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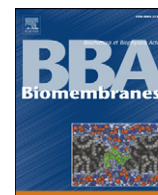

The properties and contribution of the *Corynebacterium glutamicum* MscS variant to fine-tuning of osmotic adaptation

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ABSTRACT

Based on sequence similarity, the *mscCG* gene product of *Corynebacterium glutamicum* belongs to the family of MscS-type mechanosensitive channels. In order to investigate the physiological significance of MscCG in response to osmotic shifts in detail, we studied its properties using both patch-clamp techniques and betaine efflux kinetics. After heterologous expression in an *Escherichia coli* strain devoid of mechanosensitive channels, in patch-clamp analysis of giant *E. coli* spheroplasts MscCG showed the typical pressure dependent gating behavior of a stretch-activated channel with a current/voltage dependence indicating a strongly rectifying behavior. Apart from that, MscCG is characterized by significant functional differences with respect to conductance, ion selectivity and desensitation behavior as compared to MscS from *E. coli*. Deletion and complementation studies in *C. glutamicum* showed a significant contribution of MscCG to betaine efflux in response to hypoosmotic conditions. A detailed analysis of concomitant betaine uptake (by the betaine transporter BetP) and efflux (by MscCG) under hyperosmotic conditions indicates that MscCG may act in osmoregulation in *C. glutamicum* by fine-tuning the steady state concentration of compatible solutes in the cytoplasm which are accumulated in response to hyperosmotic stress.

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1. Introduction

The first line of defense of bacterial cells against hypoosmotic stress is provided by the action of mechanosensitive channels as emergency valves [1,2]. These channels open in response to alterations of the membrane tension as a consequence of water influx caused by hypoosmotic conditions, thereby preventing cell disruption. Different classes of these proteins exist in various bacteria. In *Escherichia coli*, they have first been studied using patch-clamp techniques, which led to the definition of three types of channels, named according to their electrical properties mechanosensitive channels of large (MscL), small (MscS) and mini (MscM) conductance [3]. In the meantime, the MscL and MscS channel proteins have been isolated, purified and crystallized [4–8].

The *E. coli* MscS protein confers resistance to severe hypoosmotic shocks by mediating efflux of small solutes [1,9] thus protecting the cell against rupture of the cell membrane. These compatible solutes are synthesized or taken up during periods of hyperosmotic stress and protect the cell against dehydration. The MscCG protein from the Gram-positive soil bacterium *Corynebacterium glutamicum*, which is similar to the *E. coli* MscS protein, was previously suggested to act as a mechanosensitive channel, too [10]. Besides MscCG, *C. glutamicum* also carries an MscL type protein [10]. In patch-clamp experiments, two different electrical conductances have been observed, probably being caused by the two homologs of mechanosensitive channels found in the genome of *C. glutamicum* [11]. In view of the fact that disruption of both the *mscL* (cg1001) and the *mscCG* gene (cg1434) in *C. glutamicum*, in difference to the situation in *E. coli* [5], did not lead to a significant phenotype upon strong hypoosmotic shocks, the presence of further proteins functioning as mechanosensitive channels may be inferred. Bioinformatic analyses of the genome of *C. glutamicum*, however, do not indicate further candidate genes.

C. glutamicum is well known for its ability to excrete a number of amino acids, in particular L-glutamate and L-lysine. Recently, the *mscCG* gene product was related to the glutamate efflux activity of *C. glutamicum* [12]. Disruption of *mscCG* resulted in decreased glutamate efflux and mutations in the *mscCG* gene were shown to be correlated with constitutive glutamate secretion, but an unequivocal proof for MscCG being directly responsible for glutamate efflux

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has not yet been provided. The possible relation of MscCG-mediated betaine and glutamate efflux, respectively, will be dealt with in further publications.

We have previously studied in detail the function of compatible solute uptake in the instant response upon hyperosmotic shock, on the one hand, and also the significance of BetP, the major osmoresponsive uptake system in *C. glutamicum*, to the osmotic adaptation under continuous presence of hyperosmotic stress [13]. As a soil bacterium, *C. glutamicum* is frequently exposed to osmotic stress; furthermore its significance as a model organism in biotechnology renders it an interesting object to study osmoregulation. In response to hyperosmotic stress, we found an interesting and novel type of regulation of transport activity of BetP. In contrast to many other secondary transporters, BetP was found to be downregulated at the level of activity when a compensatory concentration of compatible solutes is reached in the cytoplasm of *C. glutamicum*. In the framework of this concept concerning mechanisms of fine-tuning of the steady state accumulation of compatible solutes, we were in particular interested whether MscCG may also contribute to osmoregulation upon hyperosmotic challenge. This has not been shown experimentally for any of the mechanosensitive channels so far, however, it may be suspected on the basis of previous suggestions [14] indicating an involvement of solute efflux to the physiological response to hyperosmotic challenge, too.

To this end, we investigated a number of *C. glutamicum* strains harboring or lacking different mechanosensitive channel proteins of the MscS-type. On the one hand, we experimentally prove that MscCG of *C. glutamicum* in fact functions as a channel whose gating depends on membrane tension, with properties roughly similar to MscS from *E. coli*. Particular aspects of gating and desensitization, however, were found to be different from those of the *E. coli* channel protein. Besides its function as a mechanosensitive channel responding to hypoosmotic stress, we also demonstrated that MscCG has, in combination with the betaine uptake carrier BetP, a pivotal physiological function in fine-tuning the steady state concentration of the compatible solute betaine which is accumulated as the major physiological response of *C. glutamicum* to hyperosmotic stress.

2. Experimental procedures

2.1. Strains, media and growth conditions

E. coli strains DH5αmcr [15] and MJF465 [5] as well as *C. glutamicum* wild-type strain ATCC13032 and its derivatives (this work) were used (strains and plasmids see Tab. S1 in the supporting material). *E. coli* was grown at 37 °C in Luria–Bertani medium, *C. glutamicum* at 30 °C in brain heart infusion (BHI, Difco, Detroit, USA) or in CgXII medium (pH 7.0) containing per l: 20 g $(NH_4)_2SO_4$, 5 g urea, 1 g KH_2PO_4, 1.6 g K_2HPO_4, 42 g MOPS, 2.9 g NaCl, 4% glucose, 0.25 g $MgSO_4$, 0.01 g $CaCl_2$, 0.2 mg biotin, 30 mg protocatechuate, 1 ml of a solution of trace elements [16]. For growth under different osmolalities MM1 medium (pH 7.0) was used containing per l: 5 g $(NH_4)_2SO_4$, 5 g urea, 2 g KH_2PO_4, 2 g K_2HPO_4, 3 g NaCl, 4% glucose, 0.25 g $MgSO_4$, 0.01 g $CaCl_2$, 0.2 mg biotin, 30 mg protocatechuate,1 ml of a solution of trace elements and 0, 200 or 400 mM NaCl. Antibiotics (see Tab. S1) were added in concentrations of 100 μg/ml for *E. coli* and 25 μg/ml for *C. glutamicum*.

