathogenic bacterium. Especially sodium bioenergetics is thought to play a crucial role in virulence because pathogens often use a sodium motive force (smf) in addition to a proton motive force (pmf) [42, 58]. The smf in turn can be established by Na^+ pumps and/or Na^+/H^+ antiporters which leads back to cation/proton antiporters as putative drug target.

For example, the motility of *Vibrio cholerae*, the causative agent of cholera, is Na^+ dependent because its flagellar motor is driven by a sodium motive force. Motility in turn is essential for colonization and thus the loss of motility results in a significant decrease of virulence [52, 59, 75]. For this reason the disturbance of Na^+ gradient formation could be an efficient way to inhibit the colonization by *V. cholerae*. Consistent with this, it could be shown that the loss of cation/proton antiporters has severe effects for many pathogens. The Gram-negative, opportunistic pathogen *Pseudomonas aeruginosa* is found in nature (soil) as well as in hospitals where it causes chronic pulmonary as well as systemic infections. The disruption of one gene of the multigene-encoded Na^+/H^+ antiporter Sha (Mrp-type) made *P. aeruginosa* highly sodium sensitive, thus reducing the capability to adapt to the Na^+ environment inside

the host cells [76]. As a result, the virulence was attenuated due to a decreased colonization of the infected organs in mice. *Yersinia pestis*, the causative agent of bubonic plague, also shows a completely attenuated phenotype and a decreased survival in blood when the two major Na^+ extruding systems NhaA and NhaB are missing [42]. These two antiporters are required for resistance to the high salt concentrations in blood (135-155 mM Na^+). But also pathogenic yeast, such as some *Candida* species which cause a wide range of infections, rely on cation/proton antiporters that mediate tolerance to high external alkali metal cation concentrations and thus ensure virulence [79].

In this work it could be shown that the lack of the two Mrp antiporters in *C. glutamicum*, predominantly Mrp1, results in a severe Na^+-sensitive phenotype (Fig. 10). This might also be true for pathogenic representatives of the *Corynebacterineae*, thus rendering the Mrp antiporters putative drug targets like in other pathogenic bacteria.

The above examples show that both, the complex Mrp-type antiporters as well as the more simple Nha-type antiporters, could be interesting drug targets. For Nha-type antiporters there are already inhibitors known. The diuretic drug amiloride and its 5-aminoalkylated derivatives are able to inhibit NhaB from *E. coli* and NhaA from *Vibrio parahaemolyticus* [30, 85]. In addition, amiloride inhibits the motility of *Vibrio alginolyticus* and thus prevents the colonization [4]. Amiloride does not work for the rather famous NhaA from *E. coli* but 2-aminoperimidine, an amiloride analog, works and also inhibits the *Y. pestis* and *V. cholerae* NhaA antiporters [24, 42]. Unfortunately, these drugs are also potent inhibitors of mammalian Na^+/H^+ antiporters from the NHE family, i.e. they are toxic for mammals [96]. For this reason, further investigation and improvement is necessary. For Mrp-type anitporters no inhibitors are known so far. Nevertheless, they represent an excellent putative drug target because Mrp proteins are not related to eukaryotic Na^+/H^+ antiporters and other eukaryotic proteins [76]. Thus a drug inhibiting Mrp antiporters would only affect the pathogenic cation homeostasis.

5.2 Does *C. glutamicum* need all its cation/proton antiporters?

Cation/proton antiporters play crucial roles in bacteria, mainly in cytoplasmic pH homeostasis at external alkaline pH and in Na^+/K^+ resistance [60, 101, 105, 125]. Because of these fundamental physiological functions most bacteria harbour several genes encoding Na^+/H^+- and/or K^+/H^+-antiporters. The genomes of most non-marine bacteria contain five to nine genes that are predicted to encode Na^+/H^+- or K^+/H^+-antiporters [83]. Generally it is thought that bacteria exceedingly exposed to a great diversity of different, often changing stress factors have more antiporters than pathogenic species that live inside host cells. For example, pathogens like *Pseudomonas aeruginosa* and *Vibrio cholerae* have to cope with different environments, inside and outside their host organisms. For this reason they have 11 and 14 (putative) antiporters, respectively [103]. In these cases the large number of antiporters correlates with the different and changeing lifestyles of the bacteria. In contrast, bacteria primary living inside other organisms often lack antiporter encoding genes, such as the aphid endosymbiont *Buchnera aphidicola*, or only have one antiporter, such as the typhus causing pathogen *Rickettsia prowazekii* [103]. However, it has to be mentioned that intracellular pathogens are rather umcommon and display a very specialized group of bacteria.
Besides the need of cation/proton antiporters to cope with lifestyle associated stress conditions, the current status is that good explanations are lacking for why so many antiporter encoding genes are maintained in the bacterial genomes.
With its four identified cation/proton antiporters, *C. glutamicum* harbours an average number of antiporter encoding genes. The biochemical characterization of these antiporters revealed that, in general, all four antiporters are able to transport sodium ions as well as potassium ions (Fig. 20). However, this fact does not necessarily reflect their physiological function. For Mrp1 and NhaP the results of the physiological and the biochemical investigations were in agreement. Mrp1 prefers Na^+ ions to K^+ ions and the vice versa situation was observed for NhaP. Due to low activities, Mrp2 and ChaA could not intensively be investigated on the biochemial level. However, the physiological data indicate a main function in potassium and sodium export, respectively. So *C. glutamicum* contains four different cation/proton antiporters with different preferences and affinities. But does *C. glutamicum* need all of them?
The physiological characterization of *C. glutamicum* antiporter deletion mutants showed that NhaP and ChaA are only of minor physiologic importance under the tested conditions. The

