Active transport of glucosylglycerol is involved in salt adaptation of the cyanobacterium *Synechocystis* sp. strain PCC 6803

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An active-transport system for the osmoprotective compound glucosylglycerol (GG) was found in the cyanobacterium *Synechocystis* sp. strain PCC 6803. Uptake assays with ^{14}C -labelled GG showed that the GG transport was enhanced in cells adapted to increasing concentrations of NaCl. Kinetic studies indicated a Michaelis–Menten relationship. The uptake of GG was energy dependent and occurred against a steep concentration gradient. It was inhibited by uncouplers as well as by a combination of darkness and KCN. The affinity of the transporter seems to be restricted to osmoprotective compounds of cyanobacteria; from a variety of compounds tested only sucrose and trehalose competed with GG for uptake. A salt-sensitive mutant of *Synechocystis* 6803 unable to synthesize GG could be complemented to salt resistance by exogenous GG. Accumulation of GG from the medium was essential for the restoration of photosynthesis and growth in mutant cells under high-salt conditions. In wild-type cells, the GG transporter probably serves to prevent GG leaking out of salt-stressed cells.

Keywords: osmoprotective compound, glucosylglycerol transport, salt adaptation, cyanobacterium, *Synechocystis* sp.

INTRODUCTION

Salinity is an important abiotic factor in aquatic biotopes. Survival and growth in habitats with high or fluctuating salinities require prokaryotes to adjust their cytoplasmic water potential. Several archaeobacteria and anaerobic heterotrophic eubacteria accumulate inorganic ions to maintain osmotic equilibrium with their surroundings. However, high concentrations of inorganic ions are toxic for most eubacteria. During adaptation to salt stress, these bacteria achieve a balanced osmotic potential by the extrusion of excess inorganic ions from the cell and the accumulation of osmoprotective compounds (compatible solutes). These low-molecular-mass hydrophilic compounds do not interfere with cell metabolism because they are largely inert and carry no net charge (Brown, 1976; Galinski & Trüper, 1994). Besides reducing the osmotic potential of the cytoplasm, osmoprotective compounds apparently prevent denaturation by helping macromolecules to retain their natural configuration (e.g. Inchar-

ensadki *et al.*, 1986). In salt-loaded cyanobacterial species, sucrose, trehalose, glucosylglycerol [2-O-(α -D-glucopyranosyl)-glycerol; GG], glycine betaine or glutamate betaine serve as the principal osmoprotective compounds (Reed & Stewart, 1988). Cyanobacteria synthesize these substances *de novo* mainly from CO_2 by photosynthesis, although limited amounts may be synthesized from stored glycogen or exogenous glucose (Tel-Or *et al.*, 1986; Erdmann *et al.*, 1989).

Unlike cyanobacteria, many heterotrophic eubacteria prefer to accumulate osmoprotective compounds from their environment. Several active-transport systems for the osmoprotective compounds proline, glycine betaine and its precursor choline have been characterized in a variety of bacteria, e.g. *Escherichia coli*, *Salmonella typhimurium* and *Bacillus subtilis* (Perroud & Le Rudulier, 1985; Csonka & Hanson, 1991; Boch *et al.*, 1994). These systems are controlled by the osmolarity of the medium at the levels of both transport activity and gene expression (Lucht & Bremer, 1994; Boch *et al.*, 1994).

E. coli under osmotic stress is able to synthesize trehalose *de novo*, but if glycine betaine is available trehalose synthesis is completely repressed and adaptation to high osmotic pressure is achieved by accumulation of glycine betaine alone (Larsen *et al.*, 1987; Giaever *et al.*, 1988). A

Abbreviations: BV, biovolume; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; 2,4-DNP, 2,4-dinitrophenol; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; GG, glucosylglycerol; MV, methyl viologen.

glycine betaine transport system has also been found in cyanobacteria. Interestingly, it was only present in strains which can synthesize this compound (Moore *et al.*, 1987).

In the present study we have used the cyanobacterium *Synechocystis* sp. strain PCC 6803, which accumulates GG and tolerates up to 1.2 M NaCl (Reed & Stewart, 1985). The osmoprotective compound GG is characteristic of moderately halotolerant cyanobacteria. It is synthesized from ADP-glucose and glycerol 3-phosphate by a salt-activated enzyme system (Hagemann & Erdmann, 1994). We show here that *Synechocystis* 6803 has an active GG uptake system, which is involved in the process of salt adaptation.

METHODS

Organisms and culture conditions. Axenic cultures of *Synechocystis* sp. strain PCC 6803 were grown photoautotrophically under continuous illumination (20 W m^{-2}) and bubbled with air containing 2.5% (v/v) CO_2 at 29°C in the growth medium of Allen & Arnon (1955). The cells were inoculated into fresh medium daily. The basal medium contained 2 mM NaCl. Cells grown at this NaCl concentration are hereafter referred to as control cells. To obtain salt-adapted cells, the cyanobacteria were precultured for at least 5 d in NaCl-enriched medium. In salt-shock experiments solid NaCl was added to the cultures. To obtain hypo-osmotically treated cells, a culture adapted to 684 mM NaCl was harvested by centrifugation, and the cells were resuspended and cultivated for 2 h in basal medium. This procedure was repeated twice. The salt-sensitive mutant 107 (unpublished) of *Synechocystis* 6803, which is unable to synthesize GG, was cultivated in the presence of $25 \mu\text{g}$ kanamycin ml^{-1} . Methods for the generation and characterization of salt-sensitive mutants are described by Hagemann & Zuther (1992). The purity of the cultures was checked by spreading 0.2 ml culture onto LB plates and by microscopic examination.

