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The *stpA* Gene from *Synechocystis* sp. Strain PCC 6803 Encodes the Glucosylglycerol-Phosphate Phosphatase Involved in Cyanobacterial Osmotic Response to Salt Shock

MARTIN HAGEMANN,[1]* ARNE SCHOOR,[1] ROBERT JEANJEAN,[2] ELLEN ZUTHER,[1] AND FRANÇOISE JOSET[2]

Universität Rostock, FB Biologie, D-18051 Rostock, Germany,[1] *and LCB-CNRS, 13402 Marseille Cedex 20, France*[2]

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Mutations in a gene, *stpA*, had been correlated with the loss of tolerance to high NaCl concentrations in the cyanobacterium *Synechocystis* sp. strain PCC 6803. Genetic, biochemical, and physiological evidence shows that *stpA* encodes glucosylglycerol-phosphate phosphatase. *stpA* mutants are salt sensitive and accumulate glucosylglycerol-phosphate, the precursor of the osmoprotectant glucosylglycerol necessary for salt adaptation of *Synechocystis*. The consensus motif present in acid phosphatases was found in StpA; however, the homology with other sugar phosphatases is very poor. The amount of *stpA* mRNA was increased by growth of the cells in the presence of NaCl concentrations above 170 mM. Expression of *stpA* in *Escherichia coli* allowed the production of a 46-kDa protein which exhibited glucosylglycerol-phosphate phosphatase activity. The StpA-specific antibody revealed a protein of similar size in extracts of *Synechocystis*, and the amount of this protein was increased in salt-adapted cells. The protein produced in *E. coli* had lost the requirement for activation by NaCl that was observed for the genuine cyanobacterial enzyme.

All living cells are able to adapt to a certain range of changes in external salt concentration. An increase in salinity enhances both external osmotic potential and the concentration of inorganic ions leading to a loss of water and to increasing internal ion concentrations. With the exception of halobacteria and some extreme halotolerant eubacteria, these changes are unfavorable or toxic for the cells. Organisms have therefore developed adaptive responses, including two main processes: the enhancement of active ion export systems and the accumulation of osmoprotective compounds (compatible solutes). Organisms tolerant of high salinities have developed such systems more effectively than low-salt-tolerant organisms.

Cyanobacteria are phototrophic prokaryotes which have successfully evolved mechanisms for salt adaptation and are therefore able to live in waters of differing salt concentrations. In 1980, glucosylglycerol [2-*O*-(α-D-glucopyranosyl)-glycerol; GG] was found as the first osmoprotective compound acting in a marine cyanobacterium, the cyanobacterium *Synechococcus* sp. strain N100 (2). After this initial report, about 130 cyanobacterial strains were screened for the ability to adapt to high salt concentrations and for the type of osmoprotectant accumulated. Sucrose has been described as the main osmoprotective compound in 66 strains, trehalose in 20, GG in 24, and glycine betaine and glutamate betaine in 18 (5, 23, 26). A correlation was found between the salt tolerance limit and the nature of the main osmolyte. Poorly halotolerant strains accumulate sucrose and trehalose, moderately halophilic strains accumulate GG, and strains exhibiting the highest salt tolerance synthesize betaines (23).

The cyanobacterium *Synechocystis* sp. strain PCC 6803 is a moderately halotolerant strain. Salt-adapted cells mainly accumulate GG and small amounts of sucrose (22). The accumulation of GG starts immediately after a salt shock. This osmolyte is provided mainly by de novo synthesis from photosynthetically fixed CO_2; only a small amount of GG is synthesized from stored glycogen (27). Direct evidence for the osmoprotective role of GG was provided from complementation assays of a class of salt-sensitive mutants of *Synechocystis*. The loss of tolerance of high salinities by these mutants is correlated with a deficiency of GG synthesis (7). These mutants, however, can grow in high salt concentrations at rates similar to those of the wild type (WT) in the presence of exogenously added GG. This molecule is accumulated via an active transport system (19). The biosynthetic pathway for GG synthesis has been recently elucidated in *Synechocystis* (8). GG is synthesized from ADP-glucose and glycerol-3-phosphate via the intermediate glucosylglycerol-phosphate (GGP) with the cooperation of GGP-synthase (GGP-S) and GGP-phosphatase (GGP-P). The GG-synthesizing enzyme system is present but inactive in cells growing in basal medium. Its activation occurs without the need for de novo protein synthesis by the addition of NaCl or other salts in vivo and in vitro (8, 9).

Little data are available on the molecular genetics of salt adaptation in cyanobacteria (13). Salt stress-induced genes from *Anabaena torulosa* have been cloned by a subtractive hybridization procedure (1). Salt-sensitive mutants of *Synechocystis* sp. strain PCC 6803 have been isolated (7, 12). A gene, *stpA* (salt tolerance protein A), capable of restoring the WT phenotype of one of these mutants has been cloned. The function of the protein encoded by this gene was not clear, since an *stpA*-null mutant showed a pleiotropic phenotype (20). The gene situated immediately upstream of *stpA*, *orf*I, shows similarities to genes encoding response regulators of bacterial two-component systems. The product of *orf*I was proposed to be involved in salt tolerance (10). Mutants impaired in *stpA* function and in that of the flanking open reading frames (ORFs) have been generated by the insertion of a resistance gene marker or by partial deletions. Characterizations of their levels of salt tolerance and their abilities to synthesize GG are de-

* Corresponding author. Mailing address: Universität Rostock, FB Biologie, Doberaner Str. 143, D-18051 Rostock, Germany. Phone: 49-381-4942076. Fax: 49-381-4942079. E-mail: mh@boserv.bio4.uni-rostock.de.