single deletion of the corresponding genes did not have any effect in regard to salt sensitivity (Fig. 7). And also the deletion of these genes in the *C. glutamicum* Δ*mrp1*Δ*mrp2* background, resulting in a complete Na^+/H^+- and K^+/H^+-antiporter deficient strain, did not increase the salt sensitivity (Fig. 10). In contrast to these results, the sole removal of the *mrp1* or the *mrp2* operon results in (severe) Na^+- or K^+-sensitivity, respectively (Fig. 5 and 6). Thus the two Mrp antiporters are the main and most important cation extrusion systems in *C. glutamicum*. ChaA and NhaP were not required in presence of the high salt concentration that were applied in the experiments. Nevertheless, it might be that they play a role in pH homeostasis. To prove this assumption, pH shock experiments should be performed in which the constructed mutant strains are subjected to a quick change of the extracellular pH. The investigation of subsequent growth would give information about the long-term effects. The short-term effects on internal pH and pH homeostasis capacity can be investigated with the fluorescence protein pHluorin [90]. This ratiometric GFP variant allows the expression-independent determination of internal pH and a setup working for *C. glutamicum* was already established [70].

As a soil bacterium *C. glutamicum* is likely to be exposed to a changing but not extreme extracellular environment. Main challenges are changes in external osmolarity and pH. During water stress cells have to cope with hypoosmotic conditions. In contrast, cells are exposed to high salt concentrations during drought stress. Normally, the concentration of dissolved K^+ is 5-10 ppm, corresponding to about 0.13-0.26 mM [Schulte and Kelling]. Others report generally low potassium concentrations of 0.1-10 mM for bacterial environments [6]. For the sodium concentrations in soil no information was found in literature but the values are thought to be higher than that of potassium. The above data show that *C. glutamicum* might be rarely exposed to salt concentrations as high as used in the experiments (300 mM NaCl, 600 mM KCl). This observation makes it difficult to answer the question whether *C. glutamicum* needs all its cation/proton antiporters in its natural habitat. However, drought stress experiments, for example, could be performed to approach this question. The experiments in this work showed that at least under experimental conditions the two Mrp systems are able, and required, to mediate sufficient resistance to high Na^+- and high K^+-concentrations. Probably *C. glutamicum* uses at least some of its cation/proton antiporters for pH homeostasis. This hypothesis was not investigated in this work. However, for other bacteria cation/proton antiporters were described to participate in pH homeostasis and thus they display a possibility for *C. glutamicum* to cope with naturally occuring changes in extracellular pH.

5.3 *C. glutamicum* not only tolerates high $[K^+]_i$ but also high $[Na^+]_i$

In a world which is relatively rich in Na^+ but rather low in K^+, living cells once made a decision to accumulate potassium but exclude the smaller sodium ions from their cytoplasm [5, 36]. So it comes that the intracellular potassium concentration is usually higher than the extracellular concentration whereas it is vice versa for sodium [133]. The exclusion of Na^+ ions is an efficient way to energize the membrane with an electrochemial sodium potential which can be used for a variety other transport processes [5]. Further, potassium accumulation can be used to maintain and regulate cytoplasmic osmotic pressure [35].

