

The *ggs* Gene from *Synechocystis* sp. Strain PCC 6803 Encoding Glucosyl-Glycerol-Phosphate Synthase Is Involved in Osmolyte Synthesis

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A salt-sensitive mutant of *Synechocystis* sp. strain PCC 6803 defective in the synthesis of the compatible solute glucosylglycerol (GG) was used to search for the gene encoding GG-phosphate synthase (GGPS), the key enzyme in GG synthesis. Cloning and sequencing of the mutated region and the corresponding wild-type region revealed that a deletion of about 13 kb occurred in the genome of mutant 11. This deletion affected at least 10 open reading frames, among them regions coding for proteins showing similarities to trehalose (*otsA* homolog)- and glycerol-3-phosphate-synthesizing enzymes. After construction and characterization of mutants defective in these genes, it became obvious that an *otsA* homolog (*sll1566*) (T. Kaneko et al., DNA Res. 3:109–136, 1996) encodes GGPS, since only the mutant affected in *sll1566* showed salt sensitivity combined with a complete absence of GG accumulation. Furthermore, the overexpression of *sll1566* in *Escherichia coli* led to the appearance of GGPS activity in the heterologous host. The overexpressed protein did not show the salt dependence that is characteristic for the GGPS in crude protein extracts of *Synechocystis*.

The accumulation of compatible solutes represents an important aspect of cellular adaptation to high-salt or high-osmotic-pressure environments. Salt-treated cells are able to accumulate these low-molecular-mass substances in high amounts, since they are hydrophilic, show no net charge, and are compatible with cellular metabolism. Osmoprotective compounds fulfil two different functions: (i) decrease of intracellular water potential to prevent water loss, and (ii) prevention of denaturation of macromolecules in a cellular environment of changed ionic composition and decreased water potential (10). Based on the principal osmoprotective compound accumulated, three salt-tolerant groups of cyanobacteria have been distinguished (25). Strains with lower salt tolerance accumulate the disaccharide sucrose or trehalose, strains with moderate halotolerance synthesize the heteroside glucosylglycerol (GG), and halophilic strains contain the quaternary ammonium compounds glycinebetaine or glutamatebetaine.

The accumulation of GG was characterized by using the moderately halotolerant strain *Synechocystis* sp. strain PCC 6803. *Synechocystis* synthesizes mainly GG and traces of sucrose, which enable it to tolerate up to 1.2 M NaCl (24). The biosynthetic pathway begins with ADP-glucose and glycerol-3-phosphate (G3P), which are used by the GG-phosphate synthase (GGPS), and proceeds via the intermediate GG-phosphate (GGP), which is dephosphorylated to GG by the GG-phosphate phosphatase (GGPP) (12). The enzyme activities were found to depend on enhanced salt concentrations in the assays. Furthermore, the GGPS and GGPP activities seem to be reversibly affected by a salt-regulated process. These enzymes could be activated in cells grown in low-salt medium

simply by adding salt during the protein extraction and, conversely, could be inhibited in extracts from cells adapted to high-salt medium by omitting salt from the homogenization and assay buffers (12). Beside NaCl, other salts were also found to be effective with regards to the activation of GG-synthesizing enzymes in *Synechocystis* extracts (15).

Genes encoding proteins involved in cyanobacterial salt adaptation were selected by using the enhanced content of their mRNAs in salt-treated cells (4) or by characterization of salt-sensitive mutants obtained after random cartridge mutagenesis (11). By use of the second strategy, 18 mutants of *Synechocystis* that were unable to tolerate salt concentrations higher than 550 mM NaCl, which is less than 50% of the salt resistance limit of the wild type (WT), were selected. The salt-sensitive mutants could be divided into two groups: (i) nine mutants that were affected in the synthesis of GG; (ii) nine mutants that showed no change in GG synthesis. The GG-defective mutants showed a larger decrease in their remaining salt tolerance than mutants of the second group. After physiological and genetic characterization of one mutant, which accumulates mainly the intermediate GGP (13), *stpA* could be identified as the GGPP-encoding gene. In addition to the direct regulation of GGPP enzyme activity by salt, it could be shown that the expression of the *stpA* gene is also salt stimulated (14).

In the present study, we describe the gene encoding GGPS, the key enzyme in GG synthesis. After cloning and partial sequencing of the mutated site from a GG-defective mutant, it was possible to define the affected region on the chromosome of *Synechocystis*, since its genome has recently been completely sequenced (17). Open reading frames (ORFs) located in the mutated region were separately mutated, and the mutants were screened for changes in salt tolerance and GG synthesis. By use of this strategy, an *otsA* homolog could be identified as the GGPS-encoding gene. After overexpression of this gene, GGPS activity was found in *Escherichia coli*. We propose the designation *ggs* for the gene encoding GGPS.

