Sucrose is involved in the diazotrophic metabolism of the heterocyst-forming cyanobacterium *Anabaena* sp.

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Abstract Sucrose synthase (SuS) expression was studied in the filamentous, nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7119. SuS activity, SusA polypeptide, and *susA* mRNA levels were lower in cells cultured diazotrophically than in the presence of combined nitrogen. An insertional *susA* mutant presented a dramatic increase in sucrose levels, whereas the disaccharide was not detectable in a *susA* overexpressing strain, indicating that SusA is involved in the cleavage of sucrose in vivo. Diazotrophic growth was impaired in the *susA* overexpressing strain, suggesting a role for sucrose in diazotrophic metabolism and the involvement of SusA in the control of carbon flux in the N_2-fixing filament.

Key words: Sucrose metabolism; Cyanobacteria; Sucrose synthase; Nitrogen metabolism; *Anabaena* sp. PCC 7119

1. Introduction

Sucrose is crucial for plant physiology as a major product of photosynthesis and export form of photoassimilate to heterotrophic tissues. It has also functions as a storage compound, in acclimation to environmental stress, and in signal transduction pathways related to metabolism, growth and development [1,2]. In higher plants, sucrose–phosphate synthase (SPS; UDP–glucose:D-fructose-6-phosphate 2-α-D-glucosyltransferase; EC 2.4.1.14) is involved in sucrose biosynthesis, sucrose synthase (SuS; UDP–Glc:D-fructose-2-α-D-glucosyl transferase; EC 2.4.1.13) can catalyze the synthesis and cleavage of sucrose, and invertase (Inv) irreversibly hydrolyze sucrose [1]. Much less is known about sucrose metabolism in microorganisms, although sucrose has been shown to accumulate in green algae [3] and some cyanobacteria [4,5] as a response to environmental stress. Recently, we have demonstrated that cyanobacteria metabolize sucrose by means of enzyme activities similar to those present in higher plants [6–10]. Also, we have reported the first characterization of a prokaryotic SuS (SS-II, renamed as SusA in this paper) and the sequence of its encoding gene, *susA*, in *Anabaena* sp. PCC 7119 [9,11]. The physiological role of sucrose metabolism in cyanobacteria has not been elucidated yet, although several lines of evidence have suggested that sucrose may play a role as a carbon carrier molecule during nitrogen fixation in filamentous, heterocyst-forming cyanobacteria [12]. Thus, (i) CO_2 fixed by vegetative cells moves into heterocysts, which have the capacity of aerobic nitrogen fixation and do not fix CO_2 [13]; (ii) the high specific activities of enzymes of the pentose–phosphate pathway found in heterocysts suggest that sugars should be supplied at a proportional rate from vegetative cells [12]; (iii) the addition of sucrose increases nitrogenase activity in isolated heterocysts [14]; (iv) SuS and Inv activities are associated to vegetative cells and heterocysts, respectively [15]; and (v) there is a lack of SuS activity in unicellular, non-nitrogen-fixing cyanobacteria [11].

The present study addressed the question of the role of SuS in *Anabaena* sp. PCC 7119 metabolism and its possible relationship with nitrogen fixation. It was found that *susA* expression levels are lower during dinitrogen fixation and that *susA* overexpression reduced *Anabaena* diazotrophic growth rate. It was also demonstrated that SusA is involved in the cleavage of sucrose in vivo.

2. Materials and methods

2.1. Bacterial strains and growth

Anabaena sp. strain PCC 7119 and derivative mutant strains were cultured in the presence of different nitrogen sources as previously described [7]. LC30 and LC60 were cultured in the presence of 1.5 μg ml^{-1} of each spectinomycin and streptomycin. *Escherichia coli* DH5α, HB101 and ED8654 were grown in Luria–Bertani medium supplemented with different antibiotics at 37°C for cloning and conjugation, or at 25°C for SusA overexpression.

The growth rate constant, μ ($\mu = \ln 2\ t_d^{-1}$, where t_d represents the doubling time), of the cultures was derived from the increase of OD_{750} at 30°C and continuous light.

2.2. Isolation, manipulation and analysis of nucleic acids

Plasmids were isolated and modified according to standard protocols [16]. Isolation of genomic DNA from cyanobacteria was performed as previously described [17]. RNA was isolated using the TRIZOL reagent (Gibco-BRL). RNA quality and PCR products were analyzed by electrophoresis in 1% agarose gels.