2.2. Construction of the deletion and complementation strains

The deletion of the *mscCG* gene (cg1434, also known as NCgl1221 or Cgl1270) was performed by the methods of cross over PCR [17] and double homologous recombination using the suicide vector pK19mobsacB_*ΔmscCG* [18], and was verified by PCR analysis. For complementation the *mscCG* gene was amplified by PCR using *C. glutamicum* ATCC 13032 chromosomal DNA as template. The amplified fragment was cleaved with *Bam*HI and *Sal*I and ligated to *Bam*HI/*Sal*I-cleaved pEKex2, resulting in pEKex2_*mscCG* or pEKex2_*mscCG*-His, respectively. Similar to this procedure *E. coli mscS* was amplified via PCR using MG1655 chromosomal DNA as template. The amplified fragment was cleaved with *Bam*HI and *Not*I and ligated to *Bam*HI/*Not*I-cleaved pEKex2, resulting in pEKex2_*mscS*-His. These plasmids were introduced into ATCC 13032 Δ*mscCG* by electroporation.

2.3. Preparation of giant spheroplasts and strain construction

E. coli strain MJF465 lacking MscL, MscS and MscK expressing the plasmid encoded *mscCG* of *C. glutamicum* was used for preparation of giant spheroplasts. Since the MJF465 strain harbours a kanamycin and chloramphenicol resistance a new vector was constructed. The pQE60 vector (ampicillin resistance) was fused to the *lacI* gene normally encoded on a second plasmid. The *mscCG* gene was amplified via PCR using ATCC 13032 chromosomal DNA as template. The amplified fragment was cleaved with *Nco*I and *Bam*HI and ligated to *Nco*I/*Bam*HI-cleaved pQE60-lacI. The procedure used for the preparation of giant spheroplasts from the new *E. coli* strain expressing MscCG protein was as described previously [19].

2.4. Electrical recording

Single-channel analysis was performed on giant *E. coli* spheroplasts [19]. Spheroplasts (1.5-3 μl) were placed in a bath containing, unless otherwise stated, 250 mM KCl, 90 mM $MgCl_2$, and 5 mM Hepes (pH 7.2). Borosilicate glass pipettes (Drummond Scientific Co., Broomall, PA) were pulled using a Flaming/Brown pipette puller (P-87, Sutter Instrument Co., Novato, CA) to a diameter which corresponded to a pipette resistance between 3.0 and 6.0 MΩ. The pipettes were filled with 200 mM KCl, 90 mM $MgCl_2$, and 5 mM Hepes (pH 7.2). All recordings were made at room temperature (19–23 °C) by applying negative pressure (suction) recorded in mm Hg to patch pipettes by using a syringe and was monitored using a piezoelectric pressure transducer (Omega Engineering, Stamford, USA). Ion currents arising from activation of the proteins using suction were recorded using an Axon 1D patch-clamp amplifier (Axon Instruments), filtered at 2 kHz and digitized at 5 kHz. Single channel analysis was done using pCLAMP10 software (Axon Instruments).

2.5. Uptake and efflux of glycine betaine

The enzymatic synthesis of [^{14}C]-labeled glycine betaine was performed as described previously [20]. For measurement of betaine efflux in response to hypoosmotic stress, *C. glutamicum* strains were grown aerobically at 30 °C at 125 rpm overnight in BHI medium containing 500 mM NaCl for hyperosmotic growth conditions. Cells were harvested by centrifugation and washed twice under hypoosmotic conditions in 100 mM Mes/Tris, pH 8.0, 4 °C, containing 5 mM Na_2HPO_4 and 5 mM K_2HPO_4. The hypoosmotic washing step results in efflux of the majority of compatible solutes which were taken up or synthesized during the growth period. Uptake of the labelled betaine was performed by incubating the cells at an OD_{600} of 4 for 80 min at 30 °C in a hyperosmotic buffer (100 mM Mes/Tris pH 8.0) containing 0.9 M NaCl, 30 mM glucose, 30 mM urea and 30 mM KCl. Shortly after transfer into the uptake buffer [^{14}C]-glycine betaine at a final concentration of 1 mM (25,000 cpm/ml) was added. After loading of the cells with labelled betaine, they were stored on ice until used. To measure the efflux, cells were centrifuged and resuspended in 100 mM Mes/Tris pH 8.0, containing various concentrations of NaCl to obtain appropriate osmolalities. After 15 s 200 μl samples were withdrawn and cells were rapidly separated from the surrounding medium by silicone oil centrifugation (oil density of 1.09 kg/l for osmolalities > 1.2 osmol/kg and 1.03 kg/l for osmolalities < 1.2 osmol/kg) and counted.

For the combined betaine uptake and efflux experiments, *C. glutamicum* strains were prepared as described above and uptake of label was followed accordingly. To visualize net betaine efflux in the steady state of transport, excess unlabeled betaine at a final concentration of 50 mM was added and samples were taken at times indicated. If cells were grown in the absence of betaine previous to the efflux experiment thus lacking internal betaine at the beginning of the experiment, the specific radioactivity of the internally accumulated betaine is identical to that added to the external medium when starting uptake, consequently the absolute efflux rate can be calculated.

2.6. Analysis of betaine uptake during osmotic compensation

C. glutamicum strains were prepared as described for betaine efflux analysis and uptake of label was monitored accordingly. A parallel culture was incubated with 4 mM unlabeled betaine. After 3, 30, 60, and 90 min an aliquot was taken and tracer-free [^{14}C]-glycine betaine (25,000 cpm/ml) was added. Samples were taken 20, 40, 60, and 80 s after addition of label and filtered rapidly through glass fibre filters. The filters were washed twice with uptake buffer, and the radioactivity was determined by liquid scintillation counting.

2.7. Determination of membrane potential

The membrane potential was determined as described earlier [21,22]. The membrane potential was calculated from the distribution of the permeant cation [^{14}C]-tetraphenylphosphonium bromide (5 µM final concentration, specific radioactivity 0.995 Ci/mol). Rapid separation of extra- and intracellular fluids was performed by using silicone oil centrifugation with perchloric acid in the bottom layer [23]. The obtained values of membrane potential were corrected for unspecific probe binding by addition of a mixture of 20 µM valinomycin and 5 µM nigericin.

2.8. Protein synthesis and Western blot analysis

To control the expression of *mscCG* mutants, cells were disrupted using a Ribolyser three times at maximum speed of 6.5 for 45 s. After separation of cell debris membranes were isolated by centrifugation and resuspended in PBS buffer, pH 7.5. Protein concentration was measured by the Bradford technique [24]. Western blot analysis was performed as described [16] using antibodies raised against 6xHis-tag (Qiagen, Hilden) and against MscCG (Eurogentech, Köln). As second antibodies anti-mouse-(AP) and anti-rabbit-(AP) (Sigma) antibodies were used.