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TABLE 1. Plasmids and *Synechocystis* sp. strain PCC 6803 mutants used and constructed in this study (see Fig. 1)

Plasmid or mutant	Description ^a
Plasmids	
pGG	pGEM7 containing <i>ggsS</i> (<i>sll1566</i>) as a 2.1-kb <i>Bam</i> HI fragment obtained by PCR with <i>ggsS5</i> and <i>ggsS3</i> primers; final size, 5.2 kb
pΔG::Km2	pGG containing an inactivated <i>ggsS</i> gene (<i>aphII</i> gene is inserted in the <i>Sna</i> BI/ <i>Stu</i> I deletion in the opposite direction of <i>ggsS</i>); final size, 6.1 kb
pΔG::Sm2	pGG containing an inactivated <i>ggsS</i> gene (<i>smr</i> gene is inserted in the <i>Sna</i> BI/ <i>Stu</i> I deletion in the opposite direction of <i>ggsS</i>); final size, 6.7 kb
pGD	pGEM7 containing <i>gpd</i> (<i>sll1085</i>) as a 1.7-kb <i>Eco</i> RI/ <i>Xho</i> I fragment obtained by PCR with <i>gpd5</i> and <i>gpd3</i> primers; final size, 4.7 kb
pGD::Km2	pGD containing an inactivated <i>gpd</i> gene (<i>aphII</i> gene is inserted at the unique <i>Bsp</i> I site in the opposite direction of <i>gpd</i>); final size, 5.9 kb
pGD::Sm2	pGD containing an inactivated <i>gpd</i> gene (<i>smr</i> gene is inserted at the unique <i>Bsp</i> I site in the opposite direction of <i>gpd</i>); final size, 6.7 kb
pGK	pGEM7 containing <i>gpk</i> (<i>slr1672</i>) as a 1.5-kb <i>Eco</i> RI/ <i>Bam</i> HI fragment obtained by PCR with <i>gpk5</i> and <i>gpk3</i> primers; final size, 4.5 kb
pGK::Km2	pGK containing an inactivated <i>gpk</i> gene (<i>aphII</i> gene is inserted at the <i>Bst</i> EII deletion in the opposite direction of <i>gpk</i>); final size, 5.6 kb
pBADGGPS	pBAD/HisB containing <i>ggsS</i> (<i>sll1566</i>) as a 1.6-kb <i>Bgl</i> II/ <i>Kpn</i> I fragment obtained by PCR with <i>ggsSE5'</i> and <i>ggsSE3'</i> used for <i>ggsS</i> overexpression in <i>E. coli</i> LMG194; final size, 5.7 kb
Mutants	
ΔGK2	Salt-sensitive <i>Synechocystis</i> mutant impaired in <i>ggsS</i> (<i>sll1566</i>) obtained after transformation of the WT with pΔG::Km2
ΔGS2	Salt-sensitive <i>Synechocystis</i> mutant impaired in <i>ggsS</i> (<i>sll1566</i>) obtained after transformation of the WT with pΔG::Sm2
DK2	Salt-tolerant <i>Synechocystis</i> mutant impaired in <i>gpd</i> (<i>sll1085</i>) obtained after transformation of the WT with pGD::Km2
ΔKK2	Salt-tolerant <i>Synechocystis</i> mutant impaired in <i>gpk</i> (<i>slr1672</i>) obtained after transformation of the WT with pGK::Km2
DS2ΔKK2	Salt-tolerant <i>Synechocystis</i> mutant impaired in <i>gpd</i> (<i>sll1085</i>) and <i>gpk</i> (<i>slr1672</i>) obtained after transformation of ΔKK2 with pGD::Sm2

^a Salt tolerance of the mutants was tested by the ability to grow in 0.55 M NaCl or to lyse at this salt concentration.

MATERIALS AND METHODS

Strains and culture conditions. A derivative of the *Synechocystis* sp. strain PCC 6803 with enhanced transforming capacity, which was used in all experiments, was obtained from S. Shestakov (Moscow State University, Moscow, Russia). Axenic cells were cultured on plates at 30°C under constant illumination by using the mineral medium C (18). Transformants were initially selected on medium containing 10 μg of kanamycin · ml⁻¹ (Sigma) or 0.5 μg of streptomycin · ml⁻¹, while the segregation of clones and cultivation of mutants was performed at 50 μg of kanamycin · ml⁻¹ or 2 μg of streptomycin · ml⁻¹ (11). For the physiological characterization, axenic cultures of the cyanobacteria were grown photoautotrophically in batch cultures as described previously (8). The *E. coli* strain TG1 was used for routine DNA manipulations (26).

