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A prokaryotic sucrose synthase gene (*susA*) isolated from a filamentous nitrogen-fixing cyanobacterium encodes a protein similar to those of plants

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Abstract. Sucrose synthase (SS), a key enzyme in plant carbohydrate metabolism, has recently been isolated from *Anabaena* sp. strain PCC 7119, and biochemically characterized; two forms (SS-I and SS-II) were detected (Porchia et al. 1999, Planta 210: 34–40). The present study describes the first isolation and characterization of a prokaryotic SS gene, *susA*, encoding SS-II from that strain of *Anabaena*. A 7 kbp DNA fragment containing an open reading frame (EMBL accession number AJ010639) with about 30–40% amino acid identity with plant SSs was isolated from an *Anabaena* subgenomic library. The putative SS gene was demonstrated to encode an SS protein by expression in *Escherichia coli*. The biochemical properties of the recombinant enzyme were identical to those of the enzyme purified from the cyanobacterial cells. The deduced amino acid sequence of the *Anabaena* SS diverged from every plant SS reported. The occurrence of SS in cyanobacteria of different taxonomic groups was investigated. The enzyme occurs in several filamentous nitrogen-fixing cyanobacteria but not in two species of unicellular, non-diazotrophic cyanobacteria.

Key words: *Anabaena* sp. (sucrose metabolism) – Cyanobacterium – Sucrose metabolism – Sucrose synthase

Introduction

Sucrose metabolism in higher plants has been studied extensively. Sucrose synthase (SS, UDP-glucose: D-fructose 2-α-D-glucosyl transferase, EC 2.4.1.13), an enzyme that catalyzes the synthesis and cleavage of sucrose, and sucrose-phosphate synthase (SPS, UDP-glucose: D-fructose-6-phosphate 2-α-D-glucosyl transferase, EC 2.4.1.14), key enzyme in the control of sucrose synthesis, are widespread in the plant kingdom (Pontis 1977; Avigad 1982). In particular, SS plays an important role in plant metabolism by providing sucrose for different pathways that are related to metabolic, structural and storage functions (Black et al. 1995; Chourey and Miller 1995). Sucrose synthase is also associated with long-distance sucrose transport, where it participates in phloem loading and unloading (Geigenberger and Stitt 1993; Martin et al. 1993; Nolte and Koch 1993; Déjardin et al. 1997; Wittich and Vreugdenhil 1998).

Much less is known about sucrose metabolism in microorganisms. Sucrose synthase and SPS have been reported to be present in several species of green algae, and were characterized biochemically (Salerno et al. 1995). The biosynthesis of sucrose through the action of SPS and sucrose-phosphate phosphatase was also shown in the filamentous, heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7119 (Porchia and Salerno 1996). A prokaryotic gene (*spsA*) encoding SPS was first identified and characterized in *Synechocystis* sp. strain PCC 6803 (Curatti et al. 1998). The occurrence of SS activity in prokaryotic organisms was reported in *Anabaena variabilis* (Schilling and Ehrnsperger 1985) and in *Anabaena* sp. strain PCC 7119 (Salerno et al. 1995). A prokaryotic SS was first isolated and biochemically characterized from *Anabaena* sp. PCC 7119 (Porchia et al. 1999). Two cyanobacterium isoforms of SS (SS-I and SS-II) were detected. Sucrose synthase II was purified; its biochemical properties showed striking differences from those of plant enzymes with respect to substrate affinities, regulation by metal ions and ATP, and the amino-acid sequence of the N-terminal region.

The present study reports the first isolation and characterization of a prokaryotic SS gene (*susA*), encoding the SS-II protein of *Anabaena* sp. strain PCC 7119. A comparison of the deduced amino acid sequence of *Anabaena* and plant SSs shows that the prokaryotic

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Abbreviations: Fru = fructose; Glc = glucose; PCR = polymerase chain reaction; SS = sucrose synthase; SPS = sucrose-phosphate synthase

enzyme clearly diverges from every plant SS reported. The presence of SS in cyanobacteria of different taxonomic groups was also investigated.