3. Results

With only one *mscL* and *mscS* type gene each in its genome, *Corynebacterium glutamicum* seems to be less well equipped with mechanosensitive channels in comparison to *E. coli*. Whereas MscL of *C. glutamicum* has a significant sequence similarity to MscL of *E. coli* (38% and 57% identical and similar amino acids, respectively, *E* value $3 \times e^{-15}$), for the *C. glutamicum* MscCG protein a rather low similarity to MscS of *E. coli* was found (26% and 46% identical and similar amino acids, respectively, *E* value $5e^{-04}$). This fact and the presence of an atypical additional C-terminal domain is the reason for the designation MscCG. The *C. glutamicum* MscCG protein consists of 533 amino acids and only the N-terminal part is similar to other MscS-type proteins. The 247 residues long C-terminal domain is absent in the homologous *mscS* gene of *E. coli* (Fig. 1). The *E. coli* MscS consists of only 286 amino acids. However, in spite of the relatively low sequence similarity a structure prediction can be derived for MscCG with MscS as template indicating the structural similarity of the two proteins (see Fig. 1). The location of the three transmembrane domains turns out to be identical, whereas the similarity decreases significantly in the C-terminal part of the two proteins. The third transmembrane helix of MscS has a conserved pattern of glycine and alanine residues putatively involved in gating of the channel. Four glycines Gly101, Gly104, Gly108 and Gly113 form a smooth surface thought to facilitate sliding of four alanines Ala98, Ala102, Ala106 and Ala110, which is required for the conformational transition leading to channel opening [25,26].

3.1. Patch clamp analysis of C. glutamicum MscCG

Patch clamp functional analysis revealed the presence of stretch-activated channels. MscCG channels opened under negative pressure between 40 and 100 mm Hg (Fig. 2). This closely resembles the range previously reported for MscS channel recorded in *E. coli* giant spheroplasts, which opens between 30 and 70 mm Hg [27] and falls well below the threshold (150–240 mm Hg) for that of MscL in spheroplasts [4]. Although comparing pressures rather than membrane tension activating the channels is less accurate, nevertheless given the overlap between the pressure range for MscS vs. MscCG this comparison is valid and largely different from the pressure activation range of MscL. This is because the shape, resistance and “bubble number” of patch pipettes in our experiments are standardized allowing the comparison. Increasing pressure (and hence membrane tension) results in successive opening of the MscS and MscL channels. Open probability vs. membrane tension for MscCG channels was not determined as some patches contained up to two hundred channels, the exact number of channels in the patch proving difficult to ascertain. Interestingly, unlike MscS [28,29] MscCG does not desensitize, as shown in Fig. 2A. The channels remained open for several minutes. Of note is the wave-like behavior seen on prolonged opening, some channels closing but after a further period, reopening.

Analagous to MscS, the channels strongly rectify, (Fig. 2B) the unitary conductance at positive voltages being 346.3 ± 22.5 pS ($n = 8$) and 99.5 ± 4.5 pS ($n = 8$) at negative voltages in asymmetric spheroplast solution (Fig. 3). Conductance at negative voltages is approximately 30% of that at positive voltages indicating strong rectification properties of the channel. This behavior is comparable to that of *E. coli* MscS, whose conductance measured at negative pipette voltages is two thirds of that obtained at positive pipette voltages [19,27].

Substitution of K^+ for Na^+ did neither influence significantly the channel conductance nor its pressure sensitivity (see Tab. S2). To examine the channel preference for the ion species we also recorded the channel behavior at symmetrical conditions (200 mM KCl, 40 mM $MgCl_2$, 5 mM HEPES, pH = 7.2) and then increased the bath concentration of KCl to 400 mM. The concentration gradient caused ions to flow from the bath to the pipette resulting in a shift of ~8.0 mV of the zero current potential in the positive direction (Fig. 3A), thus indicating the channel slight preference for cations over anions. The rightward shift of the I–V curve of ~8.0 mV indicates a selectivity ratio for potassium over chloride P_K/P_{Cl} ~ 3.0, which is comparable to the slight anionic preference of *E. coli* MscS for chloride over potassium of P_{Cl}/P_K ~ 1.5- 3.0 [19,27,30].

We further tested the conductive properties of the channel by recording its conductance at KCl concentrations from 100 mM to 400 mM (Fig. 3B). At concentrations greater than ~300 mM KCl the conductance becomes saturated at positive pipette voltages, while the conductance at negative pipette voltages was not affected. This was not observed in *E. coli* MscS for which a lack of conductance saturation up to 1.5 M KCl was reported [30].

3.2. Efflux properties of C. glutamicum MscCG

In a previous publication, basic properties of the two gene products of *C. glutamicum* similar to known *E. coli* mechanosensitive channels, namely MscL and MscCG, have been described [10]. In order