DNA manipulations. Isolation of total DNA from *Synechocystis* was done as described previously (4). All other techniques, such as plasmid isolation, transformation of *E. coli*, ligation, and restriction analysis (restriction enzymes were obtained from New England Biolabs), were standard methods (26). Labeling of DNA probes with digoxigenin for Southern hybridization was done by using the PCR DIG probe synthesis kit (Boehringer Mannheim). Sequencing was performed by the dideoxy chain termination method with [α -³⁵S]dATP (Amersham Buchler) and the Sequenase 2.0 kit (U.S. Biochemicals). Double-stranded plasmid DNA was isolated by using the QIAprep plasmid kit (Qiagen). The following synthetic primers were specifically used for sequencing the regions flanking the *aphII* gene: CAGGCCTGGTATGAGTCAGC (Kan5'); ATTTTATCTTGTGCAATGT (Kan3') (custom oligonucleotide synthesis by Pharmacia). Computer analysis of DNA and protein sequences was done by using DNASIS/PROSIS and Blast (2) software packages. The plasmid vector pGEM7 (Promega) was used to clone the ORFs of interest after PCR amplification of DNA fragments. The following primers, used for amplification of selected genes, were designed by using the complete genome sequence from *Synechocystis* sp. strain PCC 6803 (17) and contain added restriction sites (underlined): primer *ggsS5*, CGGGATCCCGAGGGGGGCAAAAACAATGTCAA; *ggsS3*, CGGGATCCCTACATTTGGGGGGGTGTCC; *gpd5*, AAAAGAATTCATGCGTAATTCCAGAA; *gpd3*, CTGATACCTCGAGTCAGTGAGACAATAGTC; *gpk5*, AAAGGATCCATGACAGCAAAAACATAAT; *gpk3*, AAAAGAATTCCTCACTGGTCAACGGATAC. Usually, the primers are complementary to the sequences behind and before the start and stop codons (shown in bold letters), respectively, of the corresponding genes. The primer *ggsS5* anneals 714 bp upstream of the start codon of the *ggsS* gene, and the primer *ggsSE3'* binds 73 bp downstream of the *ggsS* gene.

Generation of insertion mutants. For the generation of mutants in specific ORFs, the *aphII* gene (conferring kanamycin resistance) from pUC4K (30) or the *smr* gene cartridge (conferring streptomycin resistance) from pBSL130 (1) was integrated at selected unique restriction sites into the ORFs cloned into *E. coli* vectors (Table 1). Plasmid DNA of these constructs was isolated from *E. coli* by using the QIAprep spin plasmid mini kit (Qiagen). About 1 μg of DNA was used for transformation of *Synechocystis*, and antibiotic-resistant (Km^r Sm^r) clones were selected (11).

Protein overexpression. For overexpression and purification of the GGPS, the pBAD Expression System (Invitrogen), which uses the tightly controlled promoter of the arabinose operon from *E. coli*, was used. The *ggsS* ORF was

amplified from *Synechocystis* chromosomal DNA by PCR with the Elongase-Enzyme-Mix (GIBCO, Life Technologies) and the following primers: 5'-GGAAGATCTATGAATTCATCCCTGTGATC-3' (*ggsSE5'*; *Bgl*II site underlined, start codon in bold letters) and 5'-CGGGTACCCCT/AAACTCTAACTTGG-3' (*ggsSE3'*; *Kpn*I site underlined) (custom oligonucleotide synthesis by Pharmacia). The translational start codon immediately behind a *Bgl*II site was used to clone the fragment (*Bgl*II and *Kpn*I) in frame with the N-terminal polyhistidine tag into pBAD/HisB (Invitrogen). For overexpression, the recombinant plasmid carrying the *ggsS* gene (pBADGGPS) was transferred into *E. coli* LMG194 (Invitrogen). The cells were cultured at 37°C in LB medium until the suspension reached an *A*₆₀₀ of 0.5. The expression of the protein was induced by the addition of arabinose (0.0002%), and the suspension was incubated for 4 h. The proteins were extracted from *E. coli* by sonication with homogenization buffer (20 mM Na phosphate buffer, 500 mM NaCl [pH 7.8]). The extract was applied to a nickel chelate column (ProBond; Invitrogen), where the fusion protein was bound. The GGPS protein was eluted from the matrix by passing homogenization buffer with an increasing concentration of imidazole through the column (wash buffer containing 75 mM imidazole; elution buffer containing 300 mM imidazole). To reduce the salt concentration, the eluted protein was dialyzed against 20 mM Tris-maleate buffer (pH 7.8), containing 5 mM MgCl₂. The proteins were electrophoretically separated by using 12% polyacrylamide gels (14) or used for enzyme assays.

Physiological characterization. The content of low-molecular-mass carbohydrates was analyzed by high-performance liquid chromatography (27). The activities of GGPS and GGPP were determined in vitro by using the ¹⁴C-labeled substrate G3P (Amersham Buchler) and buffers containing no or enhanced NaCl contents (0 or 324 mM NaCl added). The reaction products were separated by thin-layer chromatography (TLC). The in vitro determination of these enzyme activities has been described in detail previously (12). To complete dephosphorylation of GGP in measurements of the GGPS isolated from *E. coli* after overexpression of the *ggsS* gene, 1 U of alkaline phosphatase (Boehringer Mannheim) was directly added to the assay mixtures for an additional 30 min at 30°C. Quantification of radioactive spots was done with a phosphorimager (BAS 1000; Fuji). Photosynthetic oxygen evolution was measured with a Clark-type electrode (8). Growth and cell density were monitored by determining the absorbance of diluted cyanobacterial suspensions at 750 nm (*A*₇₅₀) with a spectrophotometer (U2000; Hitachi).