Extraction and purification of GG. A culture of *Synechocystis* 6803 (1 l) adapted to 684 mM NaCl was harvested by centrifugation (6000 g at 4°C for 10 min) and stored at -20°C . GG was extracted from the cells with 200 ml 80% (v/v) ethanol for 3 h at 65°C . After centrifugation at 6000 g (20°C for 15 min) the clear supernatant was evaporated in a rotary evaporator and redissolved in water. Insoluble particles (mainly pigments) were removed by centrifugation (48000 g at 4°C for 30 min). To remove charged compounds, the clear water extract was passed through ion-exchange resins. It was applied to a column of Dowex 50Wx8 (H^+ form), rinsed with water, and then passed through a column of Dowex 1x2 (formate form). The effluent and rinse were collected, dried and again dissolved in water. These extracts were further purified by preparative TLC. TLC was performed in acetonitrile/water (85:15, v/v, three runs) on self-coated plates (cellulose MN 300, about 0.5 mm). GG was scraped off, eluted with water, and dried. The purity of the extracted GG was about 98% as determined by HPLC.

Preparation of ^{14}C -labelled GG. A culture of *Synechocystis* 6803 (500 ml) adapted to 342 mM NaCl was shocked by adding 10 g NaCl (final NaCl concentration 684 mM) to achieve an inhibition of growth and specific activation of GG synthesis. These cells were incubated with $11.1 \text{ MBq NaH}^{14}\text{CO}_3$ (specific activity $1670 \text{ MBq mmol}^{-1}$) for 5 h without gassing. The cells were harvested by centrifugation (6000 g at 4°C for 10 min), washed twice, and ^{14}C GG was extracted as described above. The purity of the labelled GG was checked by two-dimensional TLC (Feige *et al.*, 1969). After detection of labelled compounds

by autoradiography, only one spot at the position of authentic GG was visible. For the determination of the specific activity of the ^{14}C GG, the amount of GG was estimated after acid hydrolysis using an enzymic glucose assay (Erdmann *et al.*, 1992).

Transport assays. Two ^{14}C GG preparations with specific activities of $0.954 \text{ d.p.m. pmol}^{-1}$ and $0.659 \text{ d.p.m. pmol}^{-1}$, respectively, were used. $[\text{U-}^{14}\text{C}]$ sucrose (specific activity 25 GBq mmol^{-1} , Amersham) was purified from glucose and fructose by TLC on silica gel 60 plates (Merck) developed in acetone/water/chloroform/methanol (75:5:10:10, by vol., two runs). Uptake was measured under the culture conditions described above without gassing. The cell suspension (0.5 ml) was directly taken from the cultures [biovolume (BV) $1.8\text{--}2.8 \text{ mm}^3 \text{ ml}^{-1}$] and incubated for 15 min with an appropriate amount of ^{14}C GG or $[\text{U-}^{14}\text{C}]$ sucrose. The tests were stopped by adding 3 ml of medium of the same salt concentration and immediate filtration through membrane filters ($0.45 \mu\text{m}$, SM 11306, Sartorius). Subsequently, the filters were washed three times with 3 ml cultivation medium and solubilized in a scintillation cocktail [toluene/Triton X-100 (2:1, v/v), 0.4% PPO, 0.012% dimethyl-POPOP]. Radioactivity was determined by liquid scintillation counting. Modifications of this standard assay are described in the text.

Kinetic parameters were analysed using the EKI software (Raddatz, 1994). All values derive from Lineweaver–Burk plots based on the weighting method of Wilkinson (1961). Data for K_m and $V_{\max}$ are given as mean values $\pm$ SD from at least three determinations performed with independent cultures.

Complementation of mutant 107. Cells of the salt-sensitive mutant 107 grown at 87 mM NaCl were shocked by additional 513 mM NaCl (final concentration 600 mM NaCl). GG was provided at a concentration of 0.75 mM. After the times indicated samples were harvested by centrifugation and washed twice with medium of the same salt concentration.

Other methods. The GG content was estimated after extraction in 80% ethanol by HPLC (Schoor *et al.*, 1995). Cell number and diameter were determined by means of an electronic particle counter (Erdmann *et al.*, 1992). Fixation of the cells by Lugol solution led to shrinkage. Therefore the BV (product of cell number and cell volume) was underestimated by about 15%. OD_{750} was measured using a Spekol 11 instrument (Carl Zeiss Jena). Dry mass was estimated using membrane filters ($0.2 \mu\text{m}$, SM 11107, Sartorius). Total protein was determined by the Lowry method after suspending the cells in 1 M NaOH, ultrasonic treatment (90 s at 30 W), and hydrolysis at 37°C overnight. In this study BV was used as the parameter for biomass. To allow comparison with other uptake studies, the different biomass parameters were correlated to each other. For cells of *Synechocystis* 6803 from basal medium a BV of $1 \text{ mm}^3 \text{ ml}^{-1}$ is equivalent to a protein content of $240 \mu\text{g ml}^{-1}$, a dry mass of $415 \mu\text{g ml}^{-1}$, and an OD_{750} of 1.09. For cells of *Synechocystis* 6803 adapted to 684 mM NaCl, a BV of $1 \text{ mm}^3 \text{ ml}^{-1}$ is equivalent to a protein content of $216 \mu\text{g ml}^{-1}$, a dry mass of $458 \mu\text{g ml}^{-1}$, and an OD_{750} of 0.89. Photosynthesis and respiration were determined polarographically using a Clark-type oxygen electrode (Erdmann *et al.*, 1992).

