Osmotic stress in *Synechocystis* sp. PCC 6803: low tolerance towards nonionic osmotic stress results from lacking activation of glucosylglycerol accumulation

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In order to compare the molecular principles of the acclimatization of bacterial cells to salt and nonionic osmotic stress, the moderately halotolerant cyanobacterium *Synechocystis* sp. PCC 6803 was challenged by salt (NaCl), and the osmolytes sorbitol and maltose. The physiological response towards each of the three compounds was found to be different. After salt addition, the cell volume remained unchanged, and the accumulation of the osmoprotective compound glucosylglycerol (GG) was observed after activation of the key enzyme GgpS at the biochemical and gene (*ggs*) expression level. Sorbitol addition had only minor effects on the cell volume. In spite of the fact that the *ggs* expression was increased, the GgpS enzyme was not activated, resulting in the absence of GG accumulation. In contrast the cells accumulated sorbitol, which served as a compatible solute and assured a certain osmotic resistance. In comparison to NaCl and sorbitol, the addition of maltose caused a strong decrease in cell volume indicating water efflux. However, no osmolyte accumulation was observed, resulting in an osmosensitive phenotype. Consequently, a successful response of *Synechocystis* cells to an osmotic challenge is indicative of the *de novo* synthesis of GG upon salt-dependent activation of the GgpS enzyme or the uptake of external solutes.

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INTRODUCTION

Cyanobacteria are photosynthetic, oxygen-evolving prokaryotes, which can conquer all light-exposed habitats due to manifold adaptations during evolution. Today, they are widespread in fresh water and marine environments. In some extreme habitats, such as hot springs, desert soils and rice paddy fields, cyanobacteria can be dominant. As a cyanobacterial model strain for the analysis of mechanisms involved in environmental acclimatization at the molecular level, *Synechocystis* sp. PCC 6803 (henceforth referred to as *Synechocystis*) has often been used because its genome sequence has been known since 1996 and the strain is amenable for genetic manipulations (Kaneko *et al.*, 1996; Grigorieva & Shestakov, 1982).

Synechocystis belongs to the group of moderately halotolerant cyanobacteria. It is able to grow at salt concentrations ranging from fresh water to double seawater concentrations (Reed *et al.*, 1985). Salt stress is a combination of different

primary stress types. Obviously, salt-shocked cells are challenged by a changing ion concentration, as well as a changing water potential of the surroundings. Therefore, the main physiological acclimatization reactions deal with the ionic and osmotic problems. The noxious effects of high ion concentration in the cytoplasm are quickly diminished by active export. The Na⁺ ions, for example, are exported by Na⁺/H⁺ antiporters (Inaba *et al.*, 2001; Elanskaya *et al.*, 2002), which are regulated at the activity level, since their gene expression does not change in salt-shocked cells (Marin *et al.*, 2004). The internal osmotic potential of *Synechocystis* cells is balanced by uptake (Mikkat *et al.*, 1996) or *de novo* synthesis (Reed *et al.*, 1985; Marin *et al.*, 1998) of the compatible solute glucosylglycerol (GG), which enhances the internal osmotic potential, and is also able to directly protect proteins and membranes (Borges *et al.*, 2002; Hinch & Hagemann, 2004). The synthesis of GG is regulated by a salt-dependent modulation of the activity of the key enzyme glucosylglycerolphosphate synthase (GgpS), as well as by an increased expression of all genes encoding proteins involved in GG synthesis during salt acclimatization (Hagemann & Erdmann, 1994; Kanesaki *et al.*, 2002).

Abbreviation: GG, glucosylglycerol.