2.3. Enzyme assays

Crude extracts prepared from *Anabaena* cells were desalted before enzyme activity assays. SuS activity in the sucrose synthesis direction and SPS were assayed in the presence of UDP–Glc. The cleavage of sucrose by SuS was determined by adding UDP in the incubation mixture as previously reported [9]. Inv activities were measured as described [7,15].

2.4. SusA purification, polyclonal antibodies production and Western immunoanalysis

The coding region of *susA* was isolated by PCR using the oligonucleotides 5′-CGGGATCCATGTCAGAATTGATGCAAGGC-3′ (olSS-fw) and 5′-TGATGTCGACGCTTACCGATATTTATACTGTTCTAATAGTTGTTGCG-3′ (olSS-rv1) and 50 ng of *Anabaena* ge-

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nomic DNA as template. PCR products were ligated to pGEX 4T-2 (Pharmacia) to generate pSS2.4. Cultures of *E. coli* strain DH5α cells bearing pSS2.4 ($OD_{600\,nm}$ 0.5–0.7) were induced with 0.1 mM IPTG. GST::SusA was affinity purified and digested with thrombin [18]. Anti-*Anabaena* SusA polyclonal antibodies were prepared from the purified recombinant SusA protein [19]. SDS–PAGE was performed in 9% acrylamide gels as described [20]. Immunoblotting was conducted as reported [19].

2.5. RT-PCR

Competitor *susA*-specific RNA was synthesized from $pSS\Delta_{336-633}$ bearing a *Pvu*I 297-bp deletion in a *susA* 1200-bp PCR product ligated into pBluescript SK(+) (Stratagene) as described [16]. Reverse transcriptase reactions were conducted by mixing serial dilutions of the competitor RNA with 2.5 μg of *Anabaena* total RNA and 6.25 pmol of the reverse primer 5′-AGCGGTCGACAATTCTCTTTCTGATG-TCAATGGC-3′ (olSS-rv2) and incubated with M-MLV RT as recommended by the manufacturer (Promega). RT reaction products were PCR amplified using olSS-fw and olSS-rv2 and Taq-DNA polymerase (Gibco-BRL) as follows: 94°C for 1 min, followed by 23 cycles at 94°C for 30 s, 55°C for 45 s and 72°C for 1.5 min, and a single step at 72°C for 3 min, using a PTC-100 thermal cycler (Model-96V, MJ Research, Inc., MA, USA). Control reactions were incubated at 30°C during 10 min in the presence of RNaseA to ascertain RNA dependence of the RT-PCR signals. Products were resolved on 0.9% agarose gels and stained with ethidium bromide.

2.6. Insertional inactivation and overexpression of susA

A *susA*::C.S3 mutant allele was generated by disruption of *susA* from an *Anabaena* 7-kb genomic DNA fragment [11,21] and ligated into the *Spe*I site of the conjugative vector pRL278 [17] to generate plasmid pLC30, which was transferred to *Anabaena* sp. PCC 7119 by conjugation [22]. Double recombinants were identified by their Suc^R Sp^R Sm^R Nm^S phenotype (resistance to Nm is encoded in the vector portion of pRL278), and homozygosity was tested by PCR analysis using oligonucleotides olSS-fw and olSS-rv1, or olSS-fw and olSS-rv2.

For overexpression of *susA*, a 4-kb *Eco*RV genomic fragment from pSS7 [11] was ligated to pRL1404, which replicates at a high copy number in *Anabaena* sp. [23], to generate pLC60, which was transferred to *Anabaena* sp. PCC 7119 by conjugation [22].

2.7. Sucrose determination

Cells were collected by centrifugation and extracted with alkaline water [24]. Sucrose was determined after Inv hydrolysis [25]. Chlorophyll (Chl) content was quantified in methanolic extracts of the cells [26].