Under medium osmolality conditions most bacteria show an intracellular K^+ concentration of 200-500 mM whereas values increase with increasing external osmolality [5]. For example, *E. coli* normally has an intracellular potassium concentration of 210-285 mM. Under high osmotic conditions values of up tp 495 mM were observed [26, 37, 122]. For *V. alginolyticus* values of 400 mM K^+ were reported [5]. Halophilic bacteria generally show higher values of about 500-700 mM K^+ which seem to be relatively constant and do not dependent on the extracellular NaCl concentration [133]. Compared to other organisms, bacterial intracellular K^+ concentrations are quite high. The yeast *Saccharomyces cerevisiae* only accumulates 200-300 mM K^+ and plants usually contain even lower cytosolic concentrations of 50-150 mM [2, 80, 142]. Like other Gram-positive bacteria, for example *B. subtilis*, *C. glutamicum* maintains very high intracellular K^+ concentrations [137]. Under normal growth conditions up to 600 mM were observed but under osmotic stress conditions values over 700 mM are possible [98, 99]. The cytosolic potassium concentrations of about 400-500 mM which were measured for the *C. glutamicum* wild type in experiments of this study fit well into the published concentration range.

As mentioned before, in order to maintain the electrochemical sodium potential all living cells tend to keep their intracellular Na^+ concentrations very low. *E. coli* maintains sodium concentrations of maximally 60-70 mM in minimal medium [37, 122]. For *V. alginolyticus* a concentration of 80 mM was reported [5]. Plants are highly sodium sensitive and keep their cytosolic Na^+ concentrations even lower (10-30 mM) [9, 80]. So far no information was published about the intracellular sodium concentrations of *C. glutamicum*. In this study it was shown that the *C. glutamicum* wild type maintains suprisingly low intracellular Na^+ concentrations of about 4.8-8.5 mM in MMI minimal medium and about 1.2 mM in CgXII minimal

medium during the stationary phase (Fig. 5 and 13). These values are at least one order of magnitude lower than reported for other bacteria (see above). Due to the need to use Na^+/solute symporters, higher intracellular sodium concentrations in the range of 50-90 mM (after four hours) were measured for cells grown in complex medium (Fig. 13, 14 and 18). So far, the determined Na^+ concentrations for *C. glutamicum* seem to fit very well into the frame of common assumptions and resemble data published for other bacteria. Therefore it was very surprising to see that the *C. glutamicum* AQM strain expressing the *mrp1* operon could grow like the wild type in complex medium although its intracellular sodium concentration was significantly increased to about 300 mM after four hours of growth (Fig. 17 and 18). These two observations seem to be conflicting because there is the common assumption that high (intracellular) Na^+ concentrations are toxic and thus growth inhibiting [5, 9]. Nevertheless, *C. glutamicum* seems to be able to cope with this situation quite well and thus might be an exception among non-halophilic bacteria. For most halophilic bacteria intracellular sodium concentrations in the range of about 300-600 mM were reported, with some exceeding up to 1 M Na^+ [133].

5.4 Can Na^+ ions replace K^+ ions?

The surprising discovery that *C. glutamicum* tolerates high intracellular sodium concentrations also revealed another interesting fact: whenever the intracellular Na^+ concentration was increased, a decreased intracellular K^+ concentration was observed. In MMI minimal medium *C. glutamicum* AQM accumulated about 100 mM more Na^+ than the wild type, but at the same time the K^+ concentration was reduced by about 210 mM (Fig. 9). After four hours in complex medium the differences between *C. glutamicum* wild type and *C. glutamicum* AQM were as follows: the Na^+ concentration was increased from about 65 mM to about 380 mM, the K^+ concentration was reduced from about 450 mM to about 240 mM (Fig. 13). The expression of the *mrp1* operon resulted in a reduction of cytosolic sodium and a concomitant increase in potassium (Fig. 18). A similar effect was observed in *S. cerevisiae* where intracellular potassium decreases in the case of sodium influx [2].

These observations raise several questions. Do the cells actively adjust the intracellular potassium concentration in response to the sodium concentration in order to maintain a constant cytoplasmic ion strength? And is it, in general, possible that K^+ ions are, at least to a certain extent, replaced by Na^+ ions?

First of all a comparison of the properties of Na^+ ions and K^+ ions is necessary. On the structural and on the chemical level sodium and potassium are very similar. As monovalent cations they do not participate in covalent binding which makes them suitable as intracellular osmolytes [9]. All the processes inside the cell proceed in aqueous solution. For this reason, the water molecules form hydration shells around the ions. The strength of the interaction with the water molecules as well as the size and the stability of the hydration shell depends on the ion and is differnt also for similar ions such as Na^+ and K^+ [9]. Sodium ions tend to interact rather strong with surrounding water molecules whereas the hydration shell of potassium is much weaker. According to that, Na^+ ions always prefer environments that resemble this exact hydration shell and thus they tend to distort their surroundings. Potassium ions are much more flexible which is important for the interaction with proteins for example [13, 18, 19]. Thus, a replacement of potassium by sodium in enzyme reactions does not seem advisable. However, the biophysical role in the maintenance and the regulation of cytoplasmic osmotic pressure can by taken over by sodium ions. For bacteria there is no information available, but plants use this strategy under conditions of limited potassium supply. Normally, plants use K^+ accumulation for vacuole and cell expansion [9]. If potassium becomes limiting, plant cells transport potassium ions from the vacuole into the cytoplasm. The cytoplasmic potassium concentration has to be kept at a certain level in order to permit K^+-dependent processes [135]. But at the same time the osmotic pressure of the vacuole also has to be maintained. For this purpose sodium ions and other solutes are transported into the vacuole, provided that the plant is able ot take up Na^+ ions and translocate them to the shoot. So plants show that K^+ ions might be the most advantageous cations but to a given extent they can be interchanged with Na^+ ions.