In the literature, the terms salt and osmotic stress are often used synonymously. Indeed, investigations of global changes at the transcriptome level in *Synechocystis* indicated that salt and osmotic stress involved overlapping two-component systems for stress-induced alterations in gene expression (Shoumskaya *et al.*, 2005). However, different sets of genes were induced. In order to distinguish between ionic and osmotic effects, NaCl-induced salt stress and nonionic osmotic stress with sorbitol often have been compared. There is evidence that the acclimatization to sorbitol-induced osmotic stress and to NaCl-induced stress is different. Besides differences in the gene expression pattern induced by each stress, sorbitol was found to be toxic at a concentration of 700 mM, while NaCl up to 1.2 M (an equivalent of 2.4 M sorbitol in terms of osmotic stress) is easily tolerated. Therefore, we compared the effects of high NaCl concentrations and nonionic osmotic challenges on the regulation of GG synthesis and *ggsS* expression. For osmotic stress treatments the commonly used compound sorbitol was compared to maltose, which in contrast to sorbitol is a disaccharide. Neither sorbitol nor maltose are transported by the uptake system for osmoprotective compounds in *Synechocystis* (Mikkat *et al.*, 1996). This study indicates that different extracellular and particular intracellular signals are perceived by *Synechocystis* cells during salt or osmotic stress conditions. An overall low tolerance of *Synechocystis* towards nonionic osmotic shock was found. Sorbitol is taken up by the cells and inhibits the biochemical activation of GG synthesis. Therefore, it is not a valid compound to mimic nonionic osmotic stress in cyanobacteria.

METHODS

Strains and culture conditions. The wild-type of the glucose-tolerant strain of *Synechocystis* sp. PCC 6803 was kindly provided by N. Murata (NIBB, Okazaki, Japan). The reporter strain *RPggsS::luxAB* was generated by transformation of *Synechocystis* with the vector pILA (Kunert *et al.*, 2000) after insertion of the *ggsS* promoter region and a non-translated leader in front of promoterless *luxAB* genes. The DNA of the 1 kb region upstream from the translational start codon of *ggsS* was amplified by PCR using the primers *ggsSP5'* 5'-CACGGTACCCTGTTGCGGCAATTCAATC-3' and *ggsSP3'* 5'-AAAGGTACCGCGGTCTCCAAATCAAGAA-3', which include *KpnI* restriction sites (underlined) to enable insertion into pILA. Axenic cells were cultured in BG11 medium buffered by 20 mM TES at pH 8. Transformants were initially selected on media containing 10 µg kanamycin ml⁻¹ or 10 µg spectinomycin ml⁻¹, while complete segregation and continuous cultivation was performed at 50 µg ml⁻¹. For physiological characterization, cells either were grown in batch cultures using Erlenmeyer flasks (250 ml) on a shaking incubator (radius 25 mm, 150 r.p.m.) under continuous light of 25 µE (OSRAM LUMILUX 36/930) at 30 °C (growth and promoter test experiments) or were incubated in glass tubes with continuous bubbling of air enriched with 5% CO₂, under continuous light of 150 µE at 30 °C (experiments including sampling for protein and/or RNA isolation). Salt and osmotic concentrations were adjusted by adding NaCl, sorbitol or maltose to standard BG11 medium containing 18 mM NaCl. Growth and cell density were monitored by measuring OD₇₅₀ with a spectrophotometer, Ultrospec

300 (Pharmacia Biotech). Contamination by heterotrophic bacteria was analysed by spreading 200 µl culture on LB plates and following the incubation in the dark.

For all DNA manipulations the *Escherichia coli* strain DH5 α mc^r (Sambrook *et al.*, 1989) was cultured using LB media at 37 °C.

RNA techniques. Cells were harvested from 10 ml culture by centrifugation at 4000 g, for 5 min at 2 °C, and then immediately frozen in liquid nitrogen and stored at -20 °C. RNA was extracted with hot phenol/chloroform, and purified with a High Pure RNA isolation kit (Roche). Methods for separation, blotting and hybridization of RNA, such as synthesis of radioactively labelled DNA probes and the recording of signal intensities, are described by Marin *et al.* (2002). In order to quantify the data and correct errors in gel loading, all calculations were made on the basis of hybridization signals obtained after applying a radiolabelled 16S rDNA probe to the same filters.

Protein techniques. Soluble proteins were extracted using glass beads in a Fast Prep device (Thermo Savant) from a pellet of 10 ml cells grown at standard salt concentration, 18 mM NaCl. Extraction was performed in buffer A containing 20 mM Tris-maleate (pH 7.5), 0.1 mM EDTA, 10 mM MgCl₂. The enzyme assay for GgsS activity was carried out according to Schoor *et al.* (1999), whereby the indicated concentrations of NaCl, sorbitol or maltose were added directly to the enzyme-assay buffer. The amount of GG was estimated using GC analysis. Protein concentrations were determined using the Roti-Nanoquant kit (Roth).