RESULTS

The salt-sensitive mutant 11 was generated by random integration of an *aphII* gene cartridge and found to be completely defective in GG synthesis (11). To characterize the genetic basis for this defect, the mutated site was cloned (for details of the strategy, see reference 13). The cyanobacterial DNA flanking the 5' end of the *aphII* gene cartridge was partly sequenced (sequence M11Kan5) (data not shown) and used as a probe for screening a *Synechocystis* gene library to obtain the corre-

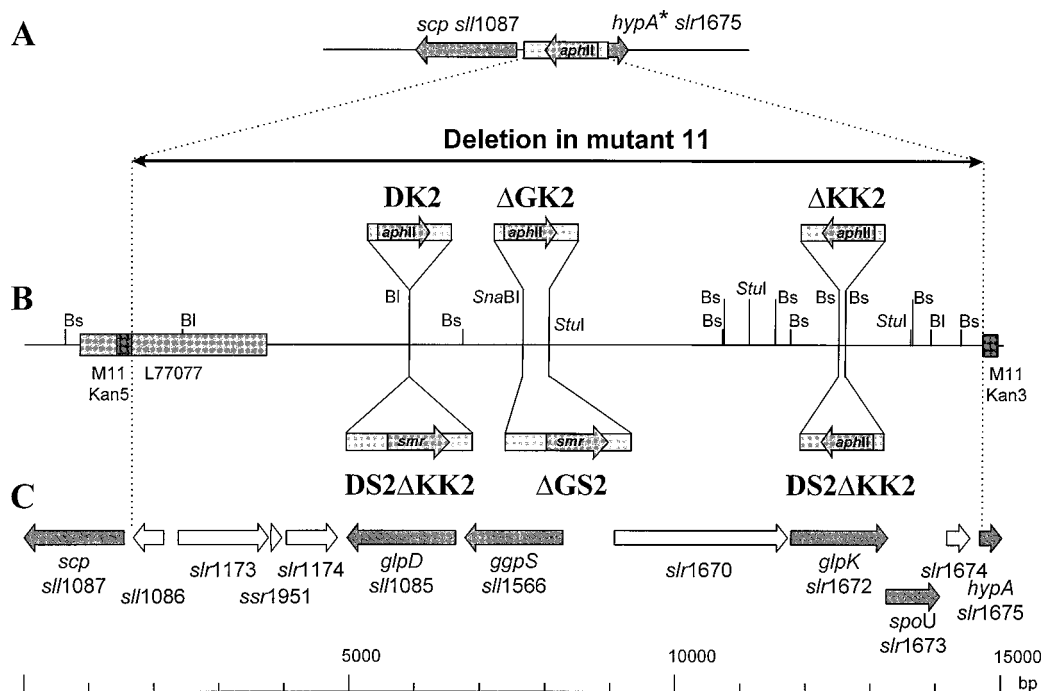


FIG. 1. Schematic drawing showing the genetic organization, restriction map, and protein-encoding regions of the chromosomal site affected in *Synechocystis* mutant 11 (A), the corresponding site of the WT of *Synechocystis* (C) (17), and the insertion sites of the *aphII* and *smr* genes in selected sites of the different genes to obtain directed mutants of different ORFs (B). Shaded arrows represent genes encoding putative proteins in *Synechocystis*; empty arrows represent genes encoding hypothetical proteins in *Synechocystis*; small arrows labeled with *aphII* represent inserted *aphII* gene cassettes; small arrows labeled with *smr* represent inserted *smr* gene cassettes; shaded boxes represent regions sequenced in this work and sequences L77077, M11Kan5, and M11Kan3; asterisks indicate partial deleted genes; a long horizontal black arrow represents the deletion that occurred during integration of an *aphII* gene in mutant 11. *Bl*, *BspI*; *Bs*, *BstEII*.

sponding WT region. From the selected phage clone, a 4.5-kb *SalI* fragment could be cloned and partly sequenced (the sequence has been deposited in GenBank under accession no. L77077 [hereafter called sequence L77077]). After comparison with the complete genome sequence of *Synechocystis* (17), it was found that the sequence L77077 corresponds to the region between bp 1941414 and bp 1944348. This sequence contains three ORFs (*sll1087*, *sll1086*, *slr1173*; designation in accordance with work of Kaneko et al. [17]) which were subsequently mutated by integration of an *aphII* gene. However, none of these single mutants showed any alteration in salt adaptation, indicating that the gene affected in mutant 11 leading to impaired GG synthesis is not harbored on the sequenced fragment (data not shown).

The cyanobacterial DNA fragment flanking the *aphII* cartridge at the 3' end in mutant 11 did not hybridize with DNA from the phage clone isolated by using the DNA fragment neighboring the 5' end. Additionally, the 200 bp of the estimated sequence of the 3' flanking fragment (sequence M11Kan3) (data not shown) exhibited no similarities to sequence L77077. These were the first indications that, during the integration of the *aphII* gene into the genome of mutant 11, a large deletion had occurred. Such deletions have already been reported from studies using this mutagenesis strategy for *Synechocystis* (7, 13). The exact size of the deletion could be estimated by comparison with the complete genome sequence (17). Sequence M11Kan3 was identical to the *Synechocystis* genome region between bp 1955270 and bp 1955316. Combined with the assigned region for sequence L77077, these comparisons clearly show that in mutant 11, about 13.0 kb of the WT genome, containing at least 10 ORFs, is missing (Fig. 1).