As known, bacteria don't have vacuoles but the cells themselves have to maintain a certain turgor pressure. Thus the opportunistical usage of sodium ions instead of potassium ions should be possible for this purpose. This strategy is relevant whenever the cytosolic sodium concentration is elevated, such as under conditions of high extracellular Na^+ concentrations, or in the case that carbon source uptake is mediated by sodium-solute symporters. The main

problem is that sodium ions are toxic and thus negatively affect cytosolic processes with increasing concentrations. A complete replacement is impossible because K^+ ions are essential for the functionality of a number of enzymes and therefore a certain intracellular concentration has to be maintained to ensure the optimal enzyme function [80]. Certainly, the topic of Na^+/K^+ interchangeability has to be investigated in more detail in bacteria.

The remaining question is how the reciprocal behavior of intracellular Na^+- and K^+ concentrations can be explained. One possibility is that both fluxes are directly linked to each other. This assumption would suppose that the cell is able to actively regulate the influx and efflux of both sodium ions and potassium ions according to the current cytoplasmic ion content and the environmental cation availability. The hypothesis of linked fluxes to explain the reciprocal relationship between Na^+ and K^+ movements has been suggested for erythrocytes and other tissues [47, 74]. However, it seems that this is not the explanation for *E. coli*, mouse ascites tumor cells, rabbit polymorphonuclear leukocytes and plant cells [34, 57, 122].

For plants it could be shown that high extracellular sodium concentrations disrupt potassium homeostasis by suppressing the influx of K^+ ions [81, 82]. In addition, a sudden osmotic shock aroused by a high extracellular NaCl concentration leads to a release of cellular contents, including potassium ions. Thus an impaired influx and an enhanced efflux of potassium ions come together and could explain the decrease of potassium in a situation of high sodium concentrations.

Whether Na^+ and K^+ fluxes are linked in *C. glutamicum* has to be investigated. The assumption of inhibited potassium uptake due to high extracellular Na^+ could be tested with the following experiment. *C. glutamicum* wild type cells from the stationary phase normally show a low intracellular potassium concentration (<100 mM) but accumulate the cation again after transfer to fresh medium (Fig. 13). Measuring the intracellular K^+ concentrations before and after the transfer to medium containing a high amount of NaCl should reveal whether potassium uptake is still possible. In addition, the radioactive measurement of K^+ uptake rates upon the application of high extracellular Na^+ concentrations could give even more detailed information.

Besides the above assumption, there is another explanation for the reciprocal behavior of the intracellular cation concentrations. In order to maintain a constant cytoplasmic ion strength, the cells might actively adjust their internal potassium concentration in response to a changed sodium concentration. Such changes occur due to passive influx at high extracellular con-

centrations or during uptake of substrates transported by Na^+/solute symporters. Prerequisite for the above assumption is that *C. glutamicum* tends to maintain a certain cytoplasmic ion strength, i.e. the sum of Na^+ and K^+ should always be the same. Figure 24 shows that this requirement is met. The *C. glutamicum* wild type and *C. glutamicum* antiporter deficient mutants show a similar sum of Na^+- and K^+ concentrations although the contribution of the two cations is different for the strains (compare to figures 5,6, 9).