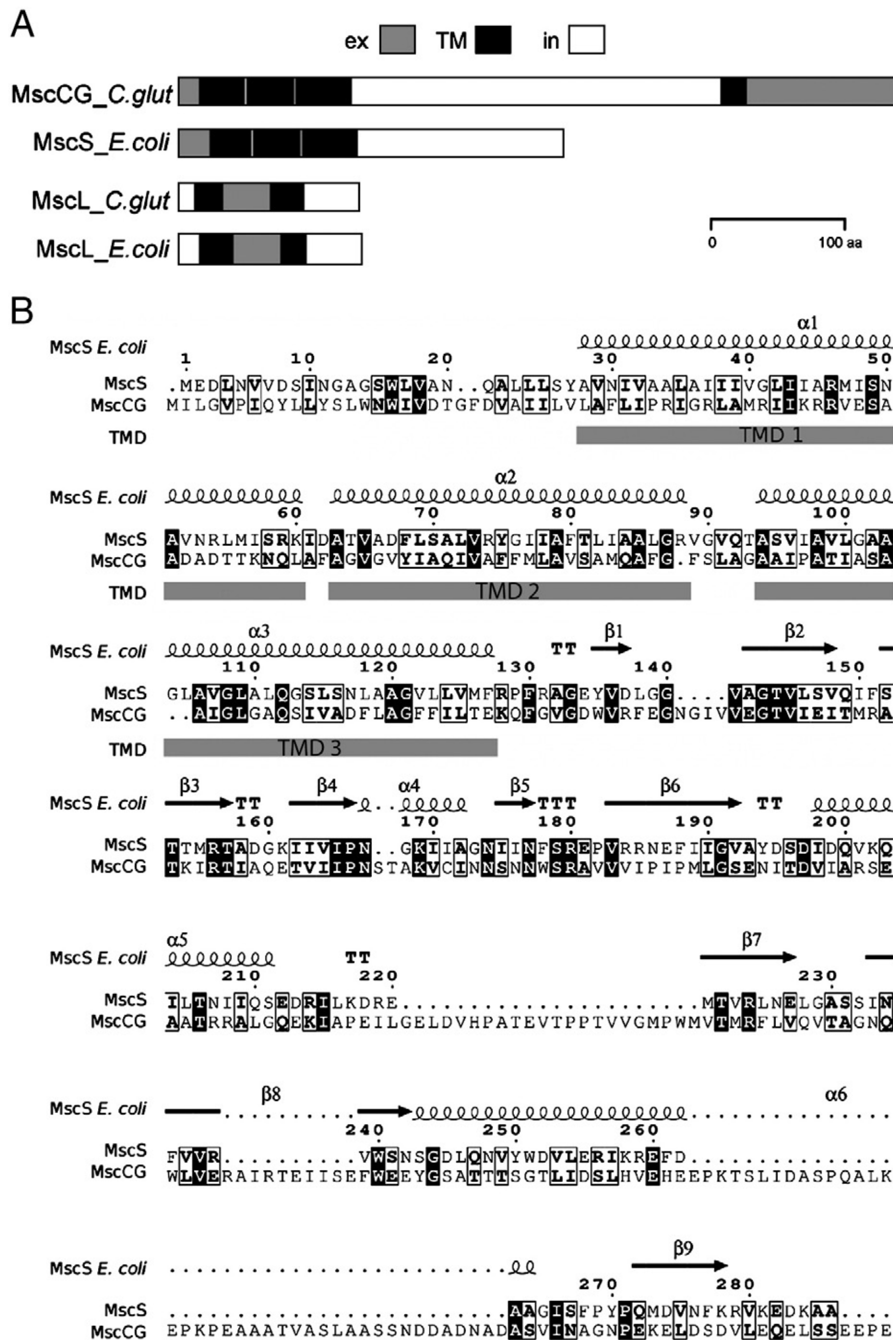

Fig. 1. (A) Comparison of length and membrane topology of MscCG and MscL of *C. glutamicum* and MscS and MscL of *E. coli*. (ex, periplasmic location; in, cytoplasmic location; TM, transmembrane domain). (B) Comparison of the primary structure of MscS of *E. coli* and MscCG of *C. glutamicum* including the secondary structure of MscS (upper line), the sequence alignment, as well as the localization of the (predicted) transmembrane domains (TMD1-2).

to define the function of MscCG in more detail, we constructed a number of recombinant *C. glutamicum* strains, including deletions of the individual *mscL* and *mscCG* genes, as well as an *mscL*, *mscCG* double deletion. In the *mscCG* deletion strain we furthermore expressed plasmid-encoded *mscCG*, a His-tagged version of the *mscCG* gene, as well as the homologous *E. coli mscS* gene. (constructions see Tab. S1). The efficiency of expression of the various *mscCG* and *mscS* constructs, respectively, depending on the concentration of IPTG added, is summarized in Fig. 4A. It should be noted that we never succeeded to visualize the MscCG protein in these blots in the wild type. This indicates a low expression of *mscCG* in *C. glutamicum*, which, however, closely resembles the situation of *mscS* expression in *E. coli*, where 20-30 MscS channels per cell have been estimated [31]. The *mscCG-His* construct was found to be strongly expressed. The extraction procedure was designed to eliminate possible contaminations by inclusion bodies.

In a test for betaine efflux upon hypoosmotic shock in intact *C. glutamicum*, cells were loaded with labeled betaine and incubated at decreasing external osmolality starting from 1.84 osmol/kg (loading buffer) down to very low values of external osmolality (Fig. 4B). As already mentioned in a previous publication [10] it cannot be fully excluded that, besides the two mechanosensitive channels known in *C. glutamicum*, further channels or even cell lysis may contribute to the observed efflux. Deletion of the *mscCG* gene resulted in a shift of the dependence of betaine efflux on external osmolality, i.e. the extent of hypoosmotic stress leading to 50% efflux is higher in the mutant

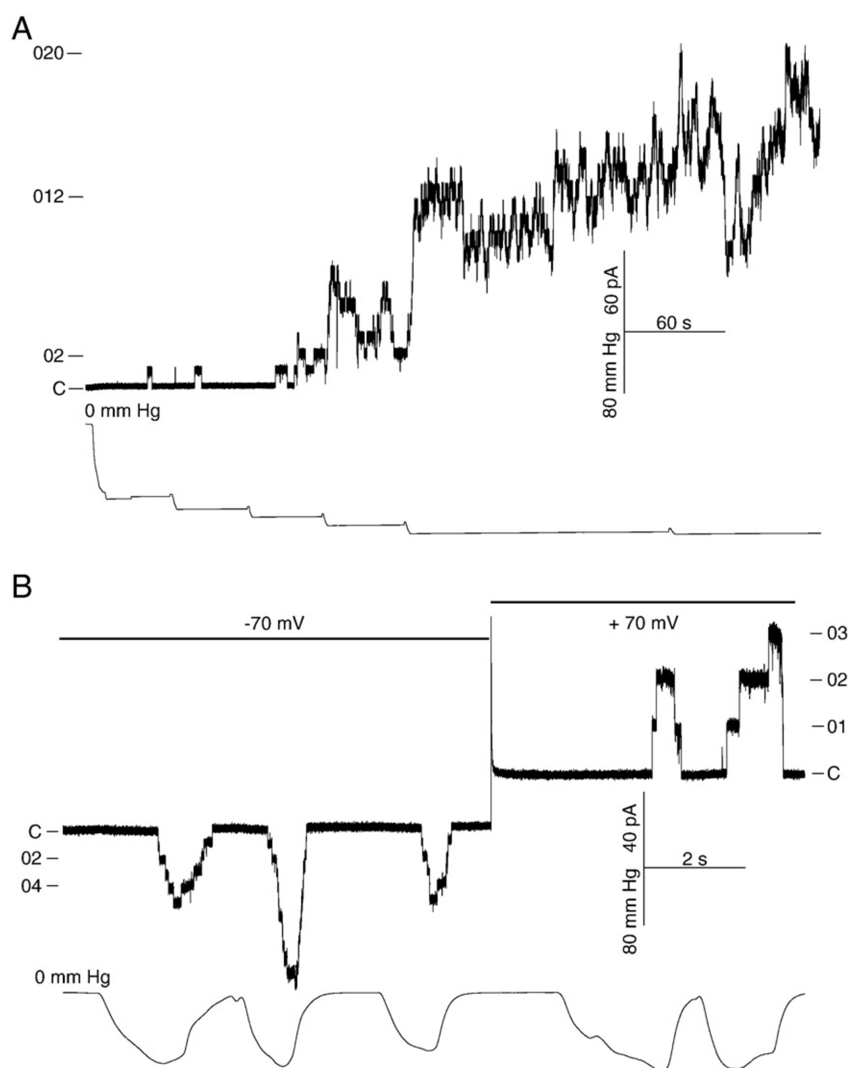

Fig. 2. (A) Gating behavior of MscCG in *E. coli* spheroplasts at +50 mV applied voltage. The first channel activates under tension (~ −80 mm Hg). Further increases in tension open more channels. The channels do not inactivate, remaining open for several minutes. *C* and O_n denote the closed and open state of *n* number of channels. There are up to 20 channels opening in this particular spheroplast membrane patch. (B) Voltage effects on YggB conductance. The channel exhibits higher conductance at positive voltages (~23 pA at +70 mV and ~7 pA at −70 mV), indicating (analogous to MscS) rectifying properties of the channel.

than in the wild type. The additional deletion of the *mscL* gene did not change the efflux pattern, indicating that *C. glutamicum* MscL protein does not seem to contribute to betaine efflux. Although larger in size, the MscL channel of *C. glutamicum* is likely not to open under these conditions for the following reason. MscL of *C. glutamicum* has a significantly higher similarity to the corresponding protein of *Mycobacterium tuberculosis* (48% and 64% identical and similar amino acids, respectively, *E* value $7 \times e^{-25}$) than to the MscL of *E. coli* (see above). It has previously been shown that MscL from *M. tuberculosis* requires about twice the tension needed to gate the *E. coli* MscL [32] with a consequence that it has been difficult to study this channel because spheroplast patches containing MscL of *M. tuberculosis* usually break at high pressure required to activate this MS channel. The phenotype upon *mscCG* deletion could be complemented by expression of *mscCG* from a plasmid. The effect on efflux depended on the expression level of the *mscCG* construct. The expression of plasmid-encoded *mscCG* at both 25 and 200 μM IPTG, respectively, as well as that of the His-tagged construct without added IPTG were similar to the wild type. Overexpression of the His-tagged *mscCG* construct at 25 μM IPTG (Fig. 4A), however, led to a betaine efflux pattern different from both the wild type and the *mscCG* complementation strain.