Materials and methods

Culture conditions

Cells of the cyanobacteria *Nostoc* sp. strain PCC 6719, *Anabaena* sp. strains PCC 7118, 7119 and PCC 7120, *Anabaena variabilis* strain ATCC 29413, *Calothrix* sp. strain PCC 7601, *Synechocystis* sp. strain PCC 6803, and *Synechococcus* strain PCC 7942, were grown in BG-11 liquid medium (Rippka et al. 1979), bubbled with air and illuminated by fluorescent lamps, and were harvested as described by Porchia et al. (1999). Cells of *Escherichia coli* strain DH5α bearing pSS7 or pBluescript SK (+) were grown in Luria-Bertani medium supplemented with 50 μg/ml carbenicillin.

Amplification of genomic DNA by polymerase chain reaction (PCR)

High-molecular-mass genomic DNA was isolated from *Anabaena* sp. strain PCC 7119 cells as described by Cai and Wolk (1990). Genomic DNA (0.1 μg) was used in a 25-μL PCR amplification reaction mixture containing 20 pmol of the following primers: forward primer (5′-GARYTNATGCARGCNATHYTNGA-3′) that corresponds to the first 8 amino acids of the *Anabaena* SS-II polypeptide (Porchia et al. 1999), and the reverse primer (5′-ACNACYTGNCCNCCNGTRTCNGG-3′) that corresponds to a conserved region of plant SSs (PDTGGQVV). The PCR conditions were 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 53 °C for 1 min and 72 °C for 1 min, and a single step of 72 °C for 3 min, using a PTC-100 thermal cycler (Model-96 V; MJ Research, Mass., USA). The PCR products were cloned in the pCRII vector (Invitrogen).

Southern blot analysis

High-molecular-mass DNA (10 μg) isolated from *Anabaena* cells was digested with different restriction endonucleases. After electrophoresis in a 0.7% agarose gel, the digested DNA fragments were transferred onto a nylon membrane (Amersham) by a standard method (Sambrook et al. 1989). Filters were prehybridized at 65 °C for 2 h in SDS buffer (0.5 M sodium phosphate buffer, pH 7.5; 7% SDS; 5 mM EDTA; 1% bovine serum albumin), and then hybridized for 12–16 h at 65 °C (high-stringency conditions) or at 55 °C (low-stringency conditions) in the same solution containing the probe (a 0.9-kbp PCR product showing similarity to plant SSs) which had been ^{32}P-labeled by random priming (DuPont, NEN). Filters were washed with 1 × saline sodium citrate buffer (SSC; 1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate) and 0.1% SDS, and then three times for 20 min in 0.1 × SSC and 0.1% SDS at 65 °C (high stringency), or with 2 × SSC and 0.1% SDS, and then three times for 20 min in 1 × SSC and 0.1% SDS at 55 °C (low stringency).

Isolation of genomic clones

Two *Anabaena* subgenomic libraries were constructed: (i) from DNA digested with *Eco*RI and size-fractionated from 5.5 to 6.5 kbp, and (ii) from DNA digested with *Spe*I and size-fractionated from 6.5 to 7.5 kbp. The screening of the libraries was performed by colony hybridization (Sambrook et al. 1989) of about 800 independent transformants in each case. Prehybridization, hybridization and washings were carried out as described above for high-stringency Southern blot hybridization.

Sequencing of DNA

Sequencing of double-stranded DNA inserts was achieved by the dideoxy sequencing method (Sambrook et al. 1989) and Sequenase kit (UBS Corp., Cleveland, Ohio, USA). Sequences were determined in both directions by further subcloning appropriate restriction fragments into pBluescript SK(+) vector and by using specific primers. The DNA sequences were compiled and analyzed using the BLASTp protocol (Altschul et al. 1990) and the Clustal W method, running the Megalign program of the DNAStar package with the PAM 250 residue weight table. Comparisons were performed with data obtained from GenBank and EMBL databases.

