The glucosylglycerol-degrading enzyme GghA is involved in acclimation to fluctuating salinities by the cyanobacterium Synechocystis sp. strain PCC 6803

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INTRODUCTION

Salinity acts as an abiotic factor that determines the distribution of organisms in aquatic habitats. To propagate in environments with high or fluctuating salinities, these organisms have to adjust their internal water potential and avoid ion toxicity. Acclimation to high external salinities involves the accumulation of compatible solutes and the active extrusion of toxic inorganic ions [1]. Compatible solutes are by definition low-molecular-mass organic molecules that are soluble in water to molar concentrations and do not interfere with cellular metabolism [2]. Compatible solutes belong to different chemical classes, such as amino acids and their derivatives (e.g. glycine betaine, glutamate, proline and ectoine), sugars (trehalose or sucrose) and polyols (e.g. glycerol, sorbitol and mannitol) (reviewed in [3]). Heterosides, including the well-characterized compatible solutes glucosylglycerol (GG; [4]), galactosylglycerol [5] and glucosylglycerate [6], combine the protective effects of sugars and polyols. With only a few exceptions, a close correlation was found between salt tolerance limits and the distribution of organisms in aquatic habitats. To propagate in environments with high or fluctuating salinities, these organisms have to adjust their internal water potential and avoid ion toxicity. Acclimation to high external salinities involves the accumulation of compatible solutes and the active extrusion of toxic inorganic ions [1]. Compatible solutes are by definition low-molecular-mass organic molecules that are soluble in water to molar concentrations and do not interfere with cellular metabolism [2]. Compatible solutes belong to different chemical classes, such as amino acids and their derivatives (e.g. glycine betaine, glutamate, proline and ectoine), sugars (trehalose or sucrose) and polyols (e.g. glycerol, sorbitol and mannitol) (reviewed in [3]). Heterosides, including the well-characterized compatible solutes glucosylglycerol (GG; [4]), galactosylglycerol [5] and glucosylglycerate [6], combine the protective effects of sugars and polyols. With only a few exceptions, a close correlation was found between salt tolerance limits and the primary compatible solute in cyanobacteria. Strains with low salt tolerance accumulate sucrose and/or trehalose, whereas marine cyanobacterial strains with moderate salt tolerance use the heteroside GG. Halophilic strains accumulate glycine betaine [1, 7]. Recently, we found the novel compatible solute homoserine betaine in the marine cyanobacterium Trichodesmium [8].

The accumulation of compatible solutes can be accomplished either by uptake from the medium or by de novo synthesis. It has been shown that the uptake of externally supplied osmolytes often represses the endogenous synthesis of compatible solutes [9, 10]. Cyanobacteria such as photoautotrophic organisms, however, prefer the de novo synthesis of compatible solutes. Nevertheless, uptake systems for compatible solutes have also been found [11–13] and are mostly used for the reuptake of compatible solutes that have diffused from the cytoplasm into the periplasm, thereby saving cellular carbon and energy. Generally, the biosynthetic pathways of compatible solutes often branch off from general metabolism and produce metabolic end products that can accumulate to high concentrations. Proline and sucrose are exceptions, because they are part of the central cellular metabolism; thus, the turnover of these compatible solutes needs to be carefully regulated [14].
GG is the characteristic compound for moderate halotolerant (marine) cyanobacteria, but it is also accumulated by some heterotrophic bacteria and fungi [7, 15, 16]. Early investigations using pulse/chase 14C-labelling of GG in salt-acclimated cells of the cyanobacterium *Microcystis firma* showed that quick GG labelling occurred during the pulse period; however, the label intensity remained constant during the subsequent chase period [17]. Therefore, we concluded that the GG pool shows no turnover under steady-state salt conditions. Moreover, when salt-acclimated cyanobacterial cells were exposed to hypo-osmotic shocks, the majority of the compatible solutes were quickly released into the external medium [18–21]. These findings were consistent with the view that the salt-stress-proportional GG accumulation was obtained solely through regulation of the activity of the GG-phosphate synthase (GgpS) due to cellular ionic relations [22]. However, there are also reports that indicate that GG might be degraded under physiological conditions. For example, it has been reported that the uptake of trehalose into *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) induced an exchange of the internal GG pool with trehalose without the release of GG [23]. Moreover, the internal GG pool of the mat-inhabiting cyanobacterium *Microcoleus chthonoplastes* was consumed during fermentation under prolonged dark conditions [24].