Since the shift in the sensitivity of betaine efflux towards hypoosmotic stress was only slightly shifted upon *mscCG* deletion, we tested whether the presence of a well-described MscS channel, i.e. the *E. coli* MscS, would lead to a comparable phenotype in *C. glutamicum*. Although we found significant expression of the *mscS* gene in *C. glutamicum*, the phenotype of the *mscCG* deletion mutant was not complemented, i.e. the shift in sensitivity to external osmolality remained at the level of the deletion mutant, as shown in Fig. 4B, indicating that *E. coli* MscS does not function properly as a mechanosensitive channel in *C. glutamicum* (not shown).

3.3. Contribution of *C. glutamicum* MscCG to fine-tuning of osmotic adaptation

Having provided evidence for efflux channel activity of MscCG under hypoosmotic conditions, we studied its significance upon hyperosmotic challenge. When cells adapt to hyperosmotic conditions, compatible solutes are accumulated up to an appropriate level corresponding to the extent of stress. Arguments have been put forward that the maintenance of a correct level of compensatory solutes in the cytoplasm may, besides the action of uptake systems, also involve transient opening of efflux channels [14]. In a basic test,

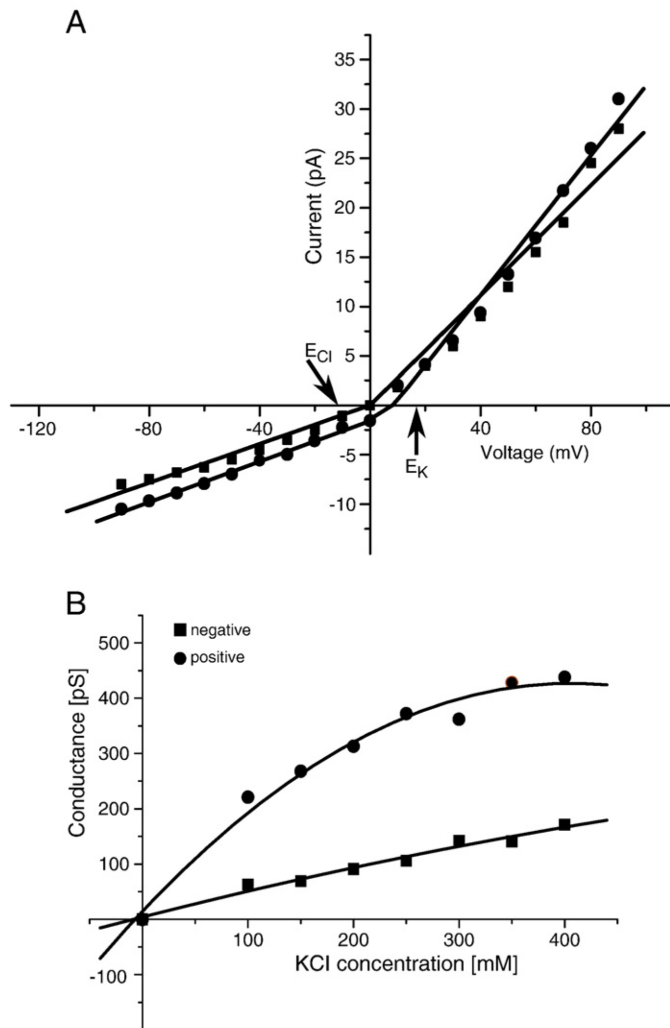

Fig. 3. Conductance and selectivity of MscCG. (A) Current/voltage relationship of MscCG in *E. coli* spheroplasts under (squares) symmetrical (200 mM KCl, 40 mM $MgCl_2$), and (circles) asymmetrical conditions (pipette 200 mM KCl, 40 mM $MgCl_2$, bath 400 mM KCl, 40 mM $MgCl_2$). Straight lines are linear regressions. Clear rectification occurs, the conductance at negative voltage approximately 30% of that at positive voltages (squares). The rightward shift of the curve (circles) indicates that the channel exhibits slight preference for cations, as evidenced by a ~8 mV shift of the zero–current potential. The reversal potentials for K^+ and Cl^- (indicated by arrows) were calculated using GHK equation. They are +17.5 mV and −13.6 mV for K^+ and Cl^+, respectively. (B) Single-channel conductance as a function of the ion concentration of the bath and pipette buffer containing 0.1, 0.2, 0.3, or 0.4 M KCl (5 mM HEPES, pH 7.2). The channel conductance at positive pipette voltages (circles) becomes saturated above 300 mM, while at negative pipette voltages (squares) is not affected in this range of ionic concentrations.