TABLE 1. Plasmids and mutants of *Synechocystis* used in and constructed for this study[a]

Designation	Size (kb)	Description
pBP4[b]	6.7	pUC18 containing a 4.0-kb *Sau*3A fragment of a *Synechocystis* library able to complement an *stpA* defect
pGE4[c]	7.6	pGEM7 containing the whole *stpA* gene on the 1,636- to 6,272-bp *Eco*RI fragment in the transcription direction of P_{lac}
pUCPE1	6.6	pUC19 containing the whole *stpA* gene on the 2,324- to 6,272-bp *Pst*I-*Eco*RI fragment
pUCEB3	6.7	pUC19 containing the whole *stpA* gene on the 1,636- to 5,674-bp *Eco*RI-*Bgl*II fragment
pGE4::Km1	8.9	pGE4 containing an inactivated *orf*I (*aph*II gene inserted at the *Pst*I site, position 2324)
pBP4::Km1[b]	8.0	pBP4 containing an inactivated *stpA* gene (*aph*II gene inserted at the *Stu*I site, position 2931)
pUCEB3::Km	8.0	pUCEB3 containing an inactivated *orf*II (*aph*II gene inserted at the *Hpa*I site, position 4968)
pGE4::Km2	8.9	pGE4 containing an inactivated *orf*III (*aph*II gene inserted at the *Bgl*II site, position 5674)
pGE4ΔPS::Km[c]	8.3	pGE4 with a partly deleted *orf*I and *stpA* gene (*aph*II gene replacing the *Pst*I-*Stu*I fragment, position 2324 to 2931)
pGE4ΔSBs::Km	8.2	pGE4 with a deleted *stpA* gene (*aph*II gene replacing the *Stu*I-*Bst*EII fragment, position 2931 to 3591)
pGEXSTPA	6.2	pGEX-4T-3 containing the *stpA* ORF (obtained by PCR) translationally fused to GST
*orf*I mutant		*Synechocystis* mutant obtained after transformation of the WT with pGE4::Km1
stpA mutant[b]		*Synechocystis* mutant obtained after transformation of the WT with pBP4::Km1
Δ*orf*I/*stpA* mutant[c]		*Synechocystis* mutant obtained after transformation of the WT with pGE4ΔPS::Km
Δ*stpA* mutant		*Synechocystis* mutant obtained after transformation of the WT with pGE4ΔSBs::Km
*orf*II mutant		*Synechocystis* mutant obtained after transformation of the WT with pUCEB3::Km
*orf*III mutant		*Synechocystis* mutant obtained after transformation of the WT with pGE4::Km2

[a] See Fig. 1. The positions of restriction sites originate from sequence U32936 (10).
[b] See reference 20.
[c] See reference 10.

scribed in this paper. In vitro evidence that *stpA* encodes a GGP-P enzyme involved in the salt-induced accumulation of GG in *Synechocystis* and data concerning the salt-dependent in vivo expression of *stpA* are presented.

MATERIALS AND METHODS

Strains and culture conditions. A derivative of *Synechocystis* sp. strain PCC 6803 with enhanced transforming capacity that was used in all experiments was obtained from S. Shestakov (Moscow State University, Moscow, Russia). Axenic cells were cultured on agar plates at 30°C under constant illumination in a mineral medium (17). Transformants were initially selected on media containing 10 μg of kanamycin (Km; Sigma) per ml, while the segregation of clones and the cultivation of mutants was performed at 50 μg of Km per ml. *Escherichia coli* JM101 (24) was used for routine DNA manipulations. For the overexpression of protein, protease-deficient *E. coli* BL21 (24) was used. *E. coli* was cultivated in Luria broth (LB) medium at 37°C. For physiological characterization, axenic cultures of the cyanobacteria were grown photoautotrophically in batch cultures as described in reference 10.

DNA manipulations. Isolation of total DNA from *Synechocystis* was done as described in reference 1. All other DNA techniques, such as plasmid isolation, transformation of *E. coli*, ligation, restriction analysis (restriction enzymes were obtained from Life Technologies), Southern hybridization analysis, and labelling of DNA probes for hybridization experiments by random priming with [α-^{32}P]dATP (Amersham Buchler) were standard methods (24). Correct integration of the *aph*II gene into plasmids was checked by restriction analysis and DNA sequencing. Sequencing was performed by the dideoxy chain termination method with α-^{35}S-dATP (Amersham Buchler) and the Sequenase 2.0 kit (U.S. Biochemicals). Double-stranded plasmid DNA was isolated with the QIAprep plasmid kit (Qiagen). The following synthetic primers were specifically used for sequencing the regions flanking the *aph*II gene: CAGGCCTGGTATGAGTC AGC (Kan5′) and ATTTTTATCTTGTGCAATGT (Kan3′) (custom oligonucleotide synthesis; Pharmacia). A computer analysis of DNA sequences was done with the DNASIS, PROSIS, GCG (4) and MOTIF software packages. The nucleotide sequence of *stpA* and neighboring genes has been deposited in GenBank under the accession number U32936 (10). Plasmid vectors pUC18/19 (30), pUC4K (29), pGEM7 (Promega), and pGEX-4T-3 (Pharmacia) were used.

Generation of insertion mutants. For the generation of mutations in specific ORFs, the *aph*II gene cartridge (aminoglycoside phosphotransferase II conferring Km resistance) from *E. coli* plasmid pUC4K (29) 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* with the QIAprep spin plasmid minikit (Qiagen). One microgram of DNA was used for the transformation of *Synechocystis*, and Km resistant (Km^r) clones were selected (7).