To clarify whether any of these ORFs plays a role in GG

synthesis, mutants impaired in only one or two of them, which are most probably responsible for the salt-sensitive phenotype, were constructed (Fig. 1; Table 1). After amplification and cloning of DNA fragments corresponding to single ORFs, antibiotic resistance gene cartridges conferring resistance to kanamycin or streptomycin were introduced into their sequences, leading to genes inactivated by insertion or partial deletion. Constructs showing inserted *aphII* and *smr* gene cartridges in an orientation opposite to the transcription direction of the affected genes (Fig. 1) were selected and transformed into *Synechocystis* WT cells. Since these plasmids do not harbor an origin of replication for *Synechocystis*, stable antibiotic-resistant clones could be obtained only after exchange of the mutated gene copy with the WT copy on the chromosome by homologous recombination. By using this strategy, single mutants affected in *sll1085*, *sll1566*, and *slr1672*, probably coding for a glycerol-3-phosphate dehydrogenase (GlpD; 51% amino acid identity with human GlpD [19]), a trehalose-phosphate synthase (OtsA) (for similarities, see Table 2), and a glycerol kinase (GlpK; 61% amino acid identity with GlpK from *E. coli* [22]), respectively, and a double mutant affected in both *sll1085* and *slr1672* were obtained (Table 1; Fig. 1).

After cultivation for several generations on increasing concentrations of the appropriate antibiotic, chromosomal DNA of selected clones was obtained and compared to the WT DNA by PCR and Southern blot analyses. In all cases, when mutant DNA was used, the PCR analyses showed fragments that were larger than the fragments obtained with WT DNA (Fig. 2). The size differences exactly corresponded to the increases expected by insertion of an *aphII* gene cassette (1.2 kb) or a *smr* gene cassette (1.8 kb). In the case of the mutants affected in *sll1566* (Δ GK2 and Δ GS2), the size increase was 0.3 kb less (Fig. 2),

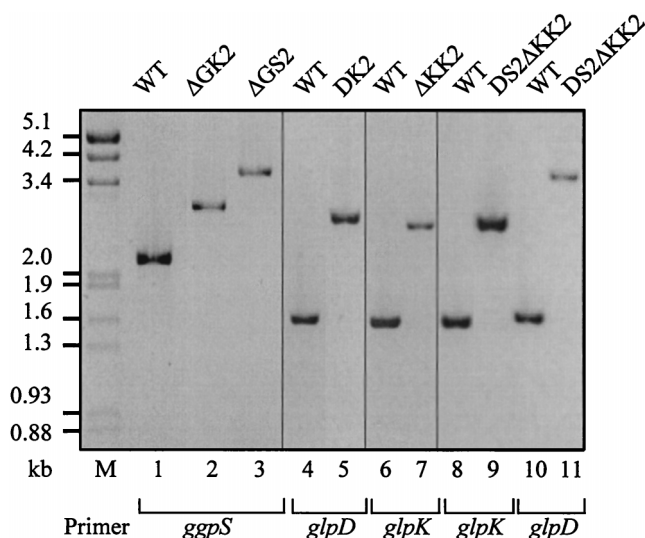


FIG. 2. PCR analyses using chromosomal DNA from the WT (lanes 1, 4, 6, 8, and 10) and mutants (Δ GK2 [lane 2], Δ GS2 [lane 3], DK2 [lane 5], Δ KK2 [lane 7], and DS2 Δ KK2 [lanes 9 and 11]) (see Table 1) of *Synechocystis* as a template and primers specific for selected genes *ggpS5* and *ggpS3* [lanes 1 to 3], *glpD5* and *glpD3* [lanes 4, 5, 10, and 11], and *glpK5* and *glpK3* [lanes 6 to 9]) in order to verify the lesions in the chromosomal DNA of the mutants. Lane M, fragment size marker, *EcoRI/HindIII*-cut λ DNA.

since before insertion of the antibiotic resistance cartridges about 0.3 kb was deleted from this gene by double digestion with *StuI/SnaBI* (Fig. 1; Table 1). Furthermore, in all PCRs with mutant DNA, the WT fragment was completely absent (Fig. 2). The results of the PCR analyses were confirmed by Southern hybridization experiments (data not shown). Additionally, the integration of the *aphII* or *smr* gene cartridge into the genome of the mutants could be verified by using DNA probes specific for these antibiotic resistance genes. Thus, the plasmid constructs used for transformation of *Synechocystis* to obtain the mutants were correctly integrated by double crossover and the mutants were completely segregated, since no WT copy remained visible.

In physiological experiments, the growth of the mutants was compared to that of WT cells under different culture conditions. Under low-salt conditions, no significant differences were observed in cultures on solid or in liquid media. But, differences became obvious after cells were transferred to media containing NaCl concentrations above 500 mM. Under those culture conditions, cells of the mutants impaired in *sll1566* (Δ GK2 and Δ GS2) were unable to grow and lysed after a few days, while all mutants impaired in *glpD* and/or *glpK* (DK2, Δ KK2, and DS2 Δ KK2) were able to adapt to high-salt concentrations (tested up to 684 mM NaCl). The degree of salt sensitivity of mutants Δ GK2 and Δ GS2 resembled the initial salt sensitivity of mutant 11, which was also unable to grow on media with NaCl concentrations above 550 mM (Table 1).