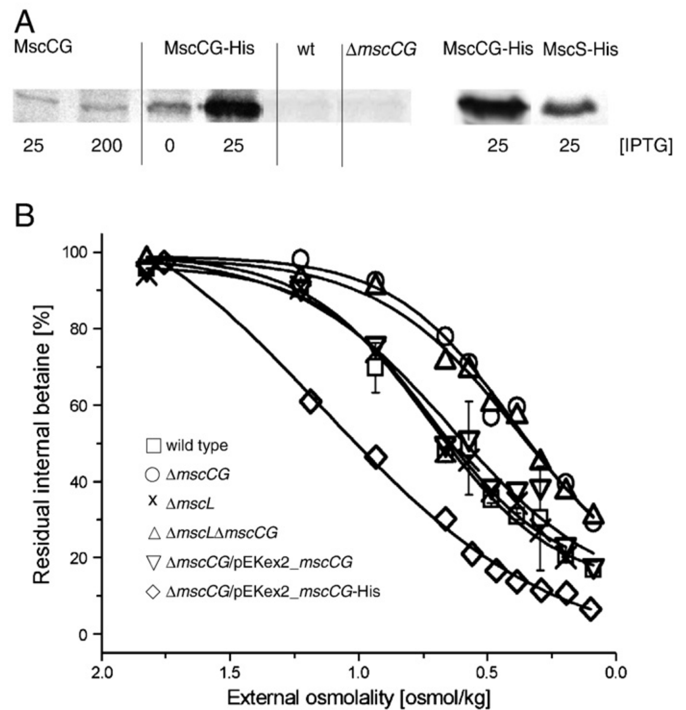

Fig. 4. (A) Western blot analysis of *mscCG* as well as His-tagged forms of *mscCG* and *mscS* expressed in *C. glutamicum* (wt, wild type strain harboring only the genomic copy of *mscCG*, ΔmscCG, *mscCG* deletion strain). 30 µg of cell membranes (extract) was loaded to each gel. Below the blot patterns, the concentration of added IPTG during growth is indicated. The six lanes at the left hand side were developed using anti-MscCG antibody, for the remaining two lanes, anti-(penta)His antibody was used. (B) Residual internal betaine after betaine efflux upon hypoosmotic shock in *C. glutamicum* wild type (squares), *mscCG* deletion strain (circles), *mscL* deletion strain (crosses), *mscCG, mscL* double deletion strain (upward triangles), and complementation strains using the *mscCG* (25 µM IPTG, downward triangles) and the *mscCG-His* gene (25 µM IPTG, diamonds), respectively. Cells were loaded with labeled betaine at an external osmolality of 1.84 osmol/kg. For a better comparison relative values for internal betaine are shown. The absolute values of labeled betaine accumulated in the loading procedure (see Methods section) were around 3000 counts/ml for all strains used, except the strain carrying the *mscCG-His* gene where it was decreased to 1800–2000 counts/ml, indicating a counteracting efflux due to high overexpression of the mscCG gene. Betaine efflux upon osmotic downshock was performed as described in Materials and methods. All experiments were carried out at least in triplicate (error bars represent standard deviation).

we analyzed growth of the various strains upon hyperosmotic stress. Only the strain with the strongly overexpressed, His-tagged *mscCG* construct had a delayed onset of growth and a slightly diminished growth rate. All other strains were indistinguishable from the wild type with respect to growth (not shown).

To analyze a possible contribution of MscCG to hyperosmotic adaptation in detail we investigated the dynamic behavior of the steady state level of internally accumulated betaine in wild type and in mutant cells. We suspected that an altered steady state level may be related to a changed balance between concomitant betaine uptake und efflux. Consequently, a different type of experiment was applied here for the kinetic analysis (Fig. 5). In the first part of the experiment, labeled betaine was taken up in response to hyperosmotic stress. After reaching a steady state of internally accumulated betaine, a large excess of unlabeled substrate was added. The betaine chase masks the subsequent influx of label due to a strongly decreased specific radioactivity and thus allows separate quantification of betaine efflux. It has to be noted that betaine is not metabolized in *C. glutamicum*. When comparing the uptake and efflux pattern of the various strains investigated here, a contribution of MscCG to betaine efflux is recognized. First, the observed efflux of label was found to be diminished upon deletion of *mscCG*. (Fig. 5A). The *C. glutamicum* strain lacking both mechanosensitive channels did not differ from the *mscCG* deletion strain in its efflux pattern (not shown). Second, efflux of label was enhanced in the strain expressing the *mscCG* gene or its His-tagged version at moderate levels and greatly enhanced upon overexpression of *mscCG-His* (Fig. 5B). Consequently, betaine efflux under steady state conditions of osmotic adaptation seems to be directly correlated to the extent of *mscCG* expression. Notably the steady state level of betaine accumulation remained constant in all strains except in the *mscCG-His* construct where it was strongly decreased in comparison to all other strains. The observation of a decreased steady state accumulation is directly correlated to the increased solute efflux through MscCG in this strain, which is observed both in Fig. 4B (see legend to this figure) and in Fig. 5B (upon the betaine chase). We also tested the *mscCG* deletion strain expressing the *E. coli mscS* gene in this type of experiment, but did not find any effect on betaine efflux, thus supporting the results from the efflux experiments described above.

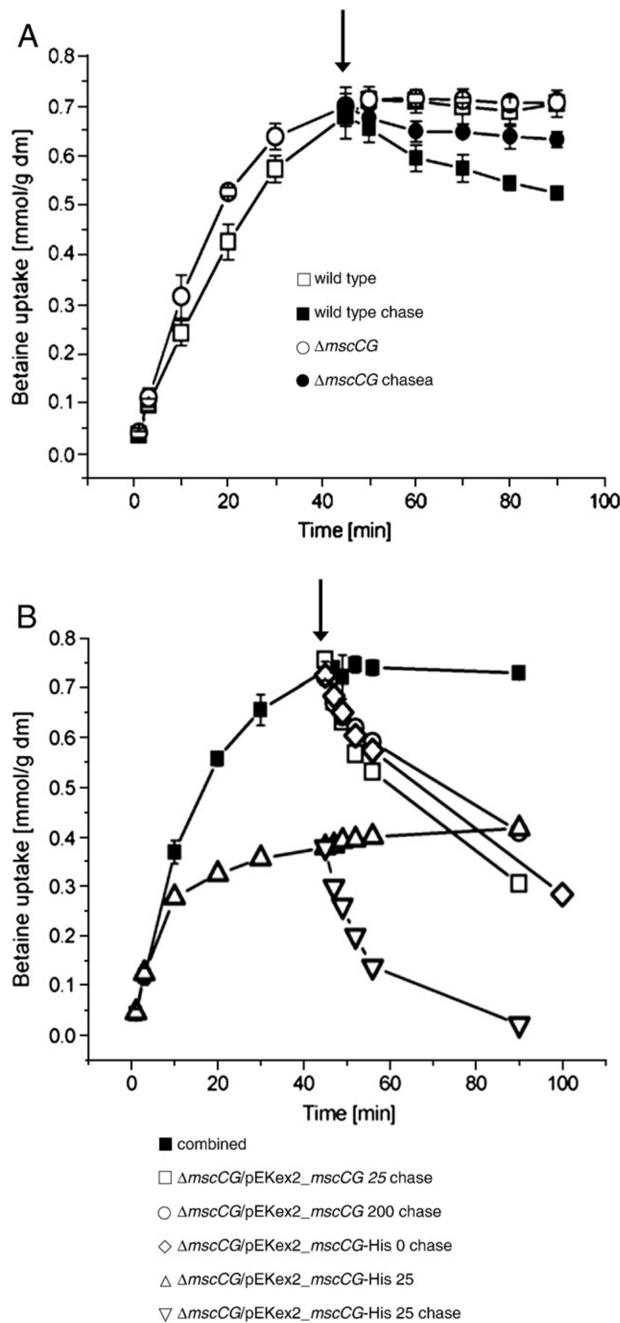

Fig. 5. Kinetic discrimination of betaine uptake and efflux in different *C. glutamicum* strains. Labeled betaine in the cytoplasm of the respective cells was measured by rapid filtration. The uptake of [^{14}C]-betaine is monitored at the beginning of the experiment. Where indicated by an arrow, an excess of unlabeled betaine was added and subsequent efflux of label was measured (chase). All experiments were carried out at least in triplicate (error bars represent standard deviation) except for strain Δ*mscCG* pEKex2_*mscCG-His* where means of duplicate measurements are shown. (A) Wild type, uptake of label (open squares) and chase (solid squares), *mscCG* deletion strain, uptake of label (open circles) and chase (solid circles). (B) Comparison of strain Δ*mscCG* pEKex2_*mscCG* upon addition of 25 (open squares) and 200 μM IPTG (circles), as well as strain Δ*mscCG* pEKex2_*mscCG-His* without addition of IPTG (diamonds), and strain Δ*mscCG* pEKex2_*mscCG-His* upon addition of 25 μM IPTG (uptake of label, upward triangles, and chase, downward triangles). The measurement of betaine uptake is summarized for the strains Δ*mscCG* pEKex2_*mscCG* (25 and 200 μM IPTG) and Δ*mscCG* pEKex2_*mscCG-His* (no IPTG) (solid squares = combined) and is shown separately for strain Δ*mscCG* pEKex2_*mscCG-His* (25 μM IPTG) (diamonds).