Expression in Escherichia coli and enzyme purification

A 7-kbp DNA fragment bearing a 2418-bp open reading frame showing similarity to plant SSs was cloned (pSS7) in pBluescript SK (+) (Stratagene) and used to transform *E. coli* strain DH5α.

Overnight cultures of *E. coli* bearing pSS7 or pBluescript SK (+) were harvested by centrifugation at 2500 *g* for 15 min. Cells were resuspended in five times their packed volume with 100 mM Hepes-NaOH buffer (pH 7.5) containing 2 mM EDTA, 20 mM $MgCl_2$, 2% ethyleneglycol, 50 μM phenylmethylsulfonylfluoride, 20 mM 2-mercaptoethanol and 20% glycerol, and broken by passage through a French press at 700 psi. Cell debris was removed by centrifugation at 27,000 *g* for 30 min at 4 °C. The supernatant, referred to as crude extract, was chromatographed through a DEAE-Sephacel column as previously described (Porchia et al. 1999).

Enzyme assays

Sucrose synthase activity in the direction of sucrose synthesis and in the reverse direction was measured as previously reported (Porchia et al 1999). Crude extracts prepared from different cyanobacterial cells were desalted through a Sephadex G-50 column before assay of enzymatic activity. Sucrose-phosphate synthase activity was determined according to Porchia and Salerno (1996).

Results

Isolation of a putative SS gene from Anabaena

The previously reported N-terminal amino acid sequence of SS from *Anabaena* sp. strain PCC 7119 (SELMQAILDS) (Porchia et al. 1999) and a conserved region of plant SSs (DTGGQVVY) were used to design degenerate oligonucleotides for SS gene cloning by PCR methodology. An amplification fragment of 900 bp was cloned and sequenced. The PCR product bore an open reading frame encoding 300 amino acids which showed about 20–25% identity with the deduced amino acid sequences reported for plant SSs (data not shown).

The *Anabaena* genomic blot was probed with the 900-bp PCR product. As shown in Fig. 1, only one major DNA fragment was detected in each lane, suggesting the presence of a single-copy gene homologous to plant SSs in the *Anabaena* genome.

To clone the full-length gene, a subgenomic library constructed from total DNA digested with *Eco*RI was probed with the cloned 900-bp fragment. Restriction

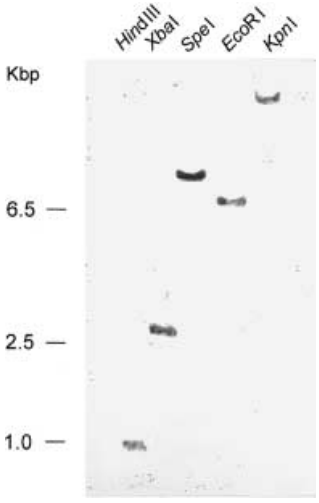

Fig. 1. Southern blot analysis of *Anabaena* genomic DNA. Blot of total genomic DNA (10 μg per lane) digested with the indicated restriction enzymes was hybridized with the 900-bp PCR fragment and washed under high-stringency conditions. The positions of the size standards are indicated on the left (in kbp)

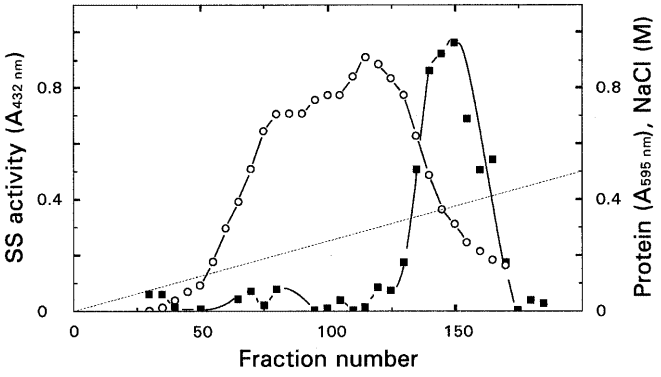

Fig. 2. Diethylaminoethyl-Sephacel chromatography of crude extracts from *E. coli* expressing *Anabaena* SS. ■–■, SS activity; ○–○, protein. The *dotted line* represents the NaCl gradient