As an indication of the *ggsS* promoter activity, the amount of LuxAB was analysed after addition of 200 µl decanal (50 mM in 50% methanol) to 1 ml cell suspension, which was directly taken from the cultures at the indicated time points. LuxAB luminescence was measured in a luminometer (Lumat LB9507, E. G. & G. Berthold). Maximum values were recorded and used for further calculation on a cell number or an OD₇₅₀ basis.

Analysis of compatible solutes. For analyses of compatible solutes, 2 ml cell suspension was harvested by centrifugation (10 000 r.p.m. for 5 min at 4 °C). Cells exposed to osmotic stress were washed three times with an iso-osmolar NaCl solution to remove all external solutes. The low molecular mass compounds were extracted from the cell pellets by incubation in 500 µl 80% ethanol for 3 h at 65 °C. The dry residue was treated with 0.5 ml chloroform and 1 ml water to remove membranes, pigments and soluble proteins. After centrifugation, the aqueous phase was taken and dried in a vacuum concentrator. In order to remove high concentrations of salts, the dry residue was redissolved in absolute ethanol. After centrifugation, the liquid phase was transferred into a new vial, dried and stored at room temperature. GC was performed on a TraceGC (ThermoFinnigan) with a FS-Supreme-5 column (length 30 m, diameter 0.25 mm; Chromatographie Service). Injector and detector temperatures were kept at 300 °C, and separation was achieved by a linear temperature gradient from 60 to 320 °C with a heating rate of 30 °C min⁻¹, and isothermal plateaus at 280 and 320 °C for 3 and 5 min, respectively. Dried samples were redissolved in pyridine containing 24 mM *O*-methoxyhydroxylamine hydrochloride and incubated for 90 min at 30 °C. Derivatization was performed after addition of 1.8 vol. *N*-methyl-*N*-trimethylsilyltrifluoroacetamide for 1 h at 65 °C. Sorbitol or ribitol served as internal standards.

Light microscopy. Microscopy pictures were taken directly after osmotic upshift by 500 mM NaCl, sorbitol or maltose, using an Eclipse E800 microscope (Nikon) equipped with a CCD camera (KY-F1030; JVC) and the Diskus software package (Hilgers).

Changes of volume and shape of *Synechocystis* cells after osmotic upshift were followed by light scattering measurements using 0.5 ml culture (OD_{750} 1.8) mixed with 1 vol. medium containing NaCl, sorbitol or maltose to reach the indicated final concentrations. Directly after mixing, the measurements were performed at 750 nm (4 nm bandpass for excitation and scattering with an angle of 90°) for 2 min using a fluorescence spectrophotometer SLM-Aminco Bowman Series 2 (SLM-Aminco) at 30°C .

Quantitative data with SDs are the mean of three independent experiments. In the other cases a typical experiment is shown, which is representative for at least three biological repetitions.

RESULTS

Resistance of *Synechocystis* towards nonionic osmotic shock

For characterization of the physiological response of *Synechocystis* cells to osmotic stress, we compared the growth of wild-type cultures treated with NaCl, sorbitol or maltose at various but iso-osmolar concentrations, i.e. 200 mM sorbitol compared with 100 mM NaCl. The addition of NaCl up to concentrations of 600 mM did not alter the growth drastically. At concentrations of 500 to 600 mM NaCl only a slightly lower growth rate was observed (Fig. 1a). Addition of sorbitol up to 300 mM did not significantly influence growth of *Synechocystis*. A further increase in sorbitol concentrations to 400 and 500 mM resulted in a reduced growth rate, while 600 mM sorbitol inhibited growth completely (Fig. 1a). In contrast, addition of 100 and 200 mM maltose strongly promoted the growth of *Synechocystis* cultures, while at concentrations higher than 200 mM no growth was observed (Fig. 1a).

The GG content was estimated in cells grown for 72 h at different external osmolyte concentrations (Fig. 1b). In cells grown at standard conditions (18 mM NaCl) only traces of GG were detectable, while increasing NaCl concentrations led to a linear increase in GG content. In contrast, cells confronted with higher sorbitol concentrations (100–600 mM) accumulated only traces of GG comparable to the amount in cells at standard conditions (Fig. 1b). *Synechocystis* cells exposed to 200 mM maltose displayed an increased GG content compared to cells grown under control conditions, while at 100 mM maltose the GG content was not increased. In non-growing cells at higher maltose concentrations GG could not be detected.