In order to exclude deleterious effects of an altered MscCG activity on the energetic situation of the cell being the reason for the observed results, we analyzed the membrane potential in *C. glutamicum* cells. The electrical potential is the major contributor to the electrochemical Na^+ potential, the driving force of betaine uptake by BetP. In all strains tested we measured electrical potentials in the range between −180 and −200 mV, independent of the presence or absence of *mscCG* (not shown). This can be taken as an indication that, even in the presence of an increased amount of MscCG channels, i.e. in the strain overexpressing the *mscCG-His* construct, membrane potential generation by the respiratory chain is far more effective than dissipation by the gating activity of the MscCG channels under these conditions.

In a final experiment, we set out to test our hypothesis on a balanced influx (via BetP) and efflux (via MscCG) being responsible for the fine-tuning of steady state betaine accumulation in response to hyperosmotic stress by analyzing the response of BetP to an artificially altered efflux activity by MscCG. Consequently, in this experiment the feedback of MscCG activity on BetP regulation is investigated. Activity regulation of BetP upon osmotic compensation in the presence of a prolonged duration of hyperosmotic stress has been analyzed before [13]. The result of these studies indicated that BetP responds to conditions of osmotic compensation by downregulation of its uptake activity. In order to study now the response of BetP to a varying activity of MscCG, i.e. to an artificial variation of the efflux component, we modified the experimental set up described in Fig. 5 to monitor BetP activity during the whole course of the experiment (Fig. 6). This complex experiment is split into two individual measurements. In the first experiment uptake of labeled betaine at high external substrate concentration (4 mM) was measured, similar to the first part of Fig. 5. In a parallel experiment, 4 mM unlabeled betaine was added under identical conditions. At given time points, indicated at the *x*-axis of Fig. 6, pure labeled betaine (label only) was added and the uptake kinetics of label was measured within a short time, taking several samples within 1 min. The actual concentration of residual external betaine at any time point of the uptake measurement within the second experiment can be derived from the first experiment (see above). This concentration can then be used to calculate the specific activity of external betaine present in the short term uptake measurements in the second experiment, resulting in absolute values of uptake rates. By this procedure, the actual betaine uptake activity of BetP can be monitored at any given time point (Fig. 6). It is important

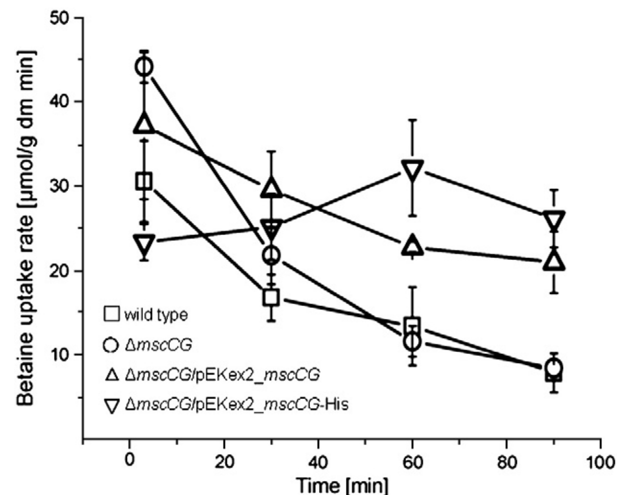

Fig. 6. Betaine uptake rates during osmotic compensation. Uptake of betaine by BetP in different *C. glutamicum* strains was measured after an initial addition of unlabeled betaine by subsequent addition of [^{14}C]-betaine (labeled betaine only) at the indicated times (for calculation of the uptake rates and for further details see text). The BetP activity indicated at the corresponding sampling times (*x*-axis) was monitored by individual, brief uptake experiments, the results of which are shown in this figure. The following strains were analyzed: wild type (squares), Δ*mscCG* (circles), Δ*mscCG* pEKex2_*mscCG* upon addition of 25 μM IPTG (upward triangles), and Δ*mscCG* pEKex2_*mscCG-His* upon addition of 25 μM IPTG (downward triangles). The standard deviation is indicated ($n = 3$).

to note that the rates shown in Fig. 6 are closely similar to those which can be derived from direct uptake measurements (Fig. 5).

The results from Fig. 6 indicate (i) that the uptake activity of BetP becomes downregulated upon increasing osmotic compensation as shown in the experiment using wild type *C. glutamicum*. This is in agreement with earlier observations [13]. (ii) Both moderate, and in particular, strong overexpression of *mscCG* leading to an increased efflux, have a significant impact on BetP activity, largely preventing downregulation of BetP activity. (iii) The effect of *mscCG* deletion, which was shown to result in a slightly decreased betaine efflux in the steady state situation (Fig. 5A), is obviously not strong enough to cause a significant responsive change in BetP activity. Based on the influence of MscCG on betaine efflux (Fig. 5), and on the responsive regulation of BetP activity (Fig. 6), the combined results indicate a regulatory interdependence of BetP and MscCG activity, respectively, in their relative contribution to flux balance leading to an appropriate dynamic steady state value of betaine accumulation in the cytoplasm.

4. Discussion

Corynebacterium glutamicum responds to hyperosmotic stress predominantly by uptake of compatible solutes, in particular of glycine betaine via the BetP transporter. Mechanosensitive channels, on the other hand, are involved in the response to hypoosmotic challenge [33]. Based on structural similarity, MscCG from *C. glutamicum* is a member of the MscS-type family of mechanosensitive channels [1,10]. By patch-clamp analysis we show that MscCG, when expressed in an *E. coli* mutant lacking all mechanosensitive channels of the MscL and MscS type, shows the typical pressure dependent gating behavior of a stretch-activated channel with a current/voltage dependence indicating rectifying behavior comparable to MscS [19].