Table 1. Kinetic parameters of the *Anabaena* SS protein purified from *Escherichia coli*

Substrate	K_m [a] (mM)	V_{max} (nkat ml^{-1})	V_{max}/K_m (nkat ml^{-1} mM^{-1})
Fru (+ UDP-Glc)	58 (52)	2	0.03
Fru (+ ADP-Glc)	4.4 (4.2)	1.6	2.75
UDP-Glc	2.2 (2.7)	1.78	0.8
ADP-Glc	0.9 (1.3)	1.56	1.73
Sucrose (+ UDP)	270 (303)	2.8	0.01
Sucrose (+ ADP)	220 (305)	2.6	0.01
UDP	0.8 (1.25)	2.8	3.5
ADP	1.0 (1.15)	2.0	2.0

[a] The K_m values in parentheses correspond to the kinetic parameters for SS-II from *Anabaena* sp. strain PCC 7119 (Porchia et al. 1999)

and partial sequence analysis of eight positive clones showed that they bore identical 6-kbp DNA fragments. After comparison with the deduced amino acid sequences of plant SSs and by considering the subunit size (92 kDa) reported for *Anabaena* SS-II (Porchia et al. 1999), it was concluded that the coding sequence lacked approximately 1,500 bp of the C-terminal portion of the putative *Anabaena* SS gene (data not shown). Therefore, a second subgenomic library was constructed from DNA digested with *Spe*I and size-fractionated from 6.5 to 7.5 kbp. As a result of the screening, three identical 7-kbp DNA fragments were cloned and sequenced, indicating the presence of a 2,418-bp open reading frame (2,418-bp *orf*) that was registered as EMBL accession number AJ010639.

Identification of the Anabaena SS-II gene

To ascertain if the 2,418-bp *orf* encodes a protein with SS activity, we constructed plasmid pSS7 containing the putative SS *orf* flanked by 2 kbp on each side. Extracts of *E. coli* bearing pSS7 harvested in late exponential phase showed an SS activity of 92 pkat (mg protein)$^{-1}$ (in the sucrose-cleavage direction) and 48 pkat (mg protein)$^{-1}$ (in the sucrose-synthesis direction). Under similar conditions, *E. coli* bearing pBluescript II SK(+) had no SS activity. We conclude that the 2,418-bp *orf* is an SS gene (*susA*).

As two SS isoforms were reported in *Anabaena* (Porchia et al. 1999), the protein encoded by *susA* was identified by chromatographing crude extracts of *E. coli* bearing pSS7 on a DEAE Sephacel column. A peak of SS activity eluted at a similar salt concentration (Fig. 2) as did activity of *Anabaena* SS-II (Porchia et al. 1999). In addition, the kinetic parameters for the enzyme partially purified from *E. coli* (Table 1) were similar to those reported for the enzyme isolated from cells of *Anabaena* (Porchia et al. 1999).

Sequence analysis of Anabaena SS protein

Anabaena susA gene encodes an 806-amino-acid polypeptide with a predicted M_r of 94,101 Da. This value is similar to the M_r reported for the SS-II polypeptide (Porchia et al. 1999). A dendogram generated by multiple alignment, using the Clustal method, shows that the *Anabaena* SS-II polypeptide clearly diverges from plant SSs (Fig. 3). As shown in Fig. 4, the most divergent portion corresponds to the N-terminal region. Sequences highly conserved during evolution are located principally in the central region of the polypeptide (Fig. 4). The amino acid identity between SS-II and plant SSs ranges between 30 and 40%.