Based on circumstantial evidence, Slr1670 was hypothesized to act as the GG hydrolase in *Synechocystis* [25]. However, the actual enzymatic activity was not demonstrated, and its physiological role during salt acclimation was not defined. Here, we investigated whether cyanobacteria can degrade the internal GG pool under physiological conditions and provide clues towards the role of GG degradation in acclimation to fluctuating salinities. Our results directly demonstrate that the gene slr1670 encodes the GG-degrading enzyme GghA (GG hydrolase A).

**METHODS**

**Mapping of transcriptional start sites in salt-stressed WT cells**

Total RNA was isolated as described [26] from cells grown in standard BG11 medium (control) and cells that were cultivated in the presence of 4% NaCl for 6 h or for 7 d (salt-acclimated cells). For sequence analysis by differential RNA-Seq [27], cDNA libraries were constructed by vertis Biotechnologie AG (Germany) as follows. The RNA samples were poly(A)-tailed using poly(A) polymerase, followed by blocking of the RNA species that carried 5′-mono-phosphate (5′P) by ligation of an RNA oligonucleotide. One half of each sample was treated with RNA 5′polyphosphatase (Epitend) to convert 5′PPP to 5′P, while the remaining sample was not treated (negative control). For both samples, a ligation reaction with the 5′ Illumina sequencing adapter was performed. First-strand cDNA synthesis was performed using an oligo(dT)-adapter primer and M-MLV (H-) reverse transcriptase. The cDNA fragments were PCR-amplified to a concentration of approximately 20–30 ng µl⁻¹ and sequenced by an Illumina sequencer as described [28]. Read mapping, data normalization and TSS definition were performed as previously described [29]. The data were extracted from an unpublished study.

**Construction of the Δslr1670 mutant**

The kanamycin resistance gene *aphII* was obtained from pUC4K after *Hin*II digestion. The slr1670 gene and its flanking sequence were obtained from DNA of the *Synechocystis* wild-type (WT) via PCR using the primers described in Table S1 (available in the online Supplementary Material). The DNA fragment was cut using *AccI*, leading to the deletion of an internal fragment of 462 bp. The resulting sticky ends were modified using the Klenow fragment and ligated with the isolated *aphII* gene. The plasmid pGslr1670::Km was transformed into WT cells of *Synechocystis*, and clones were selected by supplementation of the medium with kanamycin. Subsequent cultivation at 50 µg ml⁻¹ kanamycin resulted in complete segregation of the Δslr1670 mutant, which was confirmed by PCR (Fig. S2). Since cultures of the Δslr1670 mutant tend to appear viscous when cultivated in NaCl-free BG11 medium, samples were analysed by light microscopy (Olympus BX51). For visualization of EPS-aggregates, the samples were stained with India ink.

**Slow hypo-osmotic treatment (gradual dilution experiment)**

Cells of the *Synechocystis* WT and the Δslr1670 mutant were cultivated in a final volume of 200 ml of medium that was supplemented with 4% NaCl (w/v) in a 500 ml bioreactor, furnished with different ports that permitted aeration, sampling and the supply of NaCl-free BG11 to dilute the saline medium. Continuous mixing of the cultures was ensured by the presence of a magnetic stirrer. Cultures were grown at 30 °C under continuous illumination (50 µmol of photons m⁻² s⁻¹) and with continuous aeration with CO₂-enriched (5% v/v) air until an OD₅₇₀ of 2.0–2.5 was reached. During the dilution experiment, the cyanobacterial suspension was gradually diluted using a peristaltic pump (ISMATEC, Idex, Germany) with a continuous flow of 0.36 ml min⁻¹ NaCl-free BG11 medium. The flow was obtained using ISMATEC 2-stop tubing (Tygon-R3607-HS-tubes; Idex, Wertheim, Germany) with a diameter of 1.52 mm. A conductivity cell (WTW, Weilheim, Germany) was used to determine the salt concentration during the dilution experiment. For the calibration curve, the conductivity (μS cm⁻¹) of BG11 medium that was supplemented with different NaCl concentrations was recorded. The slope of the calibration curve was used to determine the NaCl concentration during the experiment (Fig. S5). Aliquots from the cultures were taken every 2 h and used to determine the growth GG content and resulting salinity.