The kinetics of alterations in photosynthesis and accumulation of osmoprotective compounds were investigated after applying a salt shock of 684 mM NaCl to WT and mutant cells (Fig. 3). In all cultures, an immediate inhibition of photosynthesis was observed after salt was added, which is characteristic of salt-shocked cells of *Synechocystis* (8, 13). In WT cells, as well as mutant cells affected in *glpD* and/or *glpK* (only the data for the double mutant DS2 Δ KK2 are shown, which are also representative for the corresponding single mutants), photosynthesis recovered after 4 h and became nearly adapted to the

TABLE 2. Results of amino acid sequence comparisons of *Synechocystis* protein Sll1566 and several trehalose-phosphate synthases^a

Gene	Accession no.	Organism	Sequence		Reference
			Total no. of aa	% Identical aa	
<i>otsA</i>	Q50167	<i>Mycobacterium leprae</i>	498	48	28
<i>otsA</i>	O06353	<i>Mycobacterium tuberculosis</i>	500	47	23
<i>tpsB</i>	Q00217	<i>Aspergillus niger</i>	480	46	31
<i>tpsA</i>	Q00075	<i>Aspergillus niger</i>	517	44	31
<i>tps</i>	Q92410	<i>Candida albicans</i>	478	44	32
<i>tps1</i>	Q00764	<i>Saccharomyces cerevisiae</i>	495	44	5
<i>ggs1</i>	Q07158	<i>Kluyveromyces lactis</i>	488	43	20
<i>otsA</i>	P31677	<i>Escherichia coli</i>	473	42	16
<i>tps1</i>	P40387	<i>Schizosaccharomyces pombe</i>	479	42	6
<i>otsA</i>	P55612	<i>Rhizobium</i> sp.	464	39	9

^a Amino acid sequence comparisons were performed by using BLAST (2). The sequence of *Synechocystis* protein Sll1566 (GGPS or OtsA) which is 499 amino acids (aa) in length, was compared with those of several trehalose-phosphate synthases.

high-salt environment after 48 h. On the contrary, net photosynthesis in cells of the Δ GK2 mutant decreased progressively and was completely inhibited after only 24 h (Fig. 3). Nevertheless, the cells of mutant Δ GK2 seemed not to be dead, since they continued to respire for at least 2 additional days.

The accumulation of the osmoprotective compound GG started immediately in salt-shocked WT and DS2 Δ KK2 mutant cells and reached comparable values 24 to 48 h after the salt shock. But, in salt-treated cells of mutants Δ GK2 and Δ GS2,

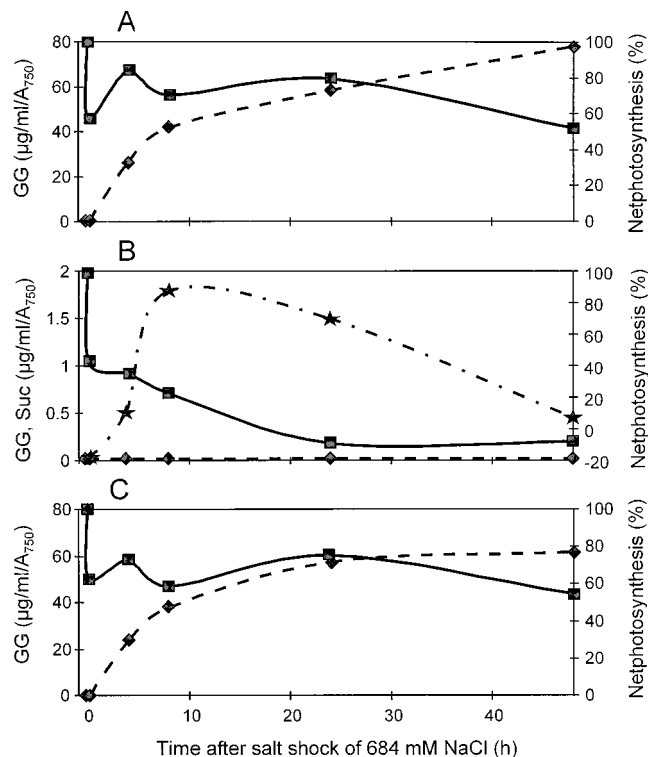


FIG. 3. Photosynthetic oxygen evolution (■) and accumulation of GG (◆) and sucrose (★) after a salt shock of 684 mM NaCl in WT (A), mutant Δ GK2 (B), and mutant DS2 Δ KK2 (C) *Synechocystis* cells. In WT and mutant DS2 Δ KK2 cells, only traces of sucrose, which were below the limit for accurate detection by the high-performance liquid chromatography systems, were present.

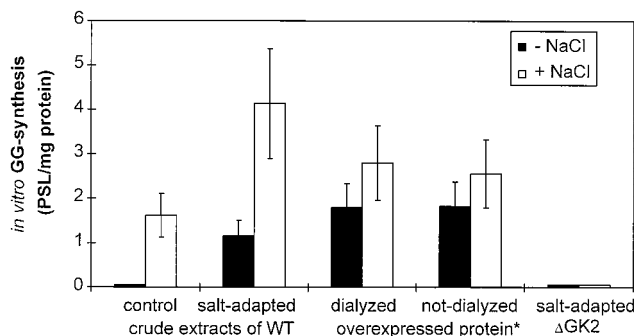


FIG. 4. Salt dependence of the specific GGPS activities in crude protein extracts from control and salt-adapted cells (342 mM NaCl) of the WT and mutant Δ GK2 of *Synechocystis* as well as GGPS protein obtained after overexpression in *E. coli*. The overexpressed GGPS protein was taken directly after purification (buffer containing 20 mM Na phosphate, 500 mM NaCl, and 300 mM imidazole [pH 7.8]) or after extensive dialysis (buffer containing 20 mM Tris-maleate and 5 mM $MgCl_2$ [pH 7.8]). Enzyme assays were performed in NaCl-free or NaCl-containing (342 mM NaCl) buffer. Radioactivity of the GG spots was determined with a phosphoimager and is expressed as pixel intensities per area (PSL). Means \pm standard deviations of values are shown. An asterisk indicates specific activities of the overexpressed protein that were divided by the factor 1,000.