RNA isolation and Northern blotting experiments. Total RNA was isolated from *Synechocystis* by a method modified from that described in reference 21. Instead of purification by ultracentrifugation, the crude RNA extract was treated with DNase (Boehringer Mannheim) to remove contaminating DNA. After denaturation, the RNA was separated by electrophoresis on 1.3% agarose gels containing 7% formaldehyde in MOPS (morpholinepropanesulfonic acid) buffer. The RNAs were transferred overnight onto nylon membranes (Hybond-N; Amersham Buchler) by capillary transfer with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate) buffer and fixed to the filters by 3 min of UV illumination. The *stpA* encoding sequence was used as a probe after amplification by PCR with the primers, which were also used for protein overexpression experiments. The probes were labelled by random priming. Hybridization with the ^{32}P-labelled DNA probe was done overnight at 53°C in high-sodium dodecyl sulfate (SDS)-concentration buffer (7% SDS, 50% formamide, 5× SSC, 2% blocking reagent, 50 mM Na phosphate buffer (pH 7.0), 0.1% *N*-lauroylsarcosine; Boehringer Mannheim). After being washed (three times in 2× SSC, 0.1% SDS at room temperature for 20 min and one time in 0.1× SSC, 0.1% SDS at 53°C for 30 min) the filters were incubated for autoradiography at −80°C. The RNA concentration was determined by absorption measurements at 260 nm in a double-beam UV/Vis spectralphotometer (U2000; Hitachi).

Detection of GGP-P activity in extracts of *E. coli*. Plasmids harboring *stpA* and flanking sequences (see Table 1) were transformed into *E. coli* JM101. These clones were cultivated in LB medium containing only 30 mM NaCl to an optical density at 500 nm of about 0.5. The *lac* promoter (P_{lac}) was then induced by addition of IPTG (isopropyl-β-D-thiogalactopyranoside; 100 μM). The cultures were further incubated for 2 h and then divided into two parts. To one suspension NaCl was added to a final concentration of 380 mM. After 30 min of further incubation, the cells from both suspensions were harvested by centrifugation and homogenized by ultrasonic treatment with the homogenization buffer used for GGP-S measurements in *Synechocystis* (8). The crude extracts were incubated with ^{14}C-labelled GGP and the assay buffer used for GGP-S measurements at 30°C for 45 min, and the reaction products were separated by thin-layer chromatography (TLC) as described in reference 8.

Protein overexpression and antibody production. For overexpression and purification of the StpA protein the glutathione *S*-transferase (GST) gene fusion system (Pharmacia) was used. The *stpA* ORF was amplified from chromosomal DNA of *Synechocystis* by PCR with the following primers: GGCCGCGGATCC **ATG**GTATTACACCAACAACGT (StpA5′) and CCGGAATTCCTACTGGG AAAAATGGACTCTTCGGCG (StpA3′) (custom oligonucleotide synthesis; Genosys). The translational start codon (boldface letters in the StpA5′ primer) was immediately behind a *Bam*HI site used to clone the fragment in frame with the GST ORF into pGEX-4T-3 (Pharmacia). Constructs showing the correct insertion were transformed into *E. coli* BL21, and a transformant was selected. The cells were cultured at 30°C in LB medium until the suspension reached an optical density at 500 nm of 1.0. The expression of the protein was induced by addition of IPTG (100 μM) and incubation for 150 min. The proteins were extracted from *E. coli* by sonication, and the fusion protein was bound on a glutathione-Sepharose slurry (Pharmacia). The StpA protein was eluted from the matrix by cleavage by the protease thrombin, for which a cleavage site had been inserted between GST and StpA. For antibody production the protein was further purified by electroelution from SDS-polyacrylamide gels. About 10 μg of purified StpA protein was injected three times into a rabbit. Serum protein was harvested after 28 days of incubation and was used without further purification in Western blotting experiments.

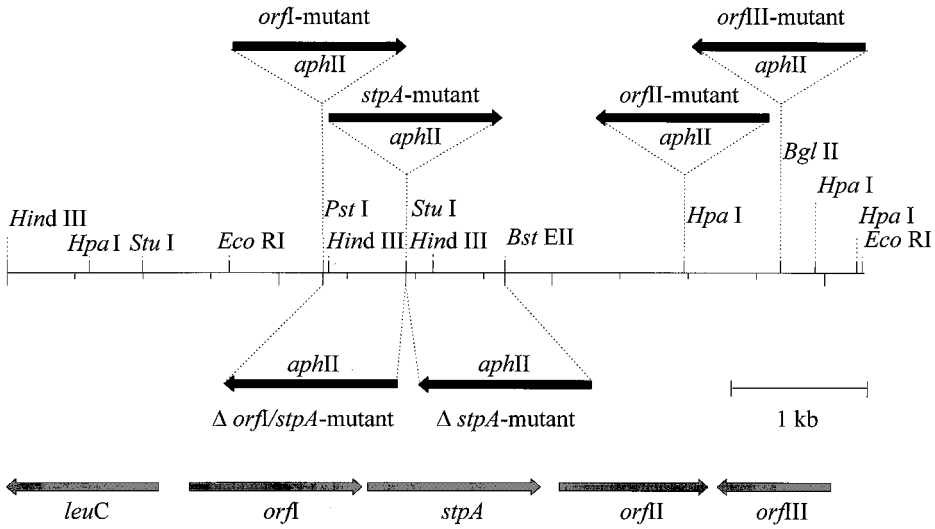

FIG. 1. Partial restriction map and protein-encoding regions (shaded arrows) of sequence U32936 (10). Integration sites of the *aph*II cartridge (black arrows) used to obtain mutants are shown above the restriction map for insertion mutants and below it for deletion mutants (see Table 1). The arrows indicate the direction of transcription.