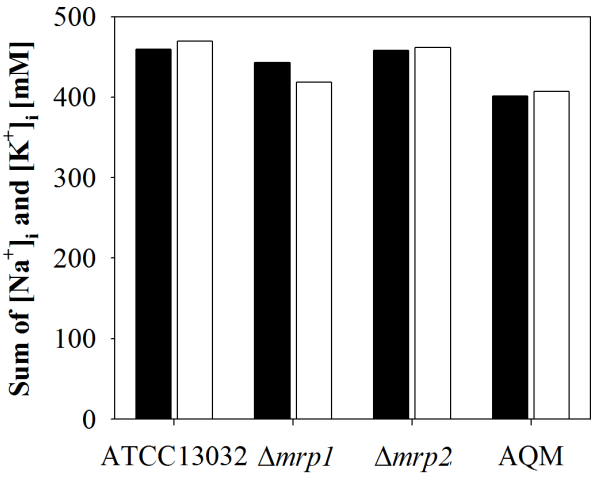

Figure 24: The sum of internal [Na^+] and internal [K^+] for *C. glutamicum* wild type and antiporter deficient mutants. To determine intracellular cation concentrations cells were incubated for one hour in MMI minimal medium (20 mM Na^+, 20 mM K^+) at indicated pH values and washed twice in 20 mM BTP (+10 mM KCl, +5 mM NaCl) at same pH and osmolality. Intracellular concentrations are the means from two independent experiments. ■ pH 7.5, □ pH 8.3

An explanation could be as follows. Whenever an increased amount of sodium ions enter the cell, for example under conditions of a high extracellular sodium concentration or growth in complex medium, the intracellular Na^+ concentration increases. But normally *C. glutamicum* already contains a high amount of K^+ ions. Thus the intracellular ion strength increases. The only possibility to change this situation is the export of either sodium or potassium. As sodium ions would directly re-enter the cell, the export of potassium ions is the best option. Supportingly, the cells should down-regulate potassium uptake to avoid a futile cycle. The fact that the described regulation mechanism also works in *C. glutamicum* AQM suggests that none of the cation/proton antiporters is responsible for potassium export. So far no other potassium exporter is known in *C. glutamicum*.

5.5 Dependency of cation/proton antiport on pH

An intersting observation that applies to all Na^+/H^+ antiporters investigated so far is their pH-dependent activity. Most antiporters show an increasing activity with increasing pH. So the shape of the pH profile curve can be bell-shaped, with decreasing activity if the pH gets too high, like in the case of *V. cholerae* NhaD, for example [53]. Another possibility is a S-shaped curve with remaining high activity at high pH like it was described for the NhaA antiporters of *V. cholerae* and *E. coli* as well as for some Mrp antiporters (Fig. 25 and [102, 126]). These antiporters are active at alkaline pH and downregulated at acidic pH. However, there is at least one cation/proton antiporter described which is only active at pH 7 and below: NhaP of *Methanococcus jannaschii* [56]. In this case the antiporter is shut off at alkaline pH. The interesting question is how the downregulation at acidic or alkaline pH works.

For a long time it was suggested, at least for the *E. coli* NhaA, that a pH-sensitive site (pH sensor) regulates the activity of the antiporter in a pH-dependent manner [46]. But electrophysiological studies with liposomes showed that there is no need for a pH sensor because the transport mechanism is self-regulating [17]. Generally, secondary active transporters are thought to function according to the alternate access model that involves a common binding site which is alternatingly accessible from both sites of the membrane [88]. Thus, in the case of a cation/proton antiporter, both ions always compete for the binding pocket although not to the exact same binding site. In the case of the *E. coli* NhaA, an acidic pH at the cytoplasmic side prohibits the effective binding of sodium ions to the transporter, effectively increasing the K_m value for Na^+. Therewith the turnover, i.e. sodium ion export, is automatically slowed down and an overacidification of the cytoplasm is prevented. This mechanism explains why NhaA is active at pH 8.5 but not at pH 6.5 [88, 102]. However, it has to be mentioned that this pH-dependent inactivation refers to conditions of symmetrical pH on both sides of the liposomes and a transport driving Na^+ gradient. Only in this case the competition of Na^+ and H^+ ions for the binding site leads to a shut off at a pH below 7. The application of symmertic sodium concentrations and a driving pH gradient (high at the Na^+ uptake side) results in NhaA activity down to pH 5 [88]. The latter observation eliminated the theory about a pH sensor shut off in NhaA.

The *Methanococcus jannaschii* NhaP antiporter belongs to the very rare cation/proton antiporters that are active at acidic pH (maximal activity at pH 6) and shut off at alkaline pH [56, 102]. The physiological function is still unclear but it was suggested that it works in both directions, using pmf or smf, and might be responsible for pH homeostasis [134]. However, this antiporter is downregulated at alkaline pH and therefore a model was proposed which is different to that described for the *E. coli* NhaA. For the case that this antiporter exports protons, a substrate depletion mechanism was suggested [17]. If the cytoplasm becomes alkaline there are less protons available to be transported, thus the transport slows down and further alkalinization is prevented. Proton-depletion as a mechanism for self-regulating antiport activity was also suggested for the mammalian NHE1 antiporter which is activated at acidic cytoplasmic pH to function as H^+ exporter [3]. So far the model explained above was only described for Na^+/H^+ antiporters. However, the general mechanism of ions competing for the same binding site could also be true for K^+/H^+ antiporters.