Occurrence of SS in other cyanobacteria

The presence of *susA* homologous sequences in cyanobacteria from different taxonomic groups (Rippka et al. 1979) was analysed by Southern blot hybridization

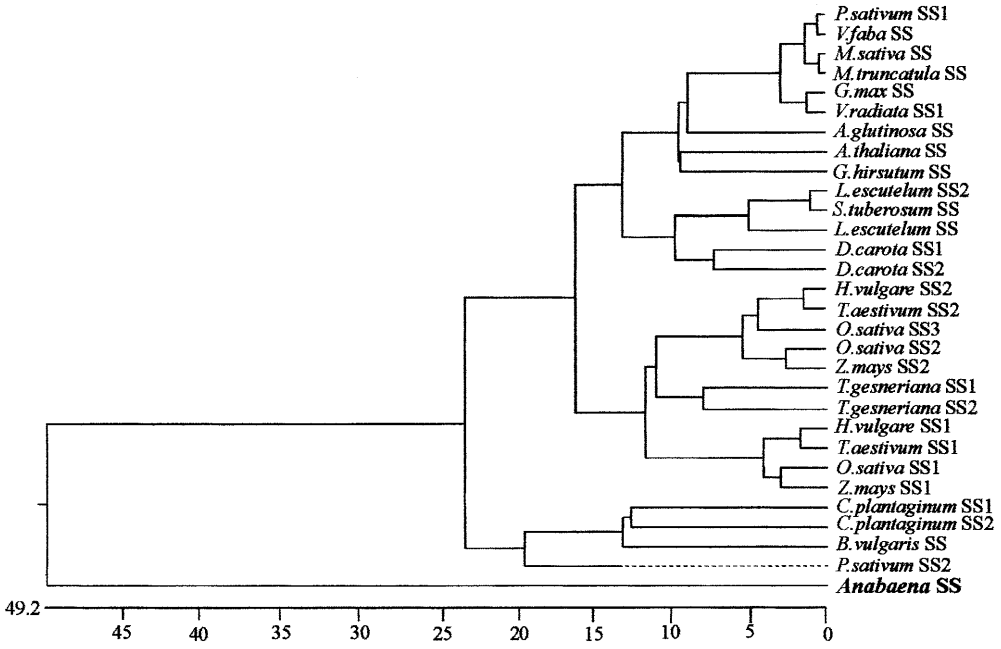

Fig. 3. Phylogenetic tree of SSs. Predicted SS sequences were obtained from the GenBank database. Accession numbers from top to base: AJ012080, Z56278, AF049487, AJ131943, AF030231, D10266, X92378, X70990, U73588, AJ011319, U24087, Y16090, Y16091, X69931, AJ000153, L03366, L33244, X96938, X96939, X65871, AJ001117, X64770, X02400, AJ131999, AJ132000, X81974, AJ001071, AJ010639 (*Anabaena* sp. PCC 7119). The tree was generated with the program Megalign of the DNAStar package, using the Clustal method with the PAM 250 residue weight table

(Fig. 5). Homologous DNA fragments are present in representative cyanobacteria of the taxonomic group IV, containing filamentous nitrogen-fixing cyanobacteria.

In addition, SS activity and SPS activity were measured in crude extracts of different representative cyanobacteria (Fig. 6). Sucrose synthase activity was detected in filamentous cyanobacteria that have the capacity for aerobic fixation of dinitrogen, but not in two species of unicellular, non-diazotrophic cyanobacteria. In contrast, SPS activity could be detected in all extracts.

Discussion

We have characterized a prokaryotic SS gene (*susA*) from *Anabaena* sp. strain PCC 7119, a filamentous nitrogen-fixing cyanobacterium. Results presented herein contribute to the knowledge of the structure and evolution of one of the key enzymes in carbohydrate metabolism in photosynthetic organisms, and will become a base of future studies of the evolution and the structural and functional properties of sucrose synthase, and physiological features of the pathway of sucrose metabolism.