Protein electrophoresis and Western blotting. The extracted proteins were separated in SDS gels containing 12% acrylamide or acrylamide gradients between 7.5 and 15% in the buffer system as described in reference 18. Proteins from the gels were transferred onto nylon membranes (Hybond-C extra; Amersham) with an electroblotting apparatus (Bio-Rad). The filters were incubated with the antibody specific for StpA at a dilution of 1:1,000 in phosphate-buffered saline containing 1% milk powder. Antibody binding was detected with the enhanced chemiluminescence system (Amersham). The protein content was estimated as described in reference 3.

Preparation of GGP and enzyme assays of GGP-S and GGP-P. The band containing ^{14}C-labelled GGP was scraped off from the TLC plates used for the separation of the products from GGP-S and GGP-P assays, and GGP was eluted with distilled water. Nonlabelled GGP was isolated from a salt-sensitive mutant of *Synechocystis* impaired in *stpA* function. Cells of the mutant were salt shocked with 500 mM NaCl for 6 h and then were extracted with 80% ethanol. The sugar phosphates were purified on ion exchange columns (Sephadex A20; Pharmacia). The concentration of low-molecular-mass carbohydrates was analyzed by high-pressure liquid chromatography (25). GGP was dephosphorylated by treatment with 1 U of alkaline phosphatase grade I from calf intestine (Boehringer Mannheim) in 20 μl of Na phosphate buffer (10 mM, pH 8.0) for at least 1 h at 37°C. The activities of GGP-S and GGP-P were determined in vitro with the ^{14}C-labelled substrate glycerol-3-phosphate (Amersham Buchler). The reaction products were separated by TLC as described in reference 8.

RESULTS

Construction and characterization of mutants. Recently it was shown that the mutation of *stpA* led to a salt-sensitive phenotype (20). In order to clarify the role of the StpA protein and the possible involvement of the three flanking ORFs in salt tolerance in more detail, several insertion and deletion mutants affected in these genes were constructed (Fig. 1; see Table 1 for the nomenclature of the mutants) and physiologically characterized. The *stpA* gene was mutated by integration of an *aph*II gene into the *Stu*I site present inside the ORF (*stpA* mutant). Besides this integration mutant, a deletion mutant, unable to revert by recombination, was constructed in order to assess the stability of the integration mutant. For this purpose the internal *Stu*I-*Bst*EII fragment of *stpA* was deleted and replaced by the *aph*II gene cartridge (Δ*stpA*-mutant) (Fig. 1). Several mutants were constructed in the ORFs neighboring *stpA*. *orf*I, situated upstream of *stpA* (10), was interrupted by introducing an *aph*II gene into the *Pst*I site (*orf*I mutant). A double mutant (Δ*stpA*/*orf*I mutant), in which *stpA* and *orf*I were partly deleted and replaced by an *aph*II gene (10), was also used in this study. Mutants whose mutations were in the two ORFs downstream of *stpA* were constructed by insertion of the resistance marker into the *Hpa*I (*orf*II) and the *Bgl*II site (*orf*III), yielding an *orf*II mutant and an *orf*III mutant, respectively (Fig. 1). Plasmids showing the correct integration of the *aph*II gene were transformed into *Synechocystis*, from which Km^r clones originating from homologous recombination with the chromosomal DNA were selected (Table 1). From all mutants chromosomal DNA was isolated and analyzed by DNA-DNA hybridization with the *aph*II gene and the genes used in the experiments as probes. In all cases, the *aph*II gene probe gave signals showing that it was introduced at the expected sites. The hybridizations with the gene probes showed that the mutants were completely segregated, since no signals of the size corresponding to the WT alleles could be observed (data not shown). These results indicated that the recombinations had occurred via a double-crossover event, with replacement of the WT alleles by the mutated copies.

Levels of salt tolerance of the mutants were compared to that of the WT by growing all clones on solid and in liquid media in the presence of 2 to 684 mM NaCl. The *stpA* integration and deletion mutants showed similar salt-sensitive phenotypes, with maximal tolerance reduced to less than 350 mM NaCl (Table 2). In contrast, the null mutants with *orf*I, *orf*II, or *orf*III grew as well as the WT in media containing 684 mM NaCl. The double mutant, Δ*stpA*/*orf*I, grew as well as the single *stpA*-deficient one.

The levels of the main osmoprotective compound, GG, were determined in salt-shocked cells of the WT and of the mutants. Clearly reduced levels of GG were detected in the *stpA* mutant. These levels could be enhanced about threefold by treatment of extracts from this clone with alkaline phosphatase, suggesting that these cells were capable of accumulating the intermediate GGP (Table 2). However, the sum of the amounts of GG and GGP (taken as the amount of GG detected after treatment with alkaline phosphatase) present in the *stpA* mutant reached only about one-third of the amount of GG accumulated in the WT. Both the *stpA* deletion mutant and the double *stpA*/*orf*I mutant showed behavior similar to that of the *stpA* integration mutant (data not shown). As expected from their salt-tolerant phenotypes, the *orf* mutants were able to accumu-

TABLE 2. Salt tolerance and GG concentrations in phosphatase-treated and non-phosphatase-treated ethanolic extracts from *Synechocystis* cells[a]

Strain	Growth at 684 mM NaCl	GG concn ($\mu g \cdot ml^{-1}$)[b]	
		−Phos	+Phos
WT	Normal	94.8 (100)	100.3 (105.9)
*orf*I mutant	Normal	85.8 (90.5)	83.6 (88.2)
stpA mutant	No growth	11.3 (11.9)	30.3 (32.0)
*orf*II mutant	Normal	88.3 (93.2)	92.3 (97.4)
*orf*III mutant	Normal	102.4 (108.1)	97.4 (102.8)

[a] WT and insertion mutant cells were subjected to a salt shock of 684 mM NaCl for 6 h.