The present work showed that all four, initially putative cation/proton antiporters of *C. glutamicum* are able to mediate Na^+/H^+- and K^+/H^+-antiport (Fig. 20). They showed different cation preferences but the activity was not always higher at alkaline pH as it is published for most of the other cation/proton antiporters investigated so far. However, among the four investigated antiporters in this study, Mrp1 showed higher activity at alkaline pH for both Na^+/H^+ antiport and K^+/H^+ antiport.

Like Mrp antiporters from other bacteria (Fig. 25A), Mrp1 also represents a pH-dependent activity profile (Fig. 25B). At pH 7 neither Na^+/H^+ nor K^+/H^+ antiport could be observed. In the range of pH 7.5 to pH 8.95 Na^+/H^+ antiport activity increases nearly linearly. The same is true for K^+/H^+ antiport although only between pH 8.0 and pH 8.95, at pH 7.5 there is no activity. So at every tested pH value the activity is much higher with sodium ions than with potassium ions. This observation supports the results of the physiological experiments which suggested Mrp1 to be rather a Na^+/H^+ antiporter than a K^+/H^+ antiporter. However, the determined apparent K_m values did not differ very much. Nevertheless, Mrp1 joins the queue of monovalent cation/proton antiporters which show high activity at alkaline pH but are shut off at acidic pH. pH profiles were not measured for Mrp2, ChaA and NhaP. But due to the fact that they are active at alkaline pH, it is likely they are also shut off at acidic pH.

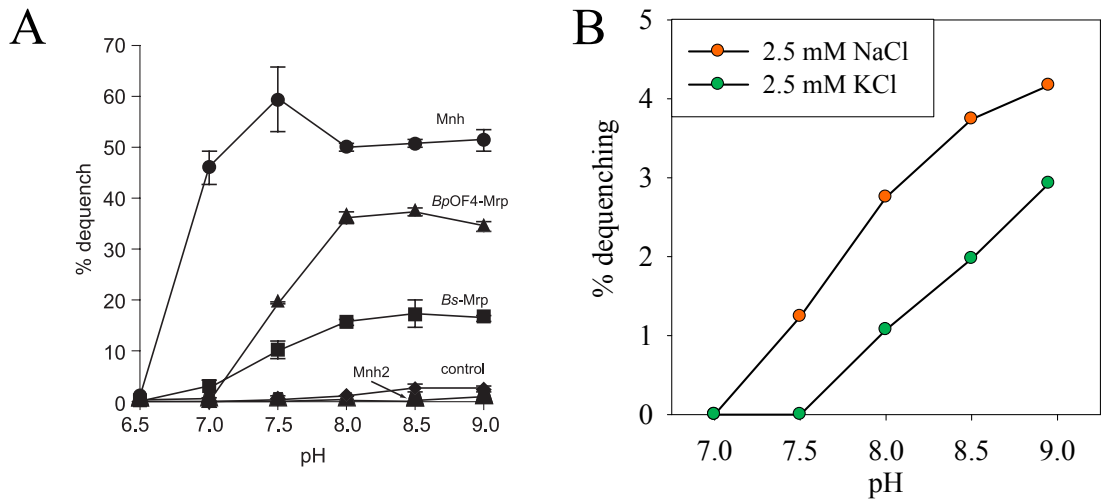

Figure 25: Na^+/H^+ (and K^+/H^+) antiport activity of different Mrp antiporters as a function of pH
Vesicles are energized by addition of succinate. Due to respiration, protons are pumped into the vesicles resulting in the development of a proton gradient (ΔpH, acid in). At the same time acridine orange molecules accumulate inside the vesicles which leads to fluorescence quenching. After the steady state is reached, the cation is added. The antiporter-mediated transport of the cations into the vesicles leads to a concomitant release of protons. This in turn results in the release of acridine orange molecules and thereby in an increase of fluorescence. The activities are assessed by the percent of dequenching after cation addition. Data are for assays with 2.5 mM NaCl, in the case of Mrp1 also for 2.5 mM KCl. **A** *B. subtilis* Mrp, *B. pseudofirmus OF4* Mrp, *S. aureus* Mnh, *S. aureus* Mnh2, taken from [126]; **B** *C. glutamcium* Mrp1

5.6 Mrp1 and NhaP from *C. glutamicum* have K_m values for K^+ which belong to the lowest ever reported

In the present work the characterization of the cation/proton antiporters of *C. glutamicum* was carried out on a physiological and on a biochemical level. The physiological characterization comprised the investigation of transporter mutant strains as well as their complementation via expression of the antiporter gene(s). A standard biochemical characterization aims, for example, for the determination of stoichiometry, regulation and kinetic parameters. In the case of cation/proton antiporters the (apparent) K_m value attracts special attention and represents the parameter to be investigated in this work. The K_m gives information about the binding strength to the substrate [112]. A high K_m value points at weak binding, a low value indicates a high affinity of the transporter for the substrate.