Impact of salt and osmotic stress on cell volume

Long-term exposure of *Synechocystis* to sorbitol concentrations higher than 500 mM and to maltose concentrations higher than 200 mM was found to be toxic. This indicates a surprisingly low resistance towards nonionic osmotic stress despite the fact that this strain displays a high salt tolerance. In order to characterize the differences in the acclimatization towards osmotic and salt stress, short-term stress treatments were analysed. First we investigated changes of cell volume and cell shape by light microscopy. After a salt

Fig. 1. (a) Growth rates of *Synechocystis* cells after inoculation in media containing different concentrations of NaCl (black bars), sorbitol (white bars) or maltose (grey bars). The OD_{750} of the cultures was adjusted to 0.01 and monitored for 5 days. (b) The content of the compatible solute GG in cells after 5 days of incubation.

shock of 500 mM NaCl no obvious changes were observed (Fig. 2a). Contrary to this, after addition of 500 mM maltose nearly all cells displayed a kidney-shaped form, indicating a significant loss of cellular volume and strong deformation of the cell envelope (Fig. 2a). Addition of 500 mM sorbitol caused a deformation of the cell envelope, as observed after maltose addition, in only a few cases (Fig. 2a).

In order to quantify the effects of osmolyte addition on cell volume and cell shape, we used light scattering at 750 nm (Fig. 2b). For cells challenged by NaCl, only a small decrease in the light scattering signal was observed, corresponding to the absence of visible changes. While after exposure to sorbitol the scattering signal was reduced up to 18 %, after maltose addition the signal decreased up to 35 %, which correlates with the observed cell deformations. We obtained hyperbolic decays after plotting the results from light scattering experiments versus osmolyte concentration. The curves were fitted by the equations given in Fig. 2. The

Fig. 2. (a) Light microscopy photographs of cells before (Con) and 2 min after application of osmotic stress by addition of 500 mM NaCl (NaCl), sorbitol (Sor) or maltose (Mal). Deformed cells are labelled by black triangles. Bars, 5 µm. (b) Results of light scattering versus osmolyte concentration for (●) NaCl-, (□) sorbitol- or (△) maltose-stressed cells. The dependency of changes in the light scattering signal on sorbitol and maltose concentrations was fitted by using the equation $y = y_0 + (a \times b) / (b + x)$, resulting in the following parameters for sorbitol $y_0 = 80$, $a = 19$, $b = 109$, and for maltose $y_0 = 45$, $a = 55$, $b = 289$. Thereby $100\% - y_0$ indicates maximum cell volume change.

maximal cell volume change inducible by sorbitol was calculated to be 20 %, and for maltose 55 %, while that of NaCl was negligible. This indicates a much higher degree of water efflux after osmotic stress induced by the disaccharide maltose in comparison to the sugar alcohol sorbitol.

Response of GG synthesis to osmotic and salt stress

In further experiments, the GG accumulation directly after osmotic- or salt-stress treatments was studied to exclude the secondary effects of long-term cultivation at high