[b] Values are the means of two independent experiments. The standard error of the estimations is about 8%. Values in parentheses are percentages of the non-phosphatase-treated WT GG concentrations. −phos, non-phosphatase-treated; +phos, phosphatase-treated.

late GG in amounts comparable to that of the WT, and no significant amounts of GGP could be detected in these cells.

Activities of enzymes involved in GG biosynthesis in the *stpA* mutant. In order to confirm the differential accumulation of GG and GGP in the *stpA* mutant, the activities of the enzymes involved in the GG biosynthetic pathway were measured in vitro. In extracts from control and salt-shocked (684 mM NaCl for 5 min) cells of *stpA* mutants only the intermediate GGP was synthesized. No phosphate-free GG could be detected in these enzyme assays (Fig. 2A), while after treatment of the test mixture with alkaline phosphatase GGP was hydrolyzed to GG (Fig. 2B). These data indicated that the activity of GGP-S remained intact, since significant amounts of GGP were synthesized, while GGP-P activity was absent, in agreement with the accumulation of GGP in cells of the mutant. In contrast, in extracts from the WT and the mutants, in which the *stpA* gene remained intact, mainly GG was synthesized in vitro (Fig. 2A) and only low levels of its intermediate GGP were detectable. In all cases, GGP-S showed its typical behavior of activation by NaCl in vitro since, in extracts from control cells grown in basal medium, GGP (mutants impaired in *stpA* function) and both GGP and (mainly) GG (WT and *orf* mutants) were synthesized when the tests were performed in the presence of 342 mM NaCl (not shown). The physiological traits, accumulation of GGP and absence of GGP-P activity in salt-shocked cells of the *stpA* mutants, gave evidence that this gene might encode GGP-P or a salt-dependent activator of this enzyme.

Nature of the StpA protein. In previous studies (10, 20) as well as in the present study no significant similarities of StpA to other proteins from the database had been detected. A consensus motif, though poorly conserved, involved in the catalytic activity of a number of acid phosphatases has been defined (Fig. 3) (28). The StpA sequence was used in a search for such a motif. Except for one mismatch, a homologous motif could be identified in the C-terminal part of the StpA protein. The two amino acid residues, a histidine and an aspartic acid residue, assumed to be essential for catalytic activity were conserved (Fig. 3). This finding in the sequence of StpA supported the hypothesis that *stpA* encodes the enzyme GGP-P.

The role of StpA was further analyzed after expression in *E. coli*. In the first series of experiments, plasmids harboring *stpA* and various lengths of flanking DNA cloned into pUC vector derivatives were transformed into *E. coli* JM101 cells. Protein extracts obtained from clones carrying plasmids pBP4, pGE4, and pUCPE4 (Table 1) containing the complete *stpA* gene were able to dephosphorylate GGP (Fig. 4). Extracts from cells of *E. coli* harboring control plasmid pUC19 (Fig. 4, lanes 1 and

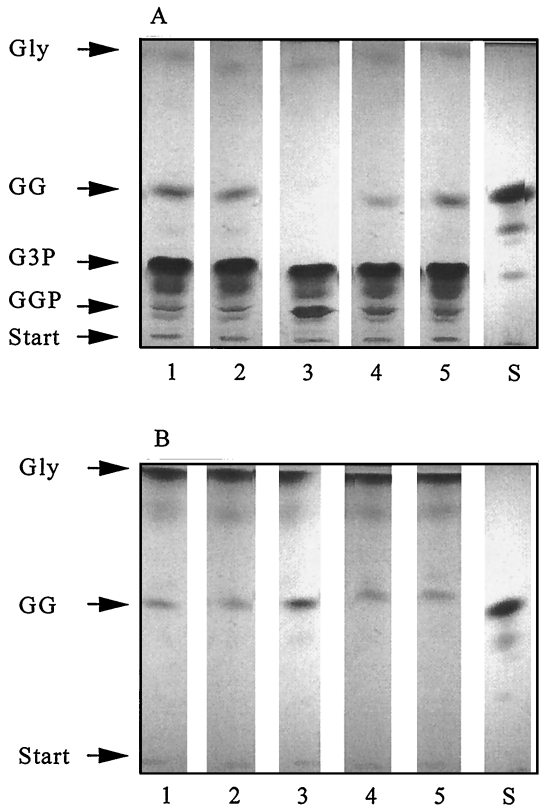

FIG. 2. Detection of GGP-S and GGP-P activities in protein extracts from WT *Synechocystis* cells (lane 1) and in those from insertion mutants. Lanes: 1, WT; 2, *orf*I mutant; 3, *stpA* mutant; 4, *orf*II mutant; 5, *orf*III mutant; S, standard (radiolabelled GG isolated from salt-adapted WT cells). Cells were shocked for 5 min with 684 mM NaCl. All tests were performed in homogenization and assay buffers containing 342 mM NaCl. TLC separation of radiolabelled products before (A) and after (B) phosphatase treatment are shown. The positions of the labelled substances are marked by arrows. Abbreviations: Gly, glycerol; G3P, glycerol-3-phosphate.