**Rapid hypo-osmotic treatment (down-shock experiment)**

To perform an abrupt osmotic down-shock, salt-adapted cultures of the WT and the Δslr1670 mutant strains were grown at 30 °C while bubbling with CO₂-enriched air (5% v/v) under continuous illumination at 75 µmol of
Expression vector pET28a (Novagen). The pET28a_}

Expression and purification of the GghA (Slr1670) and analysed by gas chromatography as described [30].

the dry cell extracts and growth media were then silylated repeated with absolute ethanol. The organic compounds in dried. To remove salts from the samples, the latter step was transferred to a new tube and freeze-dried. The cells were

Determination of GG and trehalose concentrations
Compatible solutes were quantified in cells and in the surrounding medium. For this purpose, 2 ml of the cyanobacterial cultures was taken, and the cells were harvested by centrifugation (5 min, 13,000 r.p.m., 20 °C) and frozen in liquid nitrogen. In total, 500 µl of the supernatant was transferred to a new tube and freeze-dried. The cells were extracted with ethanol (80 %, HPLC grade, Roth, Germany) for 2 h at 68 °C. Fifty µg of sorbitol was added to the samples as an internal standard. After centrifugation of the extracts, the supernatants were transferred to a new tube and freeze-dried. To remove salts from the samples, the latter step was repeated with absolute ethanol. The organic compounds in the dry cell extracts and growth media were then silylated and analysed by gas chromatography as described [30].

Expression and purification of the GghA (Slr1670) protein
The coding sequence of slr1670 was amplified using the primer combination slr1670_EcoRI/slr1670trunc_Xhol (Table S1). The resulting 2667 bp PCR product was cloned into the pGEM-T vector (Promega). After cleavage with the restriction enzymes EcoRI and Xhol, the fragment was cloned into the expression vector PET28a (Novagen). The PET28a_slr1670 plasmid was transferred into a strain of E. coli BL21(DE3) that harbours the pACYC_cpn10/cpn60 plasmid of ArcticExpress cells (Agilent). This strain constitutively expresses the Oleispira antarctica chaperons Cpn10 and Cpn60 on the ColE1-compatible, pACYC-based plasmid that harbours a gentamycin-resistance gene. Cells expressing Slr1670 (GghA) and Cpn10/Cpn60 were cultivated in LB medium at 37 °C with the following supplements: 50 µg ml⁻¹ kanamycin, 5 mM glycine betaine, 250 mM sorbitol, 2 % NaCl (w/v) and 1 % Triton-X (v/v).

Once an optical density of 0.6–0.8 at 600 nm was reached, the cultures were transferred to 10–13 °C and incubated with shaking at 220–250 r.p.m. for 30 min. After the culture equilibrated to 10–13 °C, protein expression was induced by the addition of 1 mM IPTG. Subsequently, the cultures were incubated at 10–13 °C for 16h. The cells were harvested by centrifugation at 9000 r.p.m. for 15 min at 4 °C and resuspended in ice-cold homogenization buffer (20 mM HEPES, pH 8, and 500 mM NaCl). The total cell extract was obtained by ultrasonic treatment. Soluble protein extracts were obtained after centrifugation at 40,000 g and then used for affinity chromatography on Ni-NTA columns (Pro Bond Resin, Invitrogen, Germany). The His-tagged recombinant GghA protein was eluted with 300 mM imidazole. The eluted protein fractions were combined and desalted using PD-10 columns (GE Healthcare, UK). Finally, the recombinant enzyme was eluted from the column with 20 mM HEPES, pH 8. The purity of the protein was analysed by SDS-PAGE (see Fig. S6) in 12 % polyacrylamide gels, and the protein content was determined by the method described [31].