RESULTS

Evidence for GG uptake

Cells of *Synechocystis* 6803 were able to accumulate ^{14}C GG. The rate of GG uptake was substantially higher in salt-adapted than in control cells (Fig. 1); it was linear

Fig. 1. Time course of GG uptake by *Synechocystis* 6803. Suspensions of photoautotrophically grown cells were incubated in the light (20 W m^{-2}) at 29°C in growth medium containing 1 mM $[^{14}\text{C}]\text{GG}$. Cells were adapted to 2 mM NaCl ($\blacktriangle$) or 684 mM NaCl ($\blacksquare$).

Fig. 2. Effect of adaptation of *Synechocystis* 6803 to different NaCl concentrations on the rate of GG uptake and on the intracellular accumulation of GG. Uptake was determined with a $[^{14}\text{C}]\text{GG}$ concentration of 1 mM using the standard assay (filled bars). The concentrations of accumulated GG was estimated by HPLC (open bars). Cells were adapted to the indicated salinities for at least 5 d. Data are mean values $\pm$ SD from determinations performed with independent cultures. The number of determinations (in sequence of increasing NaCl concentrations) was 9, 4, 4, 8 and 2 for GG uptake, and 3, 4, 4, 4 and 2 for GG accumulation.

for at least 30 min. Therefore, we used an incubation time of 15 min to measure the initial velocities of GG transport in the standard assays. TLC of ethanolic extracts of control and salt-adapted cells incubated for 30 min with $[^{14}\text{C}]\text{GG}$ showed a single ^{14}C -labelled compound, which co-chromatographed with authentic GG. This indicates that the exogenous GG taken up by the cells was not metabolized (not shown).

GG transport in cells adapted to different salt concentrations was investigated. Increasing transport activity was found in cells grown in medium containing increasing amounts of NaCl (Fig. 2). Cells adapted to 856 mM NaCl showed a fivefold higher uptake rate than control cells. In

Fig. 3. Effect of short-term changes of the salinity on the GG uptake by *Synechocystis* 6803. Cells were washed and resuspended in a buffer solution containing 20 mM MOPS/KOH (pH 7.5), 0.5 mM MgSO_4 and 0.5 mM CaCl_2 . Different concentrations of NaCl (a) or different salts at a concentration of 200 mM (b) were added before the uptake reaction was started by addition of $[^{14}\text{C}]\text{GG}$ (0.2 mM). The control (without added salt) for (b) is the same as in (a). Control cells (open bars) and hypo-osmotically treated cells prepared as described in Methods (filled bars) were used.

salt-adapted cells GG was synthesized *de novo* and accumulated to concentrations corresponding to the external salinity (Fig. 2). Therefore, the GG uptake took place against a steep concentration gradient.

Modulation of GG uptake by salinity

To test whether the enhanced transport activity of salt-adapted cells was due to a salt-dependent activation of pre-existing transporter molecules or to their *de novo* synthesis following an increase in salinity, GG uptake was measured after different shock treatments. If control cells were shocked with 684 mM NaCl, the uptake rate remained nearly unaltered for at least 5 h. Twenty-four hours after the shock treatment a two- to threefold increase was noticed in different experiments, while completely adapted cells took up GG about fourfold faster than control cells. In contrast, a shock of only 342 mM NaCl was followed by a shorter lag period, and the final uptake rate was reached after 24 h (data not shown). In inverse experiments, cells adapted to 684 mM NaCl were hypo-osmotically shocked using basal medium. In this case, the GG uptake remained at the high rate of salt-adapted cells for at least 5 h (data not shown). Therefore, the differences in GG uptake rates of control and salt-adapted cells, respectively, were the result of a long-term adaptation process including *de novo* synthesis of protein.

However, if hypo-osmotically treated cells were washed and resuspended in a buffer solution containing only traces of additional ions, the GG transport was immediately influenced by the addition of salts into the uptake assays (Fig. 3). The uptake rate was enhanced by increasing NaCl concentrations between 10 and 350 mM , while 700 mM NaCl led to some inhibition compared to

the optimal activation at 350 mM NaCl (Fig. 3a). Additions of NaBr, NaNO₃ and LiCl had about the same effect as NaCl, while KCl at a concentration of 200 mM was somewhat inhibitory (Fig. 3b). The same situation was found if control cells were washed and resuspended in buffer solution (Fig. 3). To exclude protein synthesis, chloramphenicol (40 µg ml⁻¹, 10 min preincubation time; Hagemann *et al.*, 1990) was used. This treatment did not affect the activation of GG uptake by short-term changes of the salinity (data not shown). These data imply that an increase of the NaCl concentration is responsible for both *de novo* synthesis of transporter proteins in a long-term process and, to a lesser degree, immediate activation of pre-existing transporters.