Anabaena SS-II is encoded by the susA gene

A DNA fragment (2,418-bp *orf*) encoding a protein homologous to plant SSs was isolated by use of the N-terminal amino acid sequence of *Anabaena* SS-II (Porchia et al. 1999) and a conserved region of plant SSs. The expression in *E. coli* of the 2,418-bp *orf* produced a fully active SS protein whose kinetic and molecular characteristics were similar to those of SS-II purified from cells of *Anabaena* (Fig. 2; Table 1; Porchia et al. 1999). Thus, the 2,418-bp *orf* is the coding region of the *Anabaena* SS-II gene (*susA*). The *susA* gene encodes an 806-amino-acid polypeptide with a predicted M_r of about 94 kDa, slightly larger than most plant SS polypeptides (Su and Preiss 1978; Chourey 1981). The difference between plant and *Anabaena* SS-II in the affinity for fructose (Fru) in the presence of UDP-glucose (UDP-Glc) or ADP-Glc (Porchia et al. 1999) was also characteristic of the protein expressed in *E. coli*. The K_m for Fru (4.4 mM) in the presence of ADP-Glc was about 13-fold lower than in the presence of UDP-Glc, and the K_m for ADP-Glc (0.9 mM) was about 2-fold lower than that of UDP-Glc (Table 1). The K_m values for the other substrates (in the direction of sucrose cleavage) are similar to those reported for plant enzymes (Pontis 1977; Avigad 1982).

Anabaena SS-II clearly diverges from plant SSs

In a previous report, an important difference between *Anabaena* and eukaryotic SSs was shown by the amino acid sequence of the first 16 residues (Porchia et al. 1999). The present results demonstrate that the divergent region extends to approximately the first 100 amino acids (Fig. 4). Interestingly, this portion of SS is fairly conserved among plant SSs and contains a serine (Ser) residue that has been proven to be phosphorylated in maize (Ser^{15}; Huber et al. 1996) and in mung bean (Ser^{11}; Nakai et al. 1998). Plant SSs were reported to occur both as a soluble and a membrane-associated enzyme (Amor et al. 1995; Chourey and Miller 1995; Winter et al. 1997). Phosphorylation of SS was suggested to be involved in the release of the membrane-bound SS form, postulated as being part of a complex with β-glucan synthases (Amor et al. 1995). Because the N-terminal region is completely different in *Anabaena* SS-II with respect to plant SSs (Fig. 4, box 1), it could be speculated that either the enzyme would not play a