GghA enzyme activity assay
The enzyme activity was determined at pH 7 in reaction mixtures that contained 20 mM HEPES, 20 mM GG and an appropriate amount of enzyme in a final volume of 200 µl. The mixtures were incubated at 30 °C for different durations. The reactions were stopped by incubation at 95 °C for 10 min. One aliquot was immediately inactivated without prior incubation to serve as a control. To detect the products of GG degradation via gas chromatography, 50 µl aliquots of the reaction mixture were mixed with ethanol (80 %, HPLC grade, Carl Roth GmbH, Germany). After the addition of 50 µg of sorbitol that functioned as an internal standard, the samples were analysed by gas chromatography as described [30]. Substrate specificity was tested after incubation with GG, trehalose, malate and sucrose, each at a concentration of 20 mM. The effects of inorganic salts on GghA activity were tested by adding 200 mM NaCl or KCl to the assay buffer. GghA activity was calculated based on the amount of glucose that was liberated by substrate cleavage (see Fig. S7 for the calibration curve). Glucose was quantified by the coupled reaction of hexokinase and glucose-6-phosphate dehydrogenase (Roche Diagnostics GmbH, Germany), which led to NADP reduction that was followed spectrophotometrically at 340 nm on a Lambda Fluoro 320 microplate fluorescence reader (MWG-Biotech AG, Germany).

RESULTS
Expression analysis identified a salt-regulated gene for a putative glucosylhydrolase
The salt-dependent expression of Synechocystis genes was investigated by differential RNA-seq [27], which permits the specific enrichment of primary 5′-RNA ends. Thus, this method not only allows transcript quantification but also...
identifies the transcriptional start sites (TSSs) of every gene or transcriptional unit that is transcribed under these conditions at single-nucleotide resolution [28, 29]. This method confirmed the salt-stimulated transcription of the \( ggpS \) gene that encodes the key enzyme for GG synthesis (Fig. 1), consistent with previous results [32]. The normalized read count for the \( ggpS \) 5’end increased approximately 50-fold, from 22,655 (under control conditions) to 1,215,205 reads, 6 h after the addition of 4% NaCl. While this strong induction was transient, 15-fold higher transcript abundance (341,668 read counts) was detected in long-term salt-acclimated cells compared to the control cells.

For \( ggpS \), the main TSS was mapped at position 19,492,020, confirming the previous start site determination that was 378 bp upstream of the GgpS translational start codon within the small \( ggpR \) gene [33]. Interestingly, the \( ggpS \) promoter overlaps with other salt-stimulated promoters that drive the transcription of genes upstream of \( ggpS \) on the opposite strand. The upstream-located gene cluster comprises three genes: \( slr1670 \) (putative glucoside hydrolase), \( glpK \) (\( slr1672 \), encoding glycerol kinase) and \( slr1673 \) (putative tRNA/rRNA methyltransferase). Instead of one TSS, we mapped three different TSSs, which were located at positions 393, 288 and 165 nt upstream of the \( slr1670 \) start codon (Fig. 1). Of these, TSS1 is intricately linked with the \( ggpS \) TSS on the reverse complementary strand; with an offset of only a single nucleotide, it uses the same sequence stretch as the −10 element. Recently, it was shown that LexA acts as a repressor for genes that are related to GG synthesis, and this implied the presence of two binding sites in the promoter of \( slr1670 \) [34]. Indeed, our data show that parts of the −10 elements and the six preceding nucleotides of TSS2 and TSS3 are each putative LexA binding sites (Fig. 1), as defined [34]. We conclude that most likely \( slr1670 \) is under the repression of LexA.