The GG uptake system was not inducible by GG, since incubation of control cells with 1 mM GG for 24 or 72 h did not increase the transport activity (data not shown).

Kinetics of GG transport

The kinetics of GG uptake in *Synechocystis* 6803 were studied in salt-adapted and control cells at various exogenous GG concentrations. Uptake of 10–4000 µM GG followed a typical Michaelis–Menten relationship (data not shown). The maximum velocity of substrate uptake was substantially higher in cells adapted to 684 mM NaCl than in control cells (269 ± 59 and 48 ± 6 pmol (mm³ BV)⁻¹ min⁻¹, respectively), as was expected from the transport experiments using cells adapted to different salinities (see Fig. 2). The apparent substrate affinity was also affected by the salinity; *K_m* values of 124 ± 17 and 52 ± 13 µM were estimated for cells adapted to 684 and 2 mM (control cells) NaCl, respectively. Beside the rather unlikely possibility of a decreased affinity for GG in salt-adapted cells, another explanation for this difference could be the leakage of intracellular GG into the medium or the periplasm. This leakage would increase the periplasmic GG concentration and decrease the specific activity of the exogenous [¹⁴C]GG, leading therefore to the apparently reduced affinity. Leakage of the osmoprotective compound trehalose into the periplasm has been reported for *E. coli* (Styrvoid & Strøm, 1991).

To differentiate between these two possibilities, kinetics of GG uptake were measured using washed and hypo-osmotically treated cells, respectively. Salt-adapted cells were washed with fresh medium of the same NaCl content to remove GG possibly present in the cultivation medium. This treatment did not reduce the apparent *K_m*. In accordance with this result, GG was not found in the medium by HPLC analysis. Hypo-osmotic treatment is supposed to completely remove GG accumulated in the cytoplasmic and periplasmic space (Reed *et al.*, 1986; Fulda *et al.*, 1990). Therefore, hypo-osmotically-treated cells were prepared as described in Methods. The treatment reduced the intracellular GG content to 3.5 nmol (mm³ BV)⁻¹, a level comparable to that observed in control cells. After continued cultivation for 2 h in medium containing 684 mM NaCl, these cells showed a *K_m* value of 58 µM, comparable to control cells. Therefore, the high

Table 1. Effect of metabolic inhibitors and incubation in the dark on the uptake of GG by *Synechocystis* 6803

Cells adapted to 684 mM NaCl were preincubated with inhibitors or in darkness for 15 min before the uptake reaction (standard assay) was started by adding [¹⁴C]GG (0.2 mM). Values are given as a percentage of GG uptake under the indicated treatments, compared to controls. If ethanol was used as solvent for the inhibitors, values were corrected for the effect of ethanol. Data are mean values ± SD. The number of experiments performed with independent cultures is indicated in parentheses.

Treatment	Concn	GG uptake (% of control)
CCCP	25 µM	54 ± 8 (4)
CCCP	100 µM	21 (1)
FCCP	25 µM	45 ± 5 (4)
FCCP	100 µM	20 (2)
DCCD	100 µM	95 ± 3 (3)
Orthovanadate	100 µM	97 (1)
DCMU	10 µM	81 ± 5 (4)
DCMU	50 µM	73 (1)
2,4-DNP	1 mM	76 ± 6 (3)
KCN	1 mM	96 ± 14 (7)
MV	1 mM	88 (2)
Darkness		77 ± 12 (5)
Darkness + KCN	1 mM	14 ± 2 (5)
MV + KCN	each 1 mM	51 (2)
DCMU + KCN	10 µM/1 mM	88 ± 7 (3)

apparent *K_m* value estimated for salt-adapted cells was related to the high GG content of these cells. Probably leakage of GG into the periplasm caused overestimation of the *K_m* in untreated cells adapted to 684 mM NaCl.

Effects of pH and metabolic inhibitors on the GG transport

GG uptake was also studied at various pH values. Acid pH inhibited GG uptake in salt-adapted cells of *Synechocystis* 6803. A linear increase in transport activity was observed between pH 4 and 7, while it remained unchanged at pH values above 7.5 (data not shown).

Several inhibitors and incubation in the light and dark were used to analyse the energetic basis of the GG uptake in salt-adapted cells of *Synechocystis* 6803 (Table 1). Addition to a final concentration of 25 µM CCCP or FCCP, which uncouple phosphorylation from photosynthetic and respiratory electron transport, diminished GG uptake by about 50%; very high uncoupler concentrations (100 µM) inhibited uptake by 80%. Transport was not affected by DCCD or orthovanadate, inhibitors of H⁺-ATPases localized in the cytoplasmic membrane, or by KCN, an inhibitor of cytochrome oxidase. Treatments which inhibit photosynthesis [addition of DCMU, MV, darkness] showed only minor effects on GG transport. A combination of darkness and KCN led to about 85% inhibition, which is comparable with the effects of the uncouplers. A combined treatment with MV (competes

Table 2. Effect of various compounds on the uptake of GG by *Synechocystis* 6803

Cells adapted to 684 or 2 mM (control cells) NaCl were incubated as described for the standard assay with [14 C]GG (0.2 mM) in the presence of the unlabelled compounds (2 mM). Values are given as a percentage of GG uptake, compared to controls without supplements. Data are mean values $\pm$ SD. The number of experiments performed with independent cultures is indicated in parentheses. ND, Not determined.