2) or plasmids carrying cyanobacterial fragments with an incomplete *stpA* gene or with no *stpA* gene (data not shown) were not able to hydrolyze GGP. Interestingly, in contrast to the situation for *Synechocystis*, GGP-P activity was found to be independent of the NaCl concentration in the growth medium and the extract buffer. Extracts from cells grown both in high-NaCl- and in low-NaCl-containing cultures showed active GGP-P (data not shown). Enzyme activities were detected at similar levels whether the assays were performed in the presence or absence of NaCl (Fig. 4).

The previous experiments in which StpA was expressed appear in *E. coli* made it very promising that this protein could be overexpressed in a heterologous host to obtain a large amount of protein for antibody production and biochemical character-

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motif of acid phosphatases:  L x L x x S H D S x L x x L x x S
                             I   I     T     T   I     I     T
                             V   V     A     A   V     V     A
                             M   M     G     N   M     M
                                 F                     F
                                 A                     Y

motif found in StpA:     396-V-A-F-P-G-G-H-D-Q-Y-V-A-A-F-K-Q-A-412
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FIG. 3. Identification of a motif characteristic of acid phosphatases (28) in the sequence of StpA with the MOTIF software package. Amino acid residues conserved in StpA are in boldface.

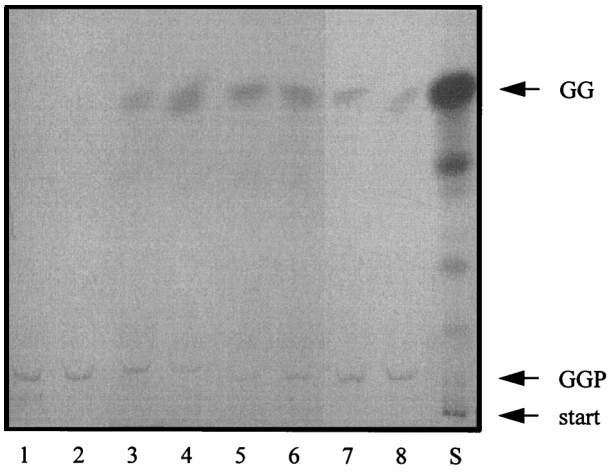

FIG. 4. Detection of GGP-P activity in protein extracts from cells of *E. coli* JM101. The strain carried the following plasmids: pUC19 (lanes 1 and 2), pUCPE1 (*stpA*$^+$) (lanes 3 and 4), pGE4 (*stpA*$^+$) (lanes 5 and 6), and pBP4 (stpA$^+$) (lanes 7 and 8). Lane S, standard, radiolabelled GG. TLC separations of the radiolabelled reaction products are shown. The tests were performed using NaCl-free buffers (lanes 1, 3, 5, 7) or buffers containing 342 mM NaCl (lanes 2, 4, 6, 8) for homogenization and assay. The positions of the labelled substances are marked by arrows.

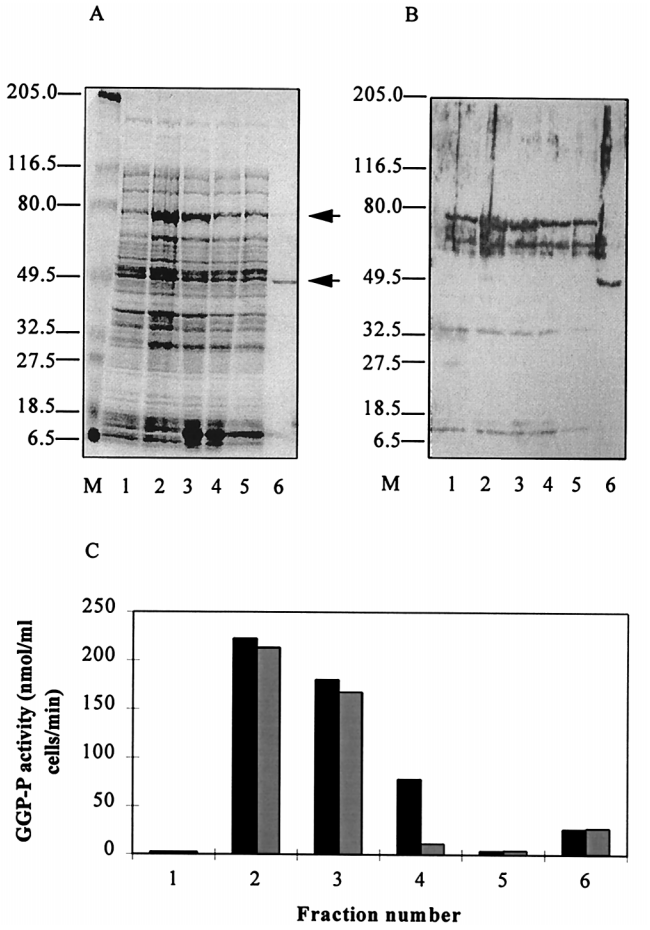

FIG. 5. Overexpression of StpA in *E. coli* BL21 using the GST gene fusion system (Pharmacia). The results of Coomassie staining (A) of total proteins and detection of StpA with specific antibodies (B) after separation by SDS-polyacrylamide gel electrophoresis are shown. Lanes: 1, extracts from *E. coli* cells before induction by IPTG; 2, extracts from *E. coli* cells after induction by IPTG; 3, crude extract after sonication; 4, crude extract after centrifugation at 25,000 × *g* for 20 min; 5, crude extract after treatment with glutathione-Sepharose and centrifugation; 6, isolated StpA after thrombin cleavage. M, prestained broad-range marker (Bio-Rad). The positions of StpA and the GST-StpA fusion protein are marked by arrows. (C) GGP-P activity in the fractions obtained during StpA overexpression. The results of assays performed under NaCl-free conditions are indicated by black bars; those of assays with 342 mM NaCl are indicated by shaded bars. The numbers on the horizontal axis correspond to the lane numbers.