All three \( slr1670 \) TSSs responded to the salt shock in a similar way (three- to eightfold induction 6 h after salt shock). However, with only a few thousand read counts for each TSS, \( slr1670 \) is transcribed less than \( ggpS \) (Fig. 1). Nevertheless, for salt-acclimated cells, the read counts remained slightly greater than those of control cells. This finding is in agreement with the previous microarray-based transcriptome data that quantified whole transcript accumulation; in these data, \( slr1670 \) and \( glpK \) showed a >twofold increased abundance in long-term salt-acclimated cells [35].

The co-localization and co-expression of this gene cluster together with \( ggpS \) imply that the \( slr1670 \) gene product is involved in salt acclimation, which is also consistent with the suggestion that Slr1670 acts as the GG hydrolase [25]. Interestingly, the \( slr1670 \) homologues are frequently co-localized with \( ggpS \) and other genes involved in salt acclimation among cyanobacteria (Fig. S1), which indicates conserved regulation and functional relation. Such a link has been shown before for the small protein GgpR, which acts as a repressor of \( ggpS \) gene expression under low-salt conditions [33]; it is also obvious for \( glpK \), located downstream of \( slr1670 \), which encodes for a glycerol kinase involved in the synthesis of the GG precursor glycerol 3-phosphate using the substrate glycerol.

**Construction and phenotype analysis of \( slr1670 \) mutant**

To perform a functional analysis of the putative glucosyl hydrolase, we first used a genetic approach. A completely...
segregated deletion mutant of the slr1670 gene was obtained (Fig. S2). This finding reveals that Slr1670 has no essential function in *Synechocystis* under standard laboratory conditions. Subsequent phenotypic characterization showed only minor differences from the WT. Interestingly, the growth rate of the mutant Δslr1670 was slower under NaCl-free conditions, whereas its growth in NaCl-supplemented BG11 was not influenced by this knock-out (Fig. S3). Mutant cells accumulated slightly higher amounts of internal GG compared to WT in 4% NaCl, but the difference was not statistically significant (Fig. S3). Furthermore, the mutant tends to excrete yet uncharacterized extracellular polysaccharides (EPS) under NaCl-free conditions, which made the medium viscous; this phenotypic difference was not observed in NaCl-supplemented media. Therefore, the appearance of WT and mutant cells was analysed under a light microscope. In contrast to WT cultures, cells of the Δslr1670 mutant were covered by large amounts of EPS, leading to the formation of cell/EPS aggregates. Moreover, we also observed cell-free EPS aggregates appearing as white clouds in the ink-stained medium of the Δslr1670 mutant, and these were not detected in the media of WT cultures (Fig. S4).

**Effect of decreasing salt concentration on the GG pool**

Since the Slr1670 protein harbours a central glucosylhydro-lase domain and is co-expressed with GgpS, we analysed its possible role in GG degradation during salt acclimation in more detail. To this end, we developed two different experimental setups that expose salt-acclimated cells of the WT and the Δslr1670 mutant strain to decreasing salinities.

First, a dilution experiment was performed in which the salinity was gradually decreased over long time periods. This treatment mimics slight fluctuations of salinity in the natural environment and is more physiological than abrupt hyp-osmotic shifts from saline into a freshwater medium. To this end, NaCl-free BG11 medium was added dropwise over 10 h. The total salt concentration showed a linear decrease from 4.5% to approximately 2.5% during the dilution period (Fig. 2). Consistent with the decrease in external salinity, the intracellular GG content decreased from 300 to 160 nmol ml\(^{-1}\) OD\(_{750}\) in cells of the WT strain. The decrease in cellular GG concentration was clearly due to GG degradation, because no GG was detected in the external medium. In contrast, the GG pool remained fairly constant at approximately 300 nmol ml\(^{-1}\) OD\(_{750}\) in cells of the Δslr1670 mutant (Fig. 2).

Second, we tested the effect of abruptly decreasing salinity on the viability of the Δslr1670 mutant by exposing cells to a rapid down-shock from 4 to 0% NaCl. As reported previously [19], this treatment leads to the rapid release of GG into the medium (Fig. 3). This behaviour was observed for cells of the WT and Δslr1670 mutant alike. In both cases, the intracellular GG quantities dropped quickly and significant amounts of GG appeared in the medium. However, whereas the WT cells were virtually free of GG just 3 h after the rapid NaCl down-shock, the cellular GG content of mutant cells remained at a constant low level of 40 nmol ml\(^{-1}\) OD\(_{750}\) for 24 h.