The acridine orange fluorescence quenching/dequenching assay is the most widely used method to determine cation/proton antiporter activity. This assay uses everted membrane vesicles which are energized by addition of an electron donor, resulting in the development of a ΔpH

(acid in) across the membrane. This process leads to quenching of the acridine orange fluorescence. The addition of a monovalent cation that is transported results in dequenching. Activities are assessed by the percent of dequenching after cation addition. In contrast to standard enzyme kinetic assays the described fluorescence assay represents an indirect measurement. Due to this fact the determined K_m is named 'apparent K_m'. In addition, the fact that the antiporter genes are not expressed in their native host but in an antiporter-deficient *E. coli* strain has to be considered. First, the expression level might be quite different for every cation/proton antiporter. Second, the antiporters of interest are not embedded in their native membranes and thus their activity might be influenced, positively or negatively.

Nevertheless, the acridine orange fluorescence method was already extensively used in preceding studies to characterize many cation/proton antiporters, not only from bacteria but also from archaea, yeast and plants. The Mrp antiporters of *B. subtilis*, *B. pseudofirmus* OF4 and *S. aureus* were desribed to exclusively catalyze Na^+/H^+ antiport [126]. All had low K_m values in the range of 0.06 mM to 0.12 mM Na^+. But there are also several antiporters that are able to mediate K^+/H^+ antiport in addition to Na^+/H^+ antiport, for example the *V. cholerae* Mrp [29]. For this antiporter K_m values of 1.3 mM and 68.5 mM for Na^+ and K^+, respectively, were reported. For Pha1 from *Sinorhizobium meliloti* K_m values of 5.47 mM Na^+ and 8.23 mM K^+ were determined and for the *B. subtilis* NhaK 24 mM and 53 mM, respectively, were published [45, 140]. Vnx1 from the yeast *S. cerevisiae* showed K_m values of 22.4 mM Na^+ and 82.2 mM K^+ [16]. But there are also antiporters, mainly from the NhaP family, that seem to be sole K^+/H^+ antiporters. Two examples are the *V. cholerae* NhaP3 with a K_m of 8.3 mM and the *Alkalimonas amylolytica* NhaP with a K_m of 0.5 mM [108, 136]. For the *V. cholerae* NhaP2 activity with both sodium and potassium ions was measured but *in vivo* this antiporter functions only as K^+/H^+ antiporter [109].

The comparison of all these K_m values reveals an intersting observation. Those transporters that are not sole Na^+/H^+ antiporters but also transport K^+ ions show a common characteristic: compared to exclusive Na^+/H^+ antiporters, the affinity for Na^+ is (much) lower. During evolution two ways could have lead to this situation. First, K^+/H^+ activity is a new feature but with the acquisition of K^+ affinity the antiporters sacrificed their high Na^+ affinity [140].

Second, there might have been the need for a highly selective Na^+/H^+ antiporter which lead to the loss of the potassium transport ability. Both possibilities are feasible. Anyway, the lower Na^+ affinity can be explained as follows. In the case that not only sodium ions but also potassium ions have to be transported, the binding site has be constructed in a way that both Na^+ ions and K^+ ions can be properly coordinated. It seems that this feature weakens the binding of Na^+ ions to the transporter, thus causing increased K_m values.

The present work includes a biochemical characterization of two cation/proton antiporters from *C. glutamicum*: Mrp1 and NhaP. For Mrp1 K_m values in the range of 0.46 mM to 0.65 mM Na^+ and 0.82 mM to 0.94 mM K^+ were determined (Fig. 22). Thus the affinity for sodium ions is not as high as for exclusive Na^+/H^+ antiporters. However, compared to other K^+/H^+ antiporters the K_m for potassium is rather low and indicates a relatively high affinity for potassium ions. The latter observation is striking because the physiological characterization of mutants lacking the *mrp1* operon did not suggest Mrp1 to be involved in potassium export. This situation resembles that of the *V. cholerae* NhaP2 antiporter which showed a K_m value of 1.04 mM for sodium ions in the biochemical assay but is only a K^+/H^+ antiporter *in vivo* [109]. For NhaP from *C. glutamicum* K_m values of 1.34 mM Na^+ and 0.6 mM K^+ were determined (Fig. 23). This result clearly shows that NhaP prefers K^+ ions to Na^+ ions as it often seems to be the case for members of the NhaP antiporter family. In addition, the K_m of 0.6 mM for potassium is very low. A similar value was determined for the *A. amylolytica* NhaP (K_m = 0.5 mM K^+) [136].