ization. Overexpression of StpA was achieved by cloning the *stpA*-encoding sequence into the pGEX plasmid (Pharmacia) and transferring the recombinant vector into *E. coli* BL21, from which the protein was purified. Large amounts of a fusion protein of the expected size, about 72 kDa, corresponding to GST (26 kDa) and StpA (46 kDa), were obtained. The StpA protein was separated from GST by thrombin cleavage (Fig. 5A). The fractions obtained during overexpression and purification were tested for GGP-P activity by an HPLC-based nonradiometric method. High levels of GGP-P activity were detected in crude extracts of *E. coli* cells containing the fusion protein after induction by IPTG (Fig. 5C). Selective binding of the fusion protein to GST-Sepharose and thrombin cleavage drastically reduced the level of enzyme activity. The relatively low level of GGP-P activity of the purified enzyme is mainly due to little recovery of the cleaved protein. Nevertheless, the amount recovered was sufficient for further analyses. As observed with the enzymes produced in *E. coli* JM101, the protein showed the expected enzyme activity at all levels of purification regardless of whether or not the testing solutions contained NaCl. The StpA protein was further purified by SDS gel electrophoresis and electroelution of the 46-kDa protein band and used for antibody production. When used with samples from the various steps of the overexpression process, the antibodies specifically recognized the purified protein as well as the fusion protein (Fig. 5B). Controls run with anti-GST antibodies (Pharmacia) reacted with the fusion protein and additional bands of lower molecular weight, revealing a degree of degradation (data not shown).

Regulation of expression of StpA in *Synechocystis* sp. strain PCC 6803. The dependence on salt treatment of the expression of the *stpA* gene was analyzed for the *Synechocystis* host. In Northern blots for which the complete ORF of the *stpA* gene obtained by PCR was used as a probe, a very weak signal could be obtained in RNA extracts from control cells (Fig. 6A). The relative level of *stpA*-specific mRNA increased with time after transfer of the cells to a medium containing 684 mM NaCl (Fig. 6A). By 20 min after the transfer a significantly enhanced hybridization signal was detectable. The intensity of the signal increased continuously up to 3 h and thereafter declined. A main band of about 1.4 kb was found. This is about the size of the *stpA* gene and indicates monocistronic RNA, but the accumulation of a stable breakdown product of a larger mRNA cannot ruled out. At the times of maximum expression, a band of higher molecular weight was also visible. In RNA extracts obtained from cells completely adapted to salt concentrations of at least 171 mM, the amount of *stpA*-specific mRNA was clearly enhanced compared to that in extracts from control cells (Fig. 6B). In contrast, in RNAs extracted from heat-shocked cells, the signal remained at the low intensity observed in control cells (data not shown).

The amount of the StpA protein was measured by Western blotting in protein extracts from salt-treated and control cells. Relatively large amounts of protein (150 μg) had to be applied to the gels to obtain a weak signal in *Synechocystis* extracts. The protein specifically recognized by the antibody showed a mo-

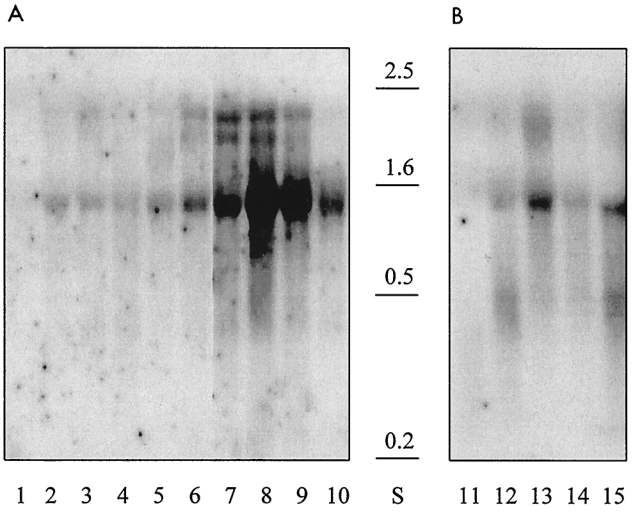

FIG. 6. Salt-dependent expression of *stpA* in WT *Synechocystis*. mRNAs were detected by Northern blotting. Total RNA extracts were from cells shocked by 684 mM NaCl (A) and from salt-adapted cells (B). The ^{32}P-labelled PCR fragment of the whole ORF of *stpA* was used as a probe. Fifteen micrograms of total RNA was applied to each lane. Panel A lanes show *stpA* expression in extracts from control cells (lane 1) or from cells shocked with 684 mM NaCl for the following times: 20 min (lane 2), 40 min (lane 3), 60 min (lane 4), 80 min (lane 5), 100 min (lane 6), 2 h (lane 7), 3 h (lane 8), 5 h (lane 9), 7 h (lane 10). Panel B shows results for control cells (lane 11), cells adapted to 171 mM NaCl (lane 12), cells adapted to 342 mM NaCl (lane 13), cells adapted to 513 mM NaCl (lane 14), and cells adapted to 684 mM NaCl (lane 15). S, molecular mass standard.

lecular mass of about 48 kDa, which corresponds well to the expected size of 46 kDa (data not shown). No antibody binding at this molecular mass was found in protein extracts from the *stpA* mutant. An increase in the amount of StpA protein was observed in protein extracts of WT cells adapted to higher salt concentrations, while in cells grown in basal medium (2 mM) only a very faint band was detectable (data not shown).