Compound	GG uptake (% of control)	
	Salt-adapted cells	Control cells
GG	22 $\pm$ 2 (4)	16 $\pm$ 5 (3)
Sucrose	28 $\pm$ 5 (4)	19 $\pm$ 5 (3)
Trehalose	61 $\pm$ 4 (3)	53 $\pm$ 7 (3)
Maltose	102 $\pm$ 4 (3)	104 (1)
Methyl α -D-glucoside	101 $\pm$ 6 (3)	ND
Phenyl α -D-glucoside	93 $\pm$ 4 (4)	93 (1)
Floridoside	98 (2)	101 (1)
Raffinose	99 $\pm$ 12 (4)	102 (1)
Glucose	96 $\pm$ 4 (4)	89 (1)
Fructose	100 $\pm$ 5 (3)	ND
Glycerol	97 (2)	ND

with cyclic photosynthetic electron transport) and KCN causes intermediate inhibition of about 50 %, while the combination of DCMU (blocks non-cyclic photosynthetic electron transport) and KCN had almost no effect (Table 1).

Specificity of the GG transporter

In order to study the specificity of the GG transport, various unlabelled potential competitors were used in 10-fold excess (Table 2). Transport was not affected by the monosaccharides glucose or fructose, nor by phenyl or methyl glucosides. The structural analogue floridoside (galactosylglycerol), an osmoprotective compound of red algae (Kirst, 1990), showed no interference with the GG uptake. However, the disaccharide sucrose inhibited the transport of [14 C]GG to nearly the same extent as unlabelled GG, indicating a similar affinity of the transporter for these two compounds. To a lesser extent trehalose also diminished the GG transport. The somewhat lower inhibition by unlabelled GG, sucrose or trehalose of GG uptake in salt-adapted cells compared to control cells is consistent with the apparently reduced affinity for GG in salt-adapted cells. The disaccharide maltose and raffinose (a trisaccharide consisting of a sucrose molecule bound by an α -1,6-glycosidic bond to galactose) did not affect GG uptake (Table 2).

Cells of *Synechocystis* 6803 took up [U- 14 C]sucrose, and the time course and rates of uptake with cultures adapted to 2 or 684 mM NaCl were similar to those for GG uptake (data not shown). With control cells, measurements of sucrose uptake at various exogenous sucrose concen-

Table 3. Kinetic parameters for competition of GG, sucrose and trehalose for uptake by *Synechocystis* 6803

Kinetics of GG and sucrose uptake were studied with cells adapted to 2 mM NaCl at various exogenous substrate concentrations ranging from 15 to 960 μ M in the absence or presence of competitive inhibitors. For GG uptake, inhibitor concentrations of 100 and 500 μ M sucrose and 500 μ M trehalose were used. For sucrose uptake, GG was added at 100 and 500 μ M and trehalose at 500 μ M. Data were analysed as described in Methods. $V_{\max} \pm$ SD from the determinations with and without inhibitors was 52 $\pm$ 4 and 45 $\pm$ 2 pmol (mm 3 BV) $^{-1}$ min $^{-1}$ for GG and sucrose uptake, respectively. Values for K_m and K_i are given in μ M.

Parameter	GG uptake	Sucrose uptake
K_m	52	120
K_i (GG)	–	77
K_i (sucrose)	96	–
K_i (trehalose)	332	298

trations ranging from 15 to 960 μ M gave the following kinetic parameters: $K_m = 106 \pm 12$ μ M, $V_{\max} = 51 \pm 7$ pmol (mm 3 BV) $^{-1}$ min $^{-1}$. In the presence of GG or trehalose at concentrations 10 times that of sucrose, the sucrose uptake was inhibited 88 % or 67 %, respectively.

The kinetics of GG and sucrose uptake were studied in the absence and presence of the inhibitory compounds (Table 3). Their addition did not decrease $V_{\max}$, while the K_m values were apparently increased. These results clearly indicate the competitive nature of inhibition. From the increased K_m values the inhibitor constants (K_i) were estimated. The inhibitor constants for GG and sucrose were in good coincidence with the corresponding K_m values for these compounds (Table 3).

Complementation of the salt-sensitive mutant 107 by exogenously supplied GG

Mutant 107 of *Synechocystis* 6803 has a salt resistance limit at about 0.5 M NaCl (about 40 % of the wild-type) caused by a defect in GG synthesis. Preliminary experiments showed that the ability to transport GG remained intact and mutant 107 grew like the wild-type in basal medium. Therefore, the mutant 107 was chosen to test whether the GG transport activity is sufficient for the accumulation of high amounts of GG to compensate a lethal salt shock. Cells of mutant 107 were shocked with 513 mM NaCl (final concentration 600 mM NaCl) in the presence or absence of 0.75 mM GG in the medium. The salt shock led to a substantial drop in photosynthetic O $_2$ production (Fig. 4a). In absence of GG, photosynthesis was completely inhibited after 24 h. No regeneration of photosynthesis, no growth and no accumulation of GG could be observed during the 96 h of the experiment. In cultures of mutant 107 supplemented with GG, photosynthetic activity started to increase about 24 h after the salt shock. At the same time significant amounts of GG were already