In addition, the extracellular GG disappeared from the medium in the WT culture within 24 h, indicating that reuptake and consumption of the previously released GG had occurred. This behaviour was not seen for the Δslr1670 culture, whose extracellular GG concentration remained quite constant for 24 h after NaCl down-shock. The growth of cells was monitored by measuring the increase in OD\(_{750}\). Interestingly, WT cells resumed growth immediately after the rapid NaCl down-shock, whereas cultures of the Δslr1670 mutant only showed a very slow increase in OD\(_{750}\) within the first 24 h after transfer from 4 to 0% NaCl. To test the viability of the cells, we performed a subsequent drop-dilution assay on solid medium. Cells of the mutant Δslr1670 survived, but the cell density was clearly lowered at 10-fold and in particular 100-fold dilution after 5 d of incubation on plates with NaCl-free BG11 (Fig. 3).

These data reveal that hypo-osmotic treatments induce a GG-degrading activity in salt-acclimated cells of the *Synechocystis* WT strain, which is clearly impaired in the cells of the mutant Δslr1670. Thus, slr1670 seems to encode the required GG hydrolase, and its activity is physiologically important for proper acclimation to gradual decreased salinities, whereas the GG release via mechanosensitive channels represents the immediate response to abrupt, large-scale hypo-osmotic treatments.

**Effect of trehalose addition on GG pool**

The decrease of the internal GG pool after the addition of trehalose to the medium represented another physiological
situation, in which case the internal GG pool becomes diminished without observing extracellular GG [23]. To test whether Slr1670 is also involved in this process, 1 mM trehalose was added to salt-acclimated cultures of the WT and the Δslr1670 mutant, and the intracellular content of GG and trehalose was measured. Consistent with the previous report, external trehalose was taken up by cells of the WT as well as the Δslr1670 mutant at similar rates (Fig. 4). Trehalose uptake is possible due to the activity of the ABC-type osmolyte uptake system [12]. The increase in cellular trehalose concentration was paralleled by a decrease in the cellular GG pool in WT cells. In contrast, the GG pool remained almost unchanged in cells of the Δslr1670 mutant, despite the accumulation of similar amounts of trehalose (Fig. 4).

The osmolyte exchange experiment provided the second hint that slr1670 encodes the corresponding GG hydrolase and further expanded its physiological relevance.

**Biochemical verification of GG hydrolase activity of GghA (Slr1670)**

These results and the circumstantial evidence presented by Savakis et al. [25] supported the idea that Slr1670 is a GG-hydrolysing enzyme. To verify this assumption directly, the

![Figure 3](image1.png)

**Fig. 3.** GG concentrations in cells of the *Synechocystis* wild-type (6803 WT) and Δslr1670 mutant exposed to rapidly decreasing external salinity (shift from 4 to 0 % NaCl). Cultures grown for 1 week in BG11 medium supplemented with 4 % NaCl were exposed to an abrupt hypo-osmotic shock. At time point 0 h, the salt-acclimated cells were harvested by centrifugation and resuspended in NaCl-free BG11 medium. The intracellular GG concentrations (circles connected by a solid line); extracellular GG concentrations (triangles connected by line) and growth expressed as an increase of OD750 (squares connected by a dotted line) were measured over 24 h after the hypo-osmotic shock. The data are the mean values±SD of three biological replicates. Inserted panels: drop-dilution assay with cells harvested from the cultures 24 h after the hypo-osmotic shock treatments. The agar plates with NaCl-free BG11 medium were incubated for 5 days.