sugar concentrations on GG synthesis in *Synechocystis*. We compared GG accumulation after addition of 200 and 500 mM (limit of sorbitol resistance) NaCl, sorbitol and maltose to avoid the toxic effects of high osmolyte concentrations observed during the growth experiments (see Fig. 1). For salt-shocked cells, we confirmed the salt-dependent accumulation of GG. At 4 h after salt addition, GG values already corresponded to the steady state levels observed in fully acclimated cells (Fig. 3a). Beside GG, only traces of sucrose were found as accumulated solute in salt-stressed cells. The addition of 200 or 500 mM maltose resulted only in a very slight increase in the GG content, up to 6 and 3 nmol GG ml⁻¹ per OD₇₅₀ unit, respectively (Fig. 3c). A small transient increase in the GG content was observed in sorbitol-shocked cells (Fig. 3b). However, 4 h after sorbitol addition GG was present only at trace amounts characteristic for cells exposed for longer times to 200 or 500 mM sorbitol (see Fig. 1b). Instead of GG, sorbitol-shocked cells accumulated high amounts of sorbitol (Fig. 3b). Thereby, the kinetics of sorbitol accumulation was comparable to the kinetics of GG accumulation after NaCl treatment. Addition of 200 mM sorbitol resulted in a rather slow accumulation, while addition of 500 mM sorbitol resulted in a fast internal accumulation of this compound. In agreement with this, long-term sorbitol-grown cells also maintained high internal sorbitol levels, which were dependent on the external sorbitol concentration (data not shown). Obviously, nonionic osmotic stress does not trigger the activation of *de novo* GG synthesis in *Synechocystis* cells in contrast to salt stress. However, cells can cope with an osmotic challenge by high external sorbitol concentrations up to 500 mM without the accumulation of GG by the uptake and stress-proportional accumulation of sorbitol. The absence of any organic solute accumulation in cells treated by high maltose concentrations explains the low stress resistance of the cells.

Expression of the *ggpS* gene in response to osmotic and salt stress

In order to investigate the basis for the missing GG accumulation in cells treated with sorbitol or maltose, *ggpS* gene expression was compared after salt, maltose and sorbitol addition. For this purpose a reporter strain RP_{ggpS}::*luxAB* was generated, in which the promoter of the *ggpS* gene was cloned upstream of the *luxAB* genes and integrated together with an antibiotic resistance gene cartridge into a neutral site on the *Synechocystis* genome. During salt and osmotic stress induced by the addition of salt or sorbitol at 0 to 600 mM, accumulation of the LuxAB proteins was analysed at different time points (Fig. 4). The addition of 200 mM NaCl had no significant influence on LuxAB activity, while addition of 400 mM NaCl led to induction. At concentrations of 500 and 600 mM NaCl a strong increase in the LuxAB activity was observed. Sorbitol stress induced the LuxAB activity in a concentration-dependent manner with the maximum induction at a concentration of 600 mM. In contrast, after addition of maltose no increase in the

Fig. 3. Low molecular mass compound content in *Synechocystis* cells after addition of 200 mM (open symbols) or 500 mM (filled symbols) NaCl (a), sorbitol (b) and maltose (c). ○, ●, Intracellular GG content; △, ▲, sorbitol content; ◇, ◆, maltose content.

amount of LuxAB was observed at any concentration, indicating a complete lack of activation of the *ggsP* promoter by nonionic osmotic stress with maltose (Fig. 4). To validate the expression data obtained by the LuxAB assay, we compared the *ggsP* mRNA content in cells challenged by 500 mM NaCl and different concentrations of sorbitol by Northern blotting (Fig. 5). In general, for NaCl addition, the results obtained with the reporter strain were verified, but the kinetics at the mRNA level were different compared to the kinetics at the protein level (LuxAB). After NaCl, as well as sorbitol addition, a fast increase in *ggsP* mRNA content with a maximum after 0.5 h and a subsequent decrease to a new steady state level at 8 h was found. However, the maximum mRNA values were lower in sorbitol-stressed cells than in cells challenged by 500 mM

NaCl, and they reached very low levels 8–24 h after the onset of the stress treatment (Fig. 5b).

In order to exclude general toxic effects of very high sorbitol concentrations on the transcription machinery, we included the gene *slr1544* in our analysis, which was described as one of the genes specifically induced by sorbitol (Kanesaki *et al.*, 2002). As expected, the *slr1544* mRNA content increased after addition of sorbitol. Higher induction ratios were observed in comparison to the *ggsP* gene at sorbitol concentrations higher than 600 mosmol (Fig. 5c). Since the maximum *slr1544* mRNA content was observed after addition of 700 mM sorbitol, a general toxic effect of short-term exposure to high sorbitol concentrations on transcriptional activity could be ruled out.

Fig. 4. Amount of LuxAB proteins expressed under the control of the *ggsP* promoter in cells of the *Synechocystis* reporter strain *RP_{ggsP}::luxAB* at standard conditions (○), or after addition of 200 (□), 400 (△), 500 (▽) or 600 mM (◇) NaCl (a), sorbitol (b) or maltose (c). RLU, relative luminescence unit.