no traces of GG could be detected. Instead of GG, these cells accumulated increased amounts of sucrose (Fig. 3). In the WT, only traces of sucrose could be detected. Enhanced sucrose accumulation has already been found in cells of another GG-defective mutant of *Synechocystis* (11). However, compared to the GG content in WT cells, the accumulated amounts of sucrose in the mutants represent less than 10%, which is not sufficient to balance the external salt concentration. Additionally, assays to detect GGPS activity were performed under different test conditions. In crude protein extracts from WT cells, GGPS activity was detected but only in tests where the assay buffer contained 340 mM NaCl. With extracts from the mutants Δ GK2 and Δ GS2, absolutely no GGPS activity was detectable regardless of whether the cells were salt adapted or the assays contained salt (Fig. 4). Therefore, the estimates of GGPS activity confirmed the data on GG accumulation in the cells.

After the sequence encoding Sll1566 was cloned in a plasmid of the pBAD series and expressed in arabinose-treated cells of

E. coli LMG194, a protein of about 58 kDa could be purified by using the polyhistidine tag added to the N terminus of the *Synechocystis* protein (Fig. 5A). This size of the purified protein corresponded very well with the theoretical molecular mass of 56 kDa deduced from the amino acid sequence of Sll1566 (17). Proteins from various steps of the purification were used in GGPS enzyme assays. Crude extracts from *E. coli* cells containing the plasmid pBADGGPS as well as the purified protein showed the expected enzyme activity, the in vitro formation of GG from ADP-glucose and G3P, while extracts from cells carrying the control plasmid pBAD/HisB showed no GG formation (Fig. 5B). The absence of dephosphorylated GG in these assays clearly demonstrates that *E. coli* does not have GGPP activity and that other phosphatases could not substitute for this activity. After treatment of aliquots of the enzyme assays with alkaline phosphatase, the end product GG was observed (Fig. 5C). The GG spots are somewhat more intensively labeled than the GGP spots, reflecting the better solubility of GG compared to that of GGP in absolute ethanol, which was used to dissolve the dried reaction products to avoid high salt contents in the TLC separations. Enzyme activity with the purified GGPS isolated from *E. coli* was obtained whether or not the test buffers contained enhanced NaCl concentrations. Furthermore, the dialyzed Sll1566 protein also showed high enzyme activity under low-salt conditions, whereas the enzyme from WT cells was completely inhibited (Fig. 4 and 5). Hence, the activity of the overexpressed protein is independent of the NaCl content in the assays, while the enzyme in crude extracts from *Synechocystis* needs enhanced salt concentrations for its activity (Fig. 4).

DISCUSSION

The fragment deleted from the chromosome of mutant 11 contains at least 10 ORFs (Fig. 1). For four ORFs, a putative function can be assigned from sequence comparisons, while the other six ORFs encode hypothetical proteins (17). After reevaluation of the sequence similarities with proteins from the database, at least two different possibilities could explain the defect in GG biosynthesis by the deletion found in mutant 11. First, it is most probable that *sll1566*, which resembles *otsA*, encodes GGPS. The amino acid sequence of Sll1566 shows significant homologies to several trehalose-phosphate synthases involved in osmotically induced trehalose synthesis in