![Figure 4](image2.png)

**Fig. 4.** GG and trehalose concentrations in cells of the *Synechocystis* wild-type (6803 WT) and Δslr1670 mutant in trehalose-supplemented BG11 medium of constant salinity (4 % NaCl – osmolyte exchange experiment). The effect of trehalose supplementation (1 mM) on the accumulation of compatible solutes in salt-acclimated cells of 6803 WT (left) and the Δslr1670 mutant (right) was investigated. The intracellular concentrations of GG (solid line) and trehalose (dashed line), and the GG concentration in a control cultures (no trehalose addition, dotted line) are shown. The data are the mean values±SD of three biological replicates.
gene was expressed in E. coli, and recombinant Slr1670 was purified (Fig. S6). The solubility of recombinant Slr1670 appeared to be low, and it tended to form inclusion bodies. Only the use of the cold-induced chaperonin system (see the Methods section) secured sufficient amounts of soluble Slr1670 for biochemical assays. Pure recombinant His-tagged Slr1670 protein of approximately 103 kDa in size was eluted from Ni columns (Fig. S6).

Following purification, recombinant Slr1670 was incubated with GG to investigate its hydrolysis activity. Gas-chromatographic analysis of the enzyme assay products showed the appearance of the cleavage products glucose and glycerol at the expense of lowered GG, which supports the notion that Slr1670 acts as sugar hydrolase (Fig. 5a). It hydrolyzed approximately 12 ng GG per min and µg protein. The recombinant Slr1670 protein did not show any glucose-releasing activity when incubated with the alternative substrates trehalose, maltose, or sucrose. Interestingly, the protein activity was clearly inhibited by salt, with NaCl having a stronger inhibitory effect than KCl (Fig. 5).

These results directly confirmed that Slr1670 specifically degrades GG; accordingly, we named this protein GG hydrolase A (GghA).

**DISCUSSION**

Our results clearly show that the salt-regulated slr1670 gene, which is localized upstream from ggpS on the opposite
DNA strand, encodes GghA, an enzyme capable of degrading GG. Biochemical experiments using the pure recombinant protein verified that GghA splits GG into glucose and glycerol. Thus, this enzyme is a GG hydrolase/glucosidase and works different from the previously described GG-degrading GG phosphorylase that was identified in Bacillus selenitireducens MLS10. This enzyme catalyzes the phosphorylase reaction that splits GG into glycerol and glucose phosphate [36].

Genetic and physiological experiments revealed that GG hydrolysis plays an important physiological role when salt-acclimated cells of Synechocystis are exposed to fluctuating salinities, or when alternative compatible solutes such as trehalose are available in the surrounding medium. Especially at decreased salinities, GghA is responsible for the proper adjustment of the cellular GG pool caused by the external salinity. The complete removal of remaining GG from cells after abrupt hypo-osmotic shocks obviously also depends on GghA and is beneficial for the fitness of the cells, since the mutant Δslr1670 needed longer times to resume growth after the hypo-osmotic treatment and showed lower survival rates.

The involvement of GghA in GG breakdown was recently indicated by circumstantial evidence. A group of authors investigated the mechanism of glycerol release from salt-acclimated Synechocystis cells [25]. They observed that inactivation of GghA abolished the glycerol release from the corresponding mutant. Furthermore, the stimulation of glycerol release from WT cells in GG-supplemented media did not occur in cells that lacked GghA. The authors also showed for the first time that external GG stimulated the growth of Synechocystis when grown at 200 mM NaCl. Modelling implied that this growth stimulation could be explained by a carbon-saving strategy [25]. However, it remained unclear whether the growth benefit from carbon saving was due to the direct utilization of GG to populate the compatible solute pool in growing cells or the utilization of the liberated glucose as carbon and energy sources. Our results tend to support the second possibility. We found that GghA stimulated the growth of WT cells in rapid hypo-osmotic stress experiments. External GG, which was probably released via mechanosensitive channels from the hypo-osmotically stressed cells [37, 38], rapidly disappeared from the media of WT cultures, while it remained in the medium of mutant Δslr1670. Most likely, GG was taken up by WT cells via the Ggt transport system [13, 23] and hydrolyzed by GghA, which eventually promoted growth via the generated glucose. In cells of the Δslr1670 mutant, uptake of external GG by the action of the Ggt transport system probably results in high quantities of internal GG, which are again released into the medium by the osmotic gradient, because GG is not degraded due to the missing GghA activity. This uptake/release cycle might explain why the amount of extra-cellular GG remains high in mutant cultures compared to WT cultures. Consistent with the previous results [25], we found significant glycerol accumulation in the medium of WT cultures, but not in the medium from Δslr1670 cultures after hypo-osmotic treatments (see Fig. S8).