Fig. 4. Effect of exogenously supplied GG on mutant 107 after a salt shock. This mutant of *Synechocystis* 6803 is unable to synthesize GG and is consequently salt sensitive. It was subjected to a salt shock in the presence (filled symbols) or absence (open symbols) of external GG, as described in Methods. (a) GG concentration in cells grown in the presence of 0.75 mM GG (▲) and photosynthesis, measured as net O₂ evolution, of cells grown with 0.75 mM GG (●) and without added GG (○), respectively. (b) Growth expressed as increase of biovolume of the culture with 0.75 mM GG (◆) and without added GG (◇), respectively.

detectable in the cells (Fig. 4a). Between 36 and 48 h after the salt shock, cultures supplemented with GG started to grow again (Fig. 4b) and photosynthetic O₂ production reached about 50 % of the initial level (Fig. 4a).

DISCUSSION

The studies presented here demonstrate the existence of an active-transport system for exogenous GG in the cyanobacterium *Synechocystis* 6803. The following results are evidence for the active nature of GG uptake: ¹⁴C-labelled GG was transported into salt-adapted cells containing GG in high concentrations against a remarkable concentration gradient; the same concentration gradient was established in a mutant which is unable to synthesize GG (in this case by means of the transport system); and furthermore, the inhibition of energy supply (by incubation under darkness and in the presence of specific inhibitors) decreased GG uptake substantially and indicated its dependence on metabolic energy.

Because of their primary photoautotrophic growth mode, little information is available on the transport of sugars and related compounds by cyanobacteria. However, *Synechocystis* 6803 does possess an active-transport system for glucose (Flores & Schmetterer, 1986; Joset *et al.*, 1988). Therefore, exogenous GG could be hydrolysed outside the cell followed by uptake of glucose and glycerol. The latter might enter the cell by passive

diffusion. In *E. coli*, where trehalose is used as an osmoprotective compound, utilization of exogenous trehalose under conditions of high osmolality takes place by hydrolysis to glucose and subsequent uptake via the phosphoenolpyruvate:sugar phosphotransferase system (Boos *et al.*, 1990). Since glucose did not interfere with GG uptake in *Synechocystis* 6803, hydrolysis of GG and subsequent uptake of glucose can be excluded. Furthermore, separation by TLC of ethanolic extracts of cells incubated with [¹⁴C]GG indicate that GG entered the cells unaltered. It was not metabolized in control or in salt-adapted cells, indicating that GG showed no turnover. The absence of a GG turnover was also postulated for salt-adapted cells of the cyanobacterium *Microcystis firma* (Hagemann *et al.*, 1987).

The transport system for GG seems to be involved in the process of salt adaptation because its activity corresponded with the external salt concentration. Compared to other processes involved in salt adaptation of *Synechocystis* 6803 (Erdmann *et al.*, 1992), the increased level of GG transport activity was reached after a rather long time following a salt shock of 684 mM NaCl. After hypo-osmotic shock it remained at the high level of salt-adapted cells for several hours. Changes of the activity of GG uptake probably represent both increased and decreased amounts of transporter protein in response to the salinity of the growth medium. Therefore, the protein(s) of this transport system seemed to have a slow turnover rate. In addition to the enhanced synthesis, the GG uptake system was activated by increasing NaCl concentrations up to 350 mM. However, activation did not increase the transport rate by more than 50%. The GG-synthesizing enzyme system was more affected by short-term changes of the salinity (Hagemann & Erdmann, 1994). Summarizing, the GG transport system seems to be regulated by the salinity at the level of both gene expression and, to a lesser degree, activity of pre-existing transport proteins.

The kinetics of the GG transporter are characteristic of bacterial uptake systems, which involve specific transport proteins. The estimated K_m value of about 50 μ M is comparable to values of the sugar uptake systems in several cyanobacterial strains, which generally exhibit weak affinities (between 100 and 1400 μ M; Smith & Moore, 1988; Joset *et al.*, 1988; Schmetterer & Flores, 1988). In contrast, the glycine betaine transporter of the cyanobacterium *Aphanothece halophytica* exhibited a higher affinity for its substrate (K_m 2 μ M; Moore *et al.*, 1987). Furthermore, this transport system showed about a 170-fold higher V_{max} than the GG transporter of *Synechocystis* 6803.

The energy dependence of the GG uptake was confirmed by the inhibitory effects of the uncouplers CCCP and FCCP in the light, as well as by KCN in the dark. KCN was ineffective in the light, and incubation in the dark without this inhibitor diminished GG uptake only slightly. Inhibition of both photosynthetic and respiratory electron transport (by the combination of darkness and KCN) or their uncoupling from phosphorylation (by CCCP or FCCP) was necessary to inhibit the GG

transport. Therefore, both processes can provide energy for uptake. It should be noted that in cyanobacteria the respiratory and photosynthetic electron transport chains are closely linked by sharing plastoquinone and cytochrome *b₆f* (e.g. Mi *et al.*, 1994). No conclusions concerning the mechanism of GG transport could be drawn from the inhibitor data.