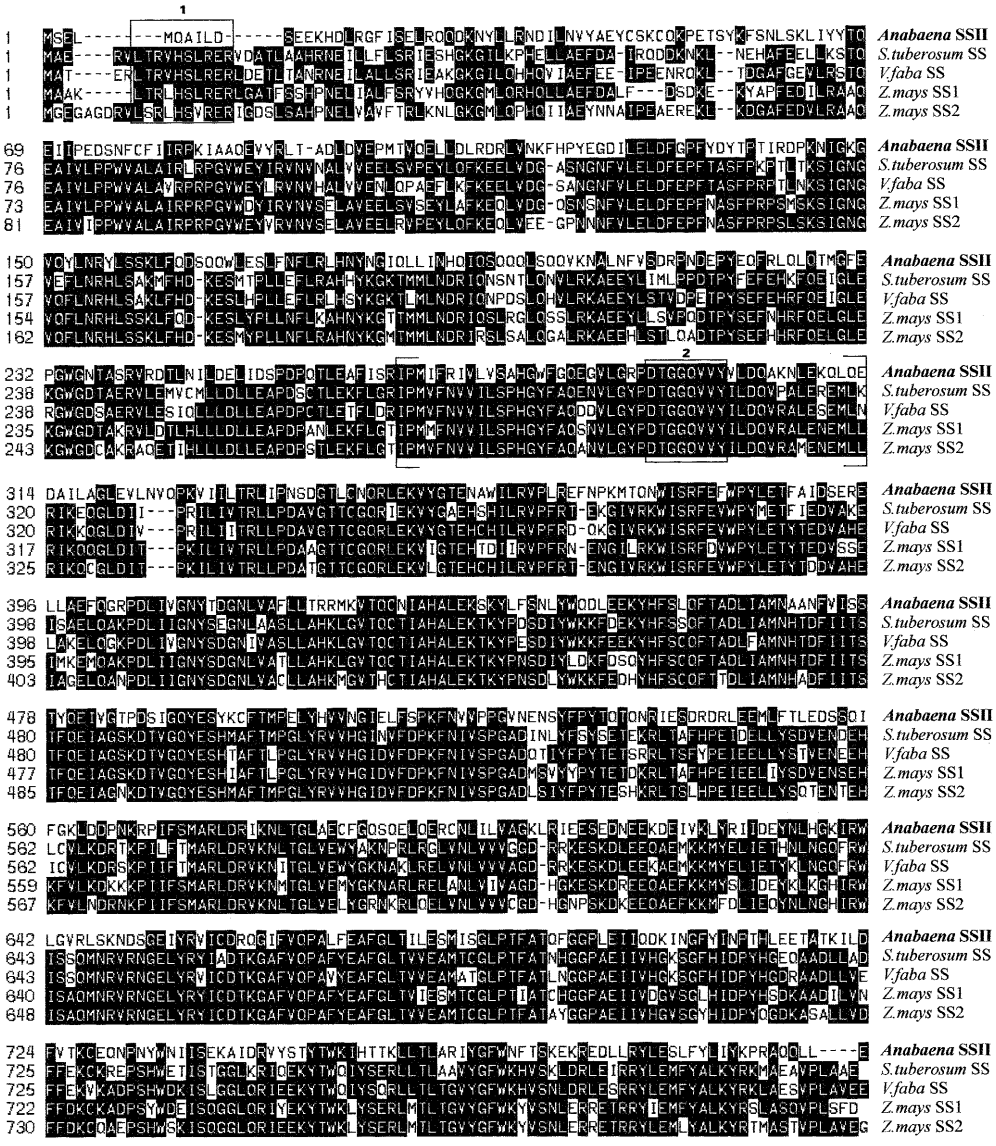

Fig. 4. Multiple alignment of representative SSs. The deduced sequences from *Anabaena* sp. PCC 7119 (residues 1–801; AJ010639), *Solanum tuberosum* SS1 (U24088), *Vicia faba* (Z56278), *Zea mays* SS1 (X02400) and *Z. mays* SS2 (L33244) were selected according to the phylogenetic clusters observed in Fig. 3. The alignment was generated with the program Megalign of the DNAStar package, using the Clustal method with the PAM 250 residue weight table

similar role in *Anabaena* or it is regulated by a different mechanism.

Conversely, *Anabaena* SS-II protein contains a highly conserved section (residues 292–299) in plant SSs and SPSs (Fig. 4, box 2). In this stretch of eight amino acids, the only difference between SS and SPS is the penultimate position: a species-invariant lysine is present in SPSs, and a valine in SSs. It was speculated that the occurrence of a non-charged residue at that position in SSs may be one of the factors influencing the selectivity for Fru rather than fructose-6-phosphate as the glycosyl acceptor (Salvucci et al. 1995). Additional support for this hypothesis is provided by the analysis of the first two reported sucrose-enzyme sequences from prokaryotic organisms. Both *Synechocystis* SPS (Curatti et al. 1998) and *Anabaena* SS-II (present report) fit with the presence of lysine or valine and substrate specificity prediction (fructose-6-phosphate or Fru). Further analysis of the adjacent region (residues 267–313; Fig. 4, box 2) indicates that while it is highly conserved in plant SSs (ca. 100%), it shares only about 50% identity with *Anabaena* SS-II protein. It is tempting to speculate that this sequence divergence may be related to the striking difference in Fru affinity of SS-II in the presence of UDP-Glc and ADP-Glc (Table 1).