Finally, it has to be emphasized that the determined K_m values for K^+ for both Mrp1 and NhaP belong to the lowest values ever reported for cation/proton antiporters. This fact might be important for organisms that do not tolerate high intracellular potassium concentrations but seems useless for *C. glutamicum* which usually accumulates K^+ ions up to concentrations of more than 500 mM. So far there is no explanation for the difference in K_m for potassium between the *C. glutamicum* antiporters, namely Mrp1 and NhaP, and other K^+/H^+ antiporters.

5.7 Concluding remarks

The present work aimed to identify and functionally characterize cation/proton antiport systems of *Corynebacterium glutamicum*. The genome contains genes for five putative cation/proton antiporters from four different families. Four of these putative antiporters, namely Mrp1, Mrp2, ChaA and NhaP, were characterized in more detail.

Biochemial investigations proved that all four transport systems are cation/proton antiporters which are able to transport Na^+ ions as well as K^+ ions. However, *in vivo* the antiporters showed different preferences.

The most information were gained for Mrp1. A *C. glutamicum* mutant lacking Mrp1 had a Na^+-sensitive phenotype while growth in presence of a high potassium concentration was not affected. In addition, expression of the *mrp1* genes restored growth of the antiporter deficient strain *C. glutamicum* AQM in complex medium and reduced the intracellular Na^+ concentration. All these results point at a function of Mrp1 as Na^+/H^+ antiporter *in vivo*.

The function of Mrp2 could only be deduced from the phenotypes of deletion mutants. The lack of Mrp2 resulted in K^+ sensitivity whereas a high amount of sodium had no effect on growth. However, deletion of the *mrp2* operon in addition to the *mrp1* operon increased sodium sensivity. Thus Mrp2 has a main role as K^+/H^+ antiporter but is also involved in sodium export.

ChaA and NhaP are of minor physiologic importance as long as the Mrp systems are present which made the *in vivo* characterization of these transporters more difficult. The investigation of triple deletion mutants revealed a higher K^+ sensitivity for *C. glutamicum* Δ*mrp1*Δ*mrp2*Δ*nhaP*, thus NhaP is likely to be a K^+/H^+ antiporter. ChaA was able to significantly improve growth of *C. glutamicum* AQM in NaCl-containing minimal medium which indicates an *in vivo* function as Na^+/H^+ antiporter.

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Danksagung

Danke an...

...Prof. Dr. Reinhard Krämer für die Möglichkeit, diese interessante Arbeit in seiner Arbeitsgruppe anfertigen zu können.

...Prof. Dr. Ulf-Ingo Flügge für die Übernahme des Zweitgutachtens.

...Gerd Seibold für die interessanten Gespräche zwischen Tür und Angel.

...meine Kollegen Natalie, Alex, Katja, Andreas, Oli, Benni, Dimitar für ein ganz tolles Arbeitsklima, viel Kuchen und die ein oder andere experimentelle Hilfe.

...Anja und Gabi für die Hilfe "drumherum".

...Denis Büchel für das tägliche Zusammen-früh-aufstehen, das Ertragen meiner Launen und die vielen Aufmunterungen.

...meine Familie, die mich immer unterstützt hat.

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Teilpublikationen liegen nicht vor.

Köln, den 16.10.2014

Unterschrift

Lebenslauf

Persönliche Daten

Name	Anna Margarida Bartsch
Anschrift	Berrenrather Str. 409
	50937 Köln
Geburtsdatum/-ort	07.11.1986 in Stuttgart
Staatsangehörigkeit	deutsch, portugiesisch
Familienstand	ledig

Schulische Ausbildung

1993-1997	Grundschule Wichterich
1997-2006	Franken Gymnasium Zülpich, Abschluss: Abitur

Studium

10/2006-09/2009	Bachelor of Science in Biologie, Universität zu Köln
10/2009-09/2011	Master of Science in Biological Sciences,
	Spezialisierung Biochemie, Universität zu Köln
09/2011-12/2014	Promotion Biochemie (biolog. Richtung), Universität zu Köln

Weitere Qualifikationen

Fremdsprachen	9 Schuljahre Englisch
	7 Schuljahre Französisch
	1 Semester Portugiesisch
EDV-Kenntnisse	10-Finger-Schreiben
	Microsoft Office (Excel, PowerPoint, Word)
	LaTeX

Köln, 16.10.2014