Fig. 5. Expression of the *ggpS* gene in *Synechocystis* cells before and after osmotic challenge of culture aliquots by 500 mM NaCl (◆), or 300 (▽), 500 (○), 700 (□) or 900 mM (△) sorbitol analysed by Northern blotting (C, control) (a) and densitometric estimation in relation to the control value, which was set to 1 (b). Levels of *ggpS* (●) and *slr1544* (○) mRNA were measured in dependence on the external osmolality adjusted by addition of sorbitol (c). RU, relative unit.

GgpS activity in response to osmotic and salt stress

Besides the salt-dependent regulation of *ggpS* gene expression, it is known that the constitutively expressed GgpS enzyme is directly activated in salt-stressed cells (Hagemann & Erdmann, 1994). This may be not the case after nonionic osmotic-stress treatment and may thus explain the observed absence of GG accumulation. Therefore, we estimated GgpS activity in protein extracts obtained from *Synechocystis* cells grown at standard conditions with and without the addition of NaCl, maltose or sorbitol to the assay buffer (Fig. 6). Under low-salt conditions no GgpS activity was measured. Addition of 200 mM NaCl activated the GgpS enzyme, while the presence of 200 mM sorbitol or maltose had no activating effect on the GgpS enzyme activity. A combined addition of NaCl with sorbitol or maltose led to an enzyme activity comparable to NaCl addition alone, thus an inhibition of the GgpS protein by high maltose or sorbitol concentrations *in vitro* could be excluded.

DISCUSSION

We intended to compare the physiological mechanisms for salt and osmotic stress response in *Synechocystis* at the level of cell volume, gene expression and protein activity regulation, such as osmolyte accumulation. Beside the salt NaCl, we used sorbitol and maltose as nonionic compounds. Interestingly, for all three types of stress we observed a different cellular response indicating clear differences in the stress situation caused by each compound.

NaCl stress includes an ionic as well as an osmotic stress component. The latter results in water efflux, which

was shown to be counteracted within milliseconds in cyanobacterial cells by the influx of ions into the cytoplasm (Blumwald *et al.*, 1983a; Reed *et al.*, 1985) and thus, we could not observe a change of cell volume using light microscopy or light scattering. Our observation is also in agreement with results of particle size analysis of *Synechocystis* cells during

Fig. 6. Activity of the GgpS enzyme was measured in the presence of different salt and osmotic supplements in a soluble protein extract of *Synechocystis* cells, which were grown under standard conditions. The GgpS activity at low salt conditions was compared to that after the addition of 200 mM NaCl, sorbitol or maltose alone and in combination (+ indicates the addition of 200 mM of the compound to the assay buffer). RU, relative unit.

NaCl stress of 500 and 1000 mosmol, which revealed a comparable small cell volume reduction of about 5 % (Reed *et al.*, 1986). In contrast, application of maltose stress caused a decrease in cell volume indicating a concentration-dependent water efflux. For the cyanobacterium *Synechococcus* sp. PCC 6311, which is closely related to *Synechocystis*, the reduction of the cell volume after addition of the disaccharide sucrose was quantified by EPR measurements (Blumwald *et al.*, 1983b). Comparable observations were also described for *E. coli* cells challenged by high sucrose concentrations. The reduction of cell volume was visualized by cryoelectron microscopy (Delamarche *et al.*, 1999). Interestingly, *Synechocystis* cells exhibited no shrinkage of the cytoplasm but the invagination of the whole cell wall after an osmotic challenge by maltose, which may reflect the different structure of the cyanobacterial envelope in comparison to other Gram-negative bacteria (Hoiczky & Hansel, 2000).

The strong water efflux after maltose addition was not counteracted by the accumulation of the compatible solute GG as is observed during the salt-stress response in *Synechocystis* cells (Figs 1 and 3). This results in a very low resistance with a maximal tolerance of 200 mM maltose, which is comparable to the remaining salt tolerance of a *Synechocystis* mutant defective in the ability to accumulate GG and sucrose during salt stress at a comparable osmolality (data not shown).