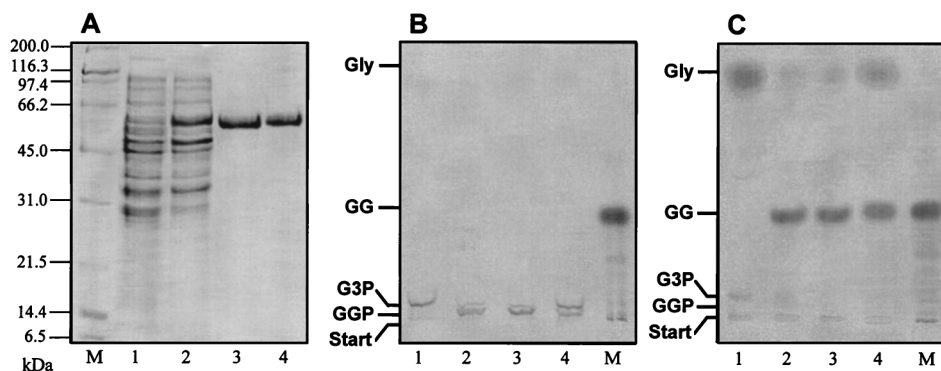


FIG. 5. Overexpression of GGPS (Sll1566) from *Synechocystis* in *E. coli*. (A) Coomassie blue-stained gel from electrophoretic separation of soluble proteins from different fractions of the purification procedure; (B and C) TLC separation of reaction products obtained after determination of GGPS activity by using soluble proteins from different fractions of the purification procedure. The reaction products were directly applied to the TLC plate (B) or pretreated with alkaline phosphatase (C). Lanes: 1, proteins extracted from *E. coli* harboring the empty pBAD/HisB vector; 2, proteins extracted from *E. coli* cells harboring the *ggs* gene on the pBADGGPS vector; 3, purified GGPS protein with the His tag; 4, purified GGPS protein that was dialyzed overnight against low-salt-containing buffer; M, marker lanes (protein broad-range marker from Bio-Rad [A] and ^{14}C -labeled GG isolated from salt-stressed *Synechocystis* cells [B and C]).

bacteria and yeasts (Table 2). These enzymes are glucosyl-transferases, which are functionally and biochemically closely related to the GGPS. But, in salt-stressed cells of *Synechocystis*, no trehalose accumulation was found (24, 27), which makes it unlikely that a trehalose-phosphate synthase exists in this strain. Second, it is possible that *sl11085* and *slr1672* encode enzymes capable of synthesizing G3P (GlpD [glycerol-3-phosphate dehydrogenase] and GlpK [glycerol kinase], respectively) (17), one of the precursors for GG biosynthesis. Their defect could lead to a depletion in G3P, which induces GG deficiency in salt-treated cells.

The experiments using the mutants affected in selected ORFs provided clear evidence that *sl11566* encodes GGPS, as was assumed as the first and most likely explanation for the defect found in mutant 11. We propose the designation *ggsS* for this gene. The GGPS shows high amino acid sequence similarities with trehalose-phosphate synthases from heterotrophic bacteria and yeasts (Table 2). By using these sequence data, an evolutionary relationship of the GG and trehalose-synthesizing enzymes can be proposed. In contrast to the situation in *E. coli*, where the two genes encoding the enzymes involved in trehalose synthesis are organized in one operon (16, 29), in *Synechocystis*, the genes encoding the enzymes involved in GG synthesis are obviously not transcribed together. The *stpA* gene encoding GGPP (14), the second enzyme involved in GG synthesis, is situated far from the *ggsS* gene on the chromosome of *Synechocystis* (*slr0746*; bp 3041493 to 3042407) (17). Mutation of only the *ggsS* gene led to a salt-sensitive phenotype, which is based on a completely impaired GG synthesis. Coupling between a defect in osmolyte synthesis and reduced salt tolerance was also reported for *E. coli*, where mutants in *otsA* as well as *otsB* (encoding trehalose-phosphate phosphatase) were found to be salt or osmosensitive (29). Diminished salt tolerance was found for yeast mutants defective in glycerol and, to a lesser extent, trehalose synthesis (21).

The characterization of the mutants strongly indicated that *sl11566* encodes the key enzyme in GG synthesis. This conclusion was finally verified by overexpression of *sl11566* in *E. coli*, leading to the appearance of GGPS activity in the heterologous host. However, GGPS purified from *E. coli* showed the enzyme activity under low-salt test conditions as well; therefore, the NaCl dependency of GGPS characteristic (12) for this enzyme in crude protein extracts from *Synechocystis* was lost. The same situation was previously found for GGPP, which was purified from *E. coli* by using a different expression and purification system (14). Therefore, in *Synechocystis* cells, an inhibitory mechanism which inhibits the GGPS and GGPP proteins under low-salt conditions should be present. This inhibition is obviously abolished by a NaCl-triggered process in *Synechocystis* and does not exist in *E. coli*.

The possibility to delete *ggsS*, *glpD*, and *glpK* from the chromosome of *Synechocystis* clearly shows that these three genes are not essential for growth under standard conditions. For *ggsS*, this is not surprising, since GG synthesis is completely absent in cells grown under low-salt conditions. The *glpD* and *glpK* genes are also dispensable for cells cultivated in high-salt media. Those cells showed an enhanced content of G3P that is probably needed for GG synthesis (15), but in addition, in control cells G3P has to be synthesized to ensure lipid and other biosyntheses. On the *Synechocystis* genome, there exists another gene encoding a putative NAD-dependent G3P dehydrogenase (*gpsA* or *slr1755*) (17), which obviously expresses sufficient enzyme to produce G3P in the mutants impaired in *glpD* and *glpK*. Nevertheless, we cannot rule out that the *glpD* gene product is significantly involved in G3P production in control and especially salt-stressed cells of the WT. The close

proximity to *ggsS* makes this possibility likely. A comparable situation was reported for *Saccharomyces cerevisiae*, where one G3P dehydrogenase (GPD1) plays a dominant role in osmo-adaptation and another enzyme (GPD2) plays a dominant role in adaptation to anoxic conditions. In single mutants, these enzymes can partly replace each other, while a double mutant was highly osmosensitive and failed to grow under anoxic conditions (3). In future studies, we will analyze how the expression of the *ggsS* gene is regulated by salt. Furthermore, by using the purified GGPS from *E. coli*, factors involved in the NaCl-mediated regulation of its enzyme activity will be isolated from *Synechocystis*.

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