DISCUSSION

Mutants defective in the synthesis of osmoprotective compounds and/or in energization of the extrusion of Na^+ cannot counterbalance the accumulation of high intracellular ion concentrations, which are toxic for cellular metabolism. The high degree of salt sensitivity of the *stpA* mutants is associated with a pleiotropic phenotype, an observation which had led to the hypothesis that StpA might be a regulator acting early in the response to high salinity (20). It is shown here that the ORFs neighboring *stpA* are not involved in GG synthesis or in any step leading to salt tolerance, since mutants in which these genes were specifically inactivated displayed behavior under high-salt conditions identical to that of the WT. This is also true for *orf*I, which shows similarities to bacterial response regulators. It had previously been speculated that the product of this gene might be involved in the regulation of GG synthesis (10).

The present results show that after salt shock, the *stpA* mutant mainly accumulates GGP (Table 2), the nonosmoprotective intermediate of GG synthesis, instead of GG. This has been confirmed by the demonstration of the presence of only GGP-S activity in the protein extracts from this mutant. However, the necessity for activation of GGP-S by NaCl in in vitro assays, observed for WT cells (8, 9), remained. The enzymatic function of the StpA protein was also directly established after expression of *stpA* in *E. coli*, in which it was possible to detect GGP-P activity. Thus, genetic, biochemical, and physiological approaches all give clear evidence that *stpA* encodes GGP-P.

Only a very weak level of similarity was found in a comparison of the amino acid sequences of StpA and functionally related enzymes. This relatively large divergence from other phosphatases could be the basis for the high degree of specificity of GGP-P for its substrate GGP. Dephosphorylation of GGP by extracts of *E. coli* cells was possible only when *stpA* was present and expressed, indicating that no other phosphatase activity present in the heterologous host was able to hydrolyze GGP. The same high degree of specificity seems to be characteristic of trehalose-phosphate phosphatase in *E. coli*. In this case also, the enzyme is activated only in salt-stressed cells (6). The substrate of the enzyme, trehalose-phosphate, has been postulated to act as a positive regulator for the genes involved in trehalose catabolism, while these genes are repressed under high-osmolarity conditions (16), establishing the exclusive function of these two metabolic pathways.

GGP-P could be activated by NaCl in vivo and in vitro in cells and protein extracts of *Synechocystis* sp. strain PCC 6803 grown in basal medium where GG synthesis is absent (8, 9), indicating salt regulation of GGP-P. In contrast, the protein overexpressed and purified from *E. coli* was also able to dephosphorylate GGP in the absence of NaCl. The permanent activity of the GGP-P isolated from *E. coli* indicates that the heterologous host lacks a regulatory factor which inactivates GGP-P under low-salt conditions in *Synechocystis*. In agreement with the molecular mass deduced from the length of the *stpA* ORF, the molecular mass of the protein obtained after expression of the gene in *E. coli* was estimated to be 46 kDa. In *Synechocystis* extracts a protein of similar size cross-reacted with the antibody specific for StpA, while in extracts of the mutants no specific binding could be observed. Functionally related enzyme OtsB from *E. coli* has a molecular mass of only 29.1 kDa (15). Surprisingly, the GST-StpA fusion protein was also enzymatically active, since *E. coli* extracts tested before thrombin cleavage displayed GGP-P activity. The smaller protein bands, which were recognized by the antibodies specific for StpA (Fig. 5) in the total protein extracts obtained after overexpression in *E. coli*, could represent cleavage products also observed by using antibodies specific for GST.

The synthesis of GGP-P is also regulated at the transcriptional level. In cells cultured at low salt concentrations only a very low level of *stpA*-specific RNA could be detected (Fig. 6). This mRNA should be sufficient to synthesize the small amounts of the StpA protein detected in non-salt-adapted cells. The proportion of this RNA increased quickly in salt-stressed cells (684 mM NaCl) and reached a maximum around 3 h after the salt shock, while the amount of StpA protein was significantly enhanced only after 5 h. The enhanced mRNA levels found in the salt-shocked cells could reflect increased transcription of the *stpA* gene under high-salt conditions. Nevertheless, stabilization or reduced degradation of the *stpA* mRNA cannot be ruled out. Higher *stpA*-specific mRNA levels are also characteristic of completely salt-adapted cells of *Synechocystis*, corresponding well to the increase in StpA protein content and to the higher GGP-P activities in those cells (9). The enzymes involved in osmotically regulated trehalose synthesis in *E. coli* showed comparable features. Besides regulation of their activities by salt concentration (6), increased transcription was detected by promoter fusions with *lacZ* as the reporter gene. The enhanced transcription seems to be controlled by alternative sigma factor RpoS, which is also involved in stationary-phase regulation (11, 14). The regulatory mechanisms responsible for the salt-induced increase of *stpA*-specific mRNA and the basis for the occurrence of transcripts

of higher molecular weight will be analyzed in the future for *Synechocystis*.

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