Apart from its basic function as a mechanosensitive channel, MscCG was characterized in the electrophysiological analysis by significant differences compared to the well-studied MscS channel from *E. coli*. Firstly, the conductance of MscCG rectifies strongly at two voltage polarities and its conductance is significantly smaller compared to that of *E. coli* MscS by being ~30% at positive and ~18% at negative pipette voltages. The way the channel rectifies, i.e. showing larger conductance at positive pipette voltages compared to that at negative pipette voltages is similar to MscS because of the right-side-out orientation of the channel in inside-out spheroplast patches, i.e. the pipette solution is facing the extracellular side of the spheroplast membrane [19]. Secondly, in contrast to MscS the MscCG conductance saturates at salt concentrations in the range of several hundred millimoles. Finally, its ion selectivity differs from that of MscS by showing a slight preference for cations (P_K/P_{Cl} ~ 3.0), whereas MscS slightly prefers anions (P_{Cl}/P_K ~ 1.5–3.0) [19,27,30]. The preference of MscCG for cations appears compatible with the presence of a large number of acidic residues in the cytoplasmically located C-terminal portion of the channel (Fig. 1). Similarly, the presence of lysine and arginine residues in the C-terminal domain of MscS have been suggested to cause a preference of this channel for anions [34].

Importantly, this study also provides evidence that mechanosensitive channels are key players in the response to hyperosmotic stress in bacteria. As a consequence, the physiological significance of mechanosensitive efflux channels seems to be twofold, both as emergency efflux valves (hypoosmotic conditions) and as regulatory elements in fine-tuning solute accumulation (hyperosmotic challenge).

Upon onset of hyperosmotic stress, compatible solutes, e.g. betaine, are accumulated until osmotic compensation is reached. The final steady state level of accumulation has to be carefully adapted to the actual extent of osmotic stress in order to optimally protect the cell against the osmotic challenge. A number of possible mechanisms are available by which an appropriate level of solute accumulation may be reached. Basically, the steady state level of solutes is directly influenced by the uptake carrier activity. Adaptation may be achieved either by downregulating the activity of the uptake carrier upon reaching a compensatory cytoplasmic level of the solute, or by a change of the carrier's function from unidirectional uptake to exchange, thus transporting betaine both inwards and outwards with the same rate. The latter concept is the common interpretation of the kinetic steady state of typical secondary carriers. A further model is the so-called pump and leak mechanism. It involves competing net fluxes via an uptake carrier and a different system, either carrier or channel, catalyzing solute efflux, leading to a fine-tuned cytoplasmic steady state concentration of the transported solute.

We have previously analyzed the involvement of the uptake carrier BetP to the steady state level of betaine upon osmotic compensation. Downregulation of BetP activity was shown to significantly contribute to the observed adaptation [13]. A certain extent of ongoing solute influx and efflux after downregulation was recognized, too, which could not be further resolved in these experiments. The present study now shows that in fact both mechanisms described above contribute to fine-tuning of the betaine pool in *C. glutamicum*. The residual efflux of label in the *mscCG* deletion mutant represents the remaining exchange activity of BetP (first model, see above) after its transport activity has been downregulated as previously recognized [13]. The difference in efflux of label between the *mscCG* deletion mutant, the wild type, and the *mscCG* overexpression strains, respectively, represents net betaine efflux via MscCG which is compensated by the residual unidirectional uptake activity of BetP (pump and leak model). Notably the first model (1:1 exchange) is not productive, i.e. it will not lead to a change in the internal betaine concentration. This is of course only true if all carriers function according to this model, and we assume that, in particular in the time frame when reaching steady state conditions, the observed betaine accumulation is possibly the sum of a mix of different functional modes. In a true steady state, however, the “pump and leak” mechanism of the combination of BetP and MscCG is the only effective means of *C. glutamicum* to fine-tune the cytoplasmic betaine concentration under steady state conditions in response to the actual extent of osmotic stress.

Here we provide evidence that the activity of the “leak” has a serious impact in the proposed pump and leak model of steady state regulation, as indicated by the reduced steady state concentration in case of strong *mscCG* overexpression. It should be emphasized that MscCG-mediated efflux under these conditions occurs in the presence of hyperosmotic stress where a significant activity of MscCG was not expected. The “pump and leak” mechanism was further approved by the finding that the previously described downregulation of BetP activity upon approaching osmotic compensation was largely prevented upon overexpression of *mscCG* which led to an increase in betaine efflux. It is important to note that the impact of a changed MscCG function on BetP activity leads in fact to a different flux balance, but not to a different steady state value of accumulation, except in the case of strong overexpression. Taken together, these results argue for a mechanism of fine-tuning the cytoplasmic solute concentration upon hyperosmotic stress by cooperation of an active and regulated uptake carrier (BetP, pump) and a passive, but also regulated efflux channel (MscCG, leak). It should be noted that betaine efflux in *Lactococcus plantarum* has previously been observed under steady state conditions of compatible solute accumulation in response to hyperosmotic stress [35]. The authors interpreted their results as a consequence of the action of an export carrier rather than a channel, however, based on the kinetics and the properties described, the observed efflux could also be mediated by a mechanosensitive channel.

In order to elaborate our model quantitatively we combined the results of efflux measurements and patch-clamp studies and calculated the change of the internal solute concentration corresponding to the pressure difference of 40–100 mm Hg, which was found necessary for MscCG channel opening. Based on the equation $c = \rho gh/RT$, where ρ is density of Hg (13.6 g/cm3), g (981 cm/s^2), and h means height of

Hg column (cm), this leads to a value of 2.2–5.4 mOsm/l. In an alternative approach we estimated whether the expected membrane stretch would be high enough to activate MscCG, according to previous investigations of MscS channels. Using the Van't Hoffs law $\Delta\Pi = RT\Delta c$ the intracellular osmolarity change Δc (Osm) that would cause swelling of a bacterial cell can be calculated, which would stretch the cell membrane and cause an increase in membrane tension [36]. The membrane tension γ in a uniformly curved spheroplast patch sufficient to activate MS channels can be estimated by using Laplace's law $\gamma = pd/4$ [2], where d is the diameter of the patch. Assuming $d = 1$ µm the pressure sufficient to activate MscCG channels in spheroplast patches of 40–100 mm Hg corresponds to a membrane tension of roughly 1.5–3.5 mN/m. Given an average volume of a bacterial cell of 0.4 μm^3 [37] and thus a diameter of roughly 0.9 µm assuming a spherical shape; this value approximates the diameter of the spheroplast patch. Consequently, the magnitude of osmotic stress that would cause membrane tension of ~1.5–3.5 mN/m corresponds to a change in osmolarity of ~2.5–5.5 mOsm/l. Obviously, the two values, calculated by two different approaches perfectly match. Fluctuations in internal osmolality of such a small extent are easily accomplished by BetP within less than a second, since BetP is able to change the internal betaine concentration by several 100 mM within short time [13,33]. Thus, the functional interaction of BetP (increasing internal osmolality) and MscCG (decreasing internal osmolality) allows the cell to adjust the internal osmolality at an optimal set point with a fluctuation of the internal solute concentration of ~5 mM only. This is remarkable because the internal betaine concentration in *C. glutamicum* cells may reach values of several 100 mM at high external salt concentrations.

The concerted activity of two opposing fluxes enables the cell to quickly respond to subtle changes of external osmolality in both directions in a very sensitive manner. Consequently, the major finding of this study is the indication of MscCG's role in fine-tuning of the steady state accumulation of compatible solutes in adaptation to hyperosmotic challenge.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2010.06.022.

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