Surprisingly, in comparison to the disaccharide maltose, addition of the sugar alcohol sorbitol induced a clearly different stress response. Equimolar amounts of sorbitol led to only minor changes of cell volume with a much lower water efflux (Fig. 2). For what is believed to be the first time, we have shown that externally added sorbitol is taken up by *Synechocystis* cells in a concentration-dependent manner (Fig. 3). Consequently, sorbitol cannot be considered as a compound causing nonionic osmotic stress. Uptake and accumulation of sorbitol is probably the reason for the stability of the cell shape, and for the survival of cells at higher concentrations of external sorbitol, because of the equalization of the internal and external water potential compared to maltose treatment. It is known that *Synechocystis* is able to take up the carbohydrates glucose (Schmetterer, 1990), GG, trehalose and sucrose. The latter three solutes are transported by the salt-regulated ABC-type transporter Ggt (Mikkat *et al.*, 1996; Mikkat & Hagemann, 2000). Because the participation of this transporter in sorbitol uptake has been excluded (Mikkat *et al.*, 1996), the transporter responsible for the uptake of this compound remains unknown. We suggest that sorbitol is penetrating the cell membrane by facilitated diffusion, since the uptake rate depends on the external sorbitol concentration over a wide range. Moreover, at low salt concentration supplementation with sorbitol at concentrations of 10 mM did not lead to sorbitol accumulation (not shown).

While maltose was obviously used as a carbon source sorbitol had no growth promoting effect on *Synechocystis* cells

(Fig. 1). Thereby, utilization of maltose can be explained by an external α -glucosidase activity of *Synechocystis* cells (Mikkat *et al.*, 1997) able to split maltose into glucose, which is taken up by the cells (Grigorieva & Shestakov, 1982). Because sorbitol was not metabolized by *Synechocystis*, it served as a compatible solute, as also reported for plant and yeast cells (Ahmad *et al.*, 1979; Shen *et al.*, 1999). However, at concentrations higher than 500 mM, sorbitol seems to be toxic for *Synechocystis*, in agreement with results obtained by Jantaro *et al.* (2003). In contrast, the natural compatible solute GG promoted growth at a very high external osmolality of 2400 mosmol (1.2 M) NaCl (Reed *et al.*, 1985). A comparable observation was made in yeast cells in which the accumulation of sorbitol was analysed in mutants lacking the key enzymes for glycerol synthesis. The protection provided by sorbitol was found to be much smaller than that provided by an equal amount of the natural compatible solute glycerol (Shen *et al.*, 1999). The direct protection of soluble and peripherally bound membrane proteins at high osmotic stress is described as a key factor for the efficiency of an osmotic protectant, which has been demonstrated for GG (Santoro *et al.*, 1992; Hinch, 1998; Borges *et al.*, 2002).

In order to address the question of how GG synthesis is affected during osmotic-stress treatment we analysed the level of *ggsS* gene expression, as well as GgsS enzyme activity. Thereby, we confirmed the induction of the *ggsS* gene in a salt-concentration-dependent manner (Marin *et al.*, 2002). However, by using the reporter gene assay we could not confirm the weak transcription of the *ggsS* gene under control conditions, resulting in the accumulation of the GgsS protein (Marin *et al.*, 2002). Sorbitol addition accompanied by sorbitol uptake also induced *ggsS* expression, while application of nonionic osmotic stress induced by maltose addition did not. We concluded that a so far unknown internal trigger, due to the accumulation of ions or sorbitol, is responsible for the transcriptional regulation.

In spite of the activation of *ggsS* expression by sorbitol, no GG was accumulated upon this treatment, indicating the hierarchically higher level of the protein activity modulation. Addition of both nonionic compounds sorbitol and maltose did not lead to an activation of the GgsS enzyme *in vitro* and most probably not *in vivo* (Figs 3 and 6). Since an inhibition of GgsS activity *in vitro* by high sorbitol concentrations was excluded, these results indicate that the GgsS protein is activated in a strictly salt-dependent manner (Schoor *et al.*, 1999).

In summary, *Synechocystis* cells are not able to acclimate to nonionic osmotic stress conditions if no osmolyte is present that can be taken up by the cell and serve as a compatible solute. The synthesis of the natural compatible solute GG is strictly salt dependent and in *Synechocystis* cells GG seems to be more effective in protection of the cellular metabolism compared to a putatively non-physiological compatible solute such as sorbitol.

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