The competition experiments indicated that sucrose is accepted by the GG transport system with a somewhat lower affinity. This was confirmed by uptake experiments with [¹⁴C]sucrose. Using control cells, uptake showed about the same $V_{\max}$ as for GG, but the K_m value was doubled compared to GG. To a lesser extent trehalose also seems to be transported by this system, since both GG and sucrose uptake were competitively inhibited by trehalose. The inhibitor constants for GG, sucrose and trehalose together with the K_m values for GG and sucrose confirm that the transport system displays the highest affinity towards GG, about half this affinity for sucrose, and a substantially lower affinity for trehalose. Surprisingly, floridoside, which is structurally closely related to GG, and all other compounds tested did not compete in uptake experiments. Interestingly, the transport system showed affinity only to substances (GG, sucrose, and trehalose) used as osmoprotective compounds by cyanobacteria. *E. coli* is also able to take up different osmolytes using its ProU and ProP systems (Csonka & Hanson, 1991; Lucht & Bremer, 1994).

The rather low activity of the GG transport was sufficient for the adaptation of the salt-sensitive mutant 107 of *Synechocystis* 6803 to a lethal salt concentration of 600 mM NaCl in the presence of 0.75 mM exogenously supplied GG. In the absence of GG, salt adaptation was not possible. The cells needed about 75 h to accumulate GG in concentrations comparable to the amount contained by wild-type cells at this salinity. *De novo* synthesis of GG in the wild-type strain reached a final value 12 h after a salt shock of 684 mM NaCl. The complementation of mutant 107 to salt tolerance by adding GG provides for the first time direct evidence that accumulation of GG is osmoprotective for the cellular metabolism of cyanobacteria. Furthermore, the kinetics indicate that GG accumulation is a prerequisite for adaptation to high salinities, since photosynthesis and growth were restored only after GG was accumulated to significant levels.

The primary function of the GG transporter in salt-adapted cells of *Synechocystis* 6803 may be the uptake of GG that has leaked out of the cell into the periplasm. Experiments carried out to estimate the K_m value gave evidence that GG is present in the periplasm of salt-grown cells. In these cells a high internal GG level has to be maintained. The avoidance of losses would save carbon and energy for the cells. This presumption is supported by the low activity of the GG uptake compared to the GG synthesis found in *Synechocystis* 6803. In natural habitats GG should be present only in traces. Significant amounts of extracellular GG may only occur in microbial mats exposed to changing salinities where GG has been detected in natural populations (Stal & Reed, 1987). Further studies should be carried out to investigate the

presence and the function of GG transport in cyanobacterial species with different salt tolerances.

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REFERENCES

- Allen, M. B. & Arnon, D. I. (1955). Studies on nitrogen-fixing blue-green algae. I. Growth and nitrogen fixation by *Anabaena cylindrica* Lemm. *Plant Physiol* **30**, 366–372.
- Boch, J., Kempf, B. & Bremer, E. (1994). Osmoregulation in *Bacillus subtilis*: synthesis of the osmoprotectant glycine betaine from exogenously provided choline. *J Bacteriol* **176**, 5364–5371.
- Boos, W., Ehmann, U., Forkl, H., Klein, W., Rimmele, M. & Postma, P. (1990). Trehalose transport and metabolism in *Escherichia coli*. *J Bacteriol* **172**, 3450–3461.
- Brown, A. D. (1976). Microbial water stress. *Bacteriol Rev* **40**, 803–846.
- Csonka, L. N. & Hanson, A. D. (1991). Prokaryotic osmoregulation: genetics and physiology. *Annu Rev Microbiol* **45**, 569–606.
- Erdmann, N., Berg, C. & Hagemann, M. (1989). Missing salt adaptation of *Microcystis firma* (cyanobacterium) in the dark. *Arch Hydrobiol* **114**, 521–530.
- Erdmann, N., Fulda, S. & Hagemann, M. (1992). Glucosylglycerol accumulation during salt acclimation of two unicellular cyanobacteria. *J Gen Microbiol* **138**, 363–368.
- Feige, G. B., Gimmler, H., Jeschke, W. D. & Simonis, W. (1969). Eine Methode zur dünnstichtchromatographischen Auftrennung von ¹⁴C- und ³²P-markierten Stoffwechselsprodukten. *J Chromatogr* **41**, 80–90.
- Flores, E. & Schmetterer, G. (1986). Interaction of fructose with the glucose permease of the cyanobacterium *Synechocystis* sp. strain PCC 6803. *J Bacteriol* **166**, 693–696.
- Fulda, S., Hagemann, M. & Libbert, E. (1990). Release of glucosylglycerol from the cyanobacterium *Synechocystis* spec. SAG 92.79 by hypoosmotic shock. *Arch Microbiol* **153**, 405–408.
- Galinski, E. A. & Trüper, H. G. (1994). Microbial behaviour in salt-stressed ecosystems. *FEMS Microbiol Rev* **15**, 95–108.
- Glaever, H. M., Styrvold, O. B., Kaasen, I. & Strøm, A. R. (1988). Biochemical and genetic characterization of osmoregulatory trehalose synthesis in *Escherichia coli*. *J Bacteriol* **170**, 2841–2849.
- Hagemann, M. & Erdmann, N. (1994). Activation and pathway of glucosylglycerol synthesis in the cyanobacterium *Synechocystis* sp. PCC 6803. *Microbiol* **140**, 1427–1431.
- Hagemann, M. & Zuther, E. (1992). Selection and characterization of mutants of the cyanobacterium *Synechocystis* sp. PCC 6803 unable to tolerate high salt concentrations. *Arch Microbiol* **158**, 429–434.
- Hagemann, M., Erdmann, N. & Wittenburg, E. (1987). Synthesis of glucosylglycerol in salt-stressed cells of the cyanobacterium *Microcystis firma*. *Arch Microbiol* **148**, 275–279.
- Hagemann, M., Wölfel, L. & Krüger, B. (1990). Alterations of protein synthesis in the cyanobacterium *Synechocystis* sp. PCC 6803 after a salt shock. *J Gen Microbiol* **136**, 1393–1399.
- Incharoensadki, A., Takabe, T. & Akazawa, T. (1986). Effect of betaine on enzyme activity and subunit interaction of ribulose-1,5-bisphosphate carboxylase/oxygenase from *Aphanethece halophytica*. *Plant Physiol* **81**, 1044–1049.