Sucrose synthase is present in filamentous cyanobacteria

Sucrose synthase activity has been shown in N_2-grown *A. variabilis*, where it was present predominately in vegetative cells (Schilling and Ehrnsperger 1985), and in *Anabaena* sp. strain PCC 7119 (Salerno et al. 1995; Porchia et al. 1999). In this report the occurrence of *susA* homologous sequences and SS activity is also demonstrated in other filamentous cyanobacteria that have the capacity for aerobic fixation of dinitrogen (taxonomic group IV). However, we find no evidence for the presence of SS in two representative strains of unicellular cyanobacteria (group I; Figs. 5, 6]. Moreover, the highly conserved sequence PDTGGQVV in all reported SSs (Fig. 4) could not be detected in the

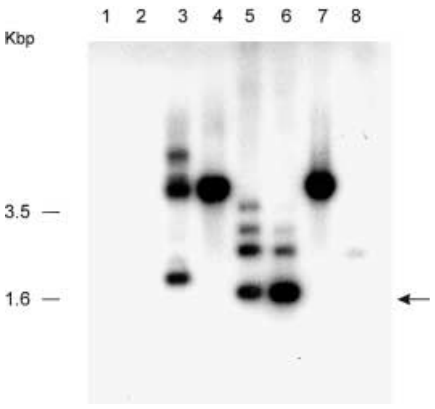

Fig. 5. Southern blot analysis of *susA* homologous sequences in cyanobacteria from different taxonomic groups. Total DNA (10 μg) from *Synechococcus* strain PCC 7942 (*lane 1*), *Synechocystis* sp. PCC 6803 (*lane 2*), *Anabaena variabilis* strain ATCC 29413 (*lane 3*), *Anabaena* sp. PCC 7119 (*lane 5*), *Anabaena* sp. PCC 7120 (*lane 6*), and *Calothrix* strain PCC 7601 (*lane 8*) were digested with *Hin*dIII, and *Anabaena* strain PCC 7118 (*lane 4*) and *Nostoc* strain 6719 (*lane 7*) with *Dra*I. A 350-bp *Eco*RI/*Hin*dIII fragment from the mid-region of the *susA* open reading frame was used as the probe. Hybridization and washings were conducted under low-stringency conditions. The *arrow* indicates the position of the *Anabaena* sp. PCC 7119 gene (*susA*). The positions of the size standards are indicated on the left (in kbp)

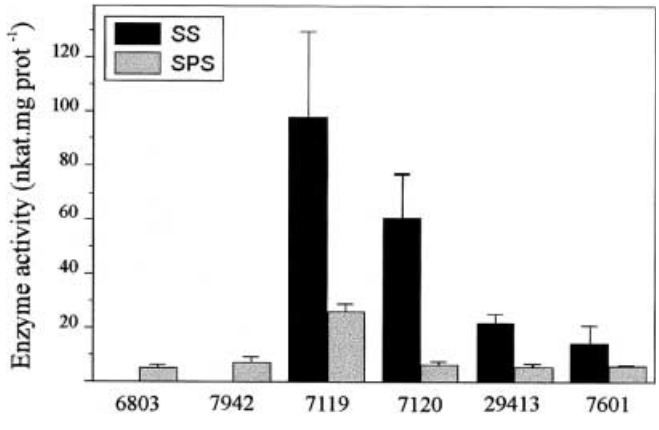

Fig. 6. Sucrose synthase (*SS*) and sucrose-phosphate synthase (*SPS*) activities in cyanobacteria from different taxonomic groups (see legend to Fig. 5 for details). Crude extracts were prepared from cells at late exponential phase. Enzyme activity was measured in the direction of sucrose synthesis

Synechocystis genome (Kaneko et al. 1996) by BLASTp analysis (Altschul et al. 1990). These results are consistent with the idea that SS may play a role in the transport of sucrose from vegetative cells to heterocysts in filamentous nitrogen-fixing cyanobacteria as proposed by Schilling and Ehrnsperger (1985). Whether the physiological role of SS is to synthesize or cleave sucrose is still unclear and must be established.

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