Our experiments indicate that the major physiological role of GghA is to adjust the GG pool under fluctuating conditions, when lowered salinities require a reduction of internal GG concentrations. Under these conditions, the degradation of GG is preferred, since it saves organic carbon and energy [25]. Our early experiments with the cyanobacterium M. firma suggested that continuous turnover of GG is unlikely [17]. However, a slight turnover of GG seems to occur in Synechocystis, since a low but continuous glycerol excretion was observed when Synechocystis WT cells were grown at 200 mM NaCl [25]. It should be mentioned that we never observed significant quantities of glycerol in media from Synechocystis cultures grown at 684 mM NaCl (4%). In contrast, cells of the marine cyanobacterium Synechococcus sp. PCC 7002 seem to exhibit rather high GG turnover rates. Stable isotope probing indicated that the GG and glycogen pools are closely connected in this cyanobacterial strain. Rather large parts of the glycogen pool seem to be synthesized from carbon that was initially found in the GG pool [39]. It could be assumed that the GghA homologue encoded by the gene synpcc7002_a2849 in the Synechococcus sp. PCC 7002 genome could be involved in this GG turnover. In this respect, it is interesting to note that in many cyanobacterial genomes, genes for GG-synthesizing enzymes as well as GghA have been found [25] (Fig. S1). This linkage points to a general capability of these strains to adjust the internal GG pool by balancing synthesis and degradation.

Regardless of whether GghA is mainly involved in GG degradation after hypo-osmotic treatments or in continuous GG turnover, its activity must be tightly regulated to ensure the salt-stress-proportional accumulation of GG inside cells. It has been shown that GgpS, the key enzyme for GG synthesis, is regulated biochemically. The GgpS activity depends on the internal ion concentrations that release GgpS from its inactivating attachment to DNA [22]. The cellular inorganic ion level is strongly increased after salt shock [40] and remains enhanced in cells that are acclimated to higher salinities [41]. These alterations in ion concentrations ensure that the GgpS activity is very high in salt-shocked cells, so that the GG pool may be filled quickly and to ensure that GgpS is gradually more active in cells acclimated to higher salinities, so that GG synthesis activity is balanced [22]. In contrast to GgpS, the GghA activity was negatively influenced by increasing amounts of inorganic ions. Na⁺ seems to have greater inhibitory effects than K⁺, which would result in the inhibition of GghA in salt-shocked cells (when GG degradation should be avoided). Hypo-osmotic shocks not only resulted in the release of GG, but also other low-molecular-mass compounds [19]; thus, these cells have rather low internal ion concentrations, supporting the activity of GghA. Nevertheless, the regulatory interplay between GghA and GgpS needs to be studied further.

Finally, it was interesting to observe that the mutant Δslr1670 only grew more slowly under NaCl-free conditions, whereas its growth was similar in NaCl-supplemented BG11 medium. Moreover, the viscous appearance of media
from mutant cells, which probably resulted from the massive release of EPS, was not observed when these cells grew in NaCl-enriched BG11 medium. These observations point to a function of GG degradation via GghA under low-salt conditions in the synthesis and/or attachment of EPS to *Synechocystis* cells. In this regard, it is interesting to note that GG is a scaffold for teichoic acids in several Gram-positive bacteria [42] that form a polysaccharide network at the cell wall. It has been also shown that membrane-derived oligosaccharides in the periplasm of Gram-negative bacteria contain glucose linked to glycerol phosphate [43]. The possible roles of GG and its turnover, separate from salt acclimation, need to be addressed in the future.

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### Conflicts of interest

The authors declare that there are no conflicts of interest.

### Ethical statement

The study did not involve experiments on humans or animals.

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