- Joset, F., Buchou, T., Zhang, C.-C. & Jeanjean, R. (1988).** Physiological and genetic analysis of the glucose-fructose permeation system in two *Synechocystis* species. *Arch Microbiol* **149**, 417–421.
- Kirst, G. O. (1990).** Salinity tolerance of eukaryotic marine algae. *Annu Rev Plant Physiol Plant Mol Biol* **41**, 21–53.
- Larsen, P. I., Sydnès, L. K., Landfald, B. & Strøm, A. R. (1987).** Osmoregulation in *Escherichia coli* by accumulation of organic osmolytes: betaines, glutamic acid, and trehalose. *Arch Microbiol* **147**, 1–7.
- Lucht, J. M. & Bremer, E. (1994).** Adaptation of *Escherichia coli* to high osmolarity environments: osmoregulation of the high-affinity glycine betaine transport system ProU. *FEMS Microbiol Rev* **14**, 3–20.
- Mi, H., Endo, T., Schreiber, U., Ogawa, T. & Asada, K. (1994).** NAD(P)H dehydrogenase-dependent cyclic electron flow around photosystem I in the cyanobacterium *Synechocystis* PCC 6803: a study of dark-starved cells and spheroplasts. *Plant Cell Physiol* **35**, 163–174.
- Moore, D. J., Reed, R. H. & Stewart, W. D. P. (1987).** A glycine betaine transport system in *Aphanothece halophytica* and other glycine betaine-synthesising cyanobacteria. *Arch Microbiol* **147**, 399–405.
- Perroud, B. & Le Rudulier, D. (1985).** Glycine betaine transport in *Escherichia coli*: osmotic modulation. *J Bacteriol* **161**, 393–401.
- Raddatz, G. (1994).** EKI-Programm. In *Enzymkinetik, Theorie und Methoden*. Edited by H. Bisswanger. Weinheim: VCH Verlagsgesellschaft.
- Reed, R. H. & Stewart, W. D. P. (1985).** Osmotic adjustment and organic solute accumulation in unicellular cyanobacteria from freshwater and marine habitats. *Mar Biol* **88**, 1–9.
- Reed, R. H. & Stewart, W. D. P. (1988).** The response of cyanobacteria to salt stress. In *Biochemistry of the Algae and Cyanobacteria*, vol. 12, pp. 217–231. Edited by L. J. Rogers & J. R. Gallon. Oxford: Clarendon Press.
- Reed, R. H., Warr, S. R. C., Kerby, N. W. & Stewart, W. D. P. (1986).** Osmotic shock-induced release of low molecular weight metabolites from free-living and immobilized cyanobacteria. *Enzyme Microb Technol* **8**, 101–104.
- Schmetterer, G. & Flores, E. (1988).** Uptake of fructose by the cyanobacterium *Nostoc* sp. ATCC 29150. *Biochim Biophys Acta* **942**, 33–37.
- Schoor, A., Erdmann, N., Effmert, U. & Mikkat, S. (1995).** Determination of the cyanobacterial osmolyte glucosylglycerol by high-performance liquid chromatography. *J Chromatogr A* **704**, 89–97.
- Smith, A. J. & Moore, J. E. (1988).** Transport of carbohydrates into cyanobacteria. *Methods Enzymol* **167**, 551–556.
- Stal, L. J. & Reed, R. H. (1987).** Low-molecular mass carbohydrate accumulation in cyanobacteria from a marine microbial mat in response to salt. *FEMS Microbiol Ecol* **45**, 305–312.
- Styrvoid, O. B. & Strøm, A. R. (1991).** Synthesis, accumulation, and excretion of trehalose in osmotically stressed *Escherichia coli* K-12 strains: influence of amber suppressors and function of the periplasmic trehalase. *J Bacteriol* **173**, 1187–1192.
- Tel-Or, E., Spath, S., Packer, L. & Mehlhorn, R. J. (1986).** Carbon-13 NMR studies of salt shock-induced carbohydrate turnover in the marine cyanobacterium *Agmenellum quadruplicatum*. *Plant Physiol* **82**, 646–652.
- Wilkinson, G. N. (1961).** Statistical estimations of enzyme kinetics. *Biochem J* **80**, 324–332.

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