3. Results

3.1. Expression analysis of susA in Anabaena cells grown on different nitrogen sources

SuS activity measured in both reaction directions was about 40% lower in protein extracts from *Anabaena* sp. PCC 7119 cells cultured in dinitrogen than in those from nitrate- or ammonium-grown cells (Fig. 1A). Patterns of expression similar to those observed for SuS activity were also observed when SusA polypeptide levels were immunoanalyzed (Fig. 1B) or *susA* mRNA content was determined by competitive RT-PCR (Fig. 2). Note that, for a 10^{-2} dilution, some amplification of the synthetic competitor was observed in the presence of the N_2-RNA sample, but not in the presence of those from nitrate- or ammonium-grown cells (Fig. 2C), indicating the presence of a lower amount of *susA* transcript in the N_2-RNA sample. Additional experiments showed that the decrease in SuS activity was already evident 24 h after nitrogen step-down (not shown).

3.2. Genetic modification of susA levels in Anabaena sp.

An insertion mutant strain was constructed by disrupting *susA* with a Sp/Sm resistance cartridge (Fig. 3A) and transfering the resulting construct to *Anabaena* sp. PCC 7119 by conjugation. Mutant homozygous clones were obtained, and one of these clones was named strain LC30 (Fig. 3B). Neither SusA polypeptide (Fig. 3C, lane 2) nor SuS activity (Table 1) was detected in LC30. A second open reading frame, *susB* (EMBL access. no. AJ316584), encoding a deduced amino acid sequence 58% identical to SusA, was identified in the genome of *Anabaena* sp. PCC 7120 (http://www.kazusa.or.jp/cyano/anabaena.html). Neither His_{6x}::SusB nor GST::SusB from both *Anabaena* sp. strains PCC 7119 or PCC 7120 expressed in *E. coli* showed SuS activity (not shown).

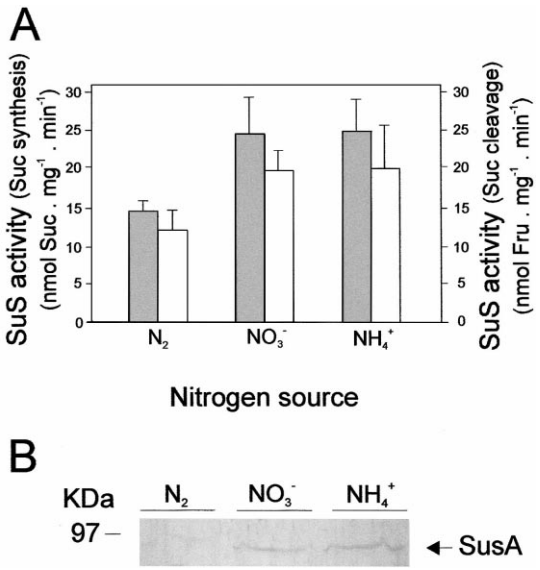

Fig. 1. Effect of the nitrogen source on SuS activity and SusA polypeptide levels in *Anabaena* sp. PCC 7119. A: SuS activity in the sucrose synthesis (filled boxes) or sucrose cleavage (open boxes) direction. Data are the mean and standard deviation of four independent experiments. B: Immunoanalysis of protein extracts (40 μg per lane) from *Anabaena* sp. PCC 7119 cell homogenates were revealed using anti-*Anabaena* SusA.

On the other hand, SusA polypeptide and SuS activity levels increased about 10-fold in LC60, a *susA* overexpressing strain (Fig. 3C, lane 3, and Table 1).

3.3. Physiological effects of genetic modification of susA expression

Sucrose levels were about 10-fold higher in mutant LC30 than in *Anabaena* sp. PCC 7119 cells, but sucrose could not be detected in strain LC60 (Table 1). Sucrose was not accumulated in dinitrogen-grown *Anabaena* sp. PCC 7119 cells, which presented an increased Inv activity in comparison with nitrate-grown cells. Sucrose contents were higher in the presence of NO_3^- than in the presence of NH_4^+ (Table 1) and were even higher when the cells were grown in the presence of a combination of both nitrogen sources (776 ± 18 and 4359 ± 484 nmol Suc mg^{-1} Chl, in strains PCC 7119 and LC30, respectively). The high sucrose content was paralleled by a decrease in Inv activity (Table 1), whereas SPS activity was similar under the three nitrogen conditions assayed. SPS and Inv activities were barely modified in strains LC30 and LC60. As compared to the wild type, no difference was observed in the growth rate of LC30 and LC60 in the presence of NO_3^- or NH_4^+. However, LC60 growth was severely affected when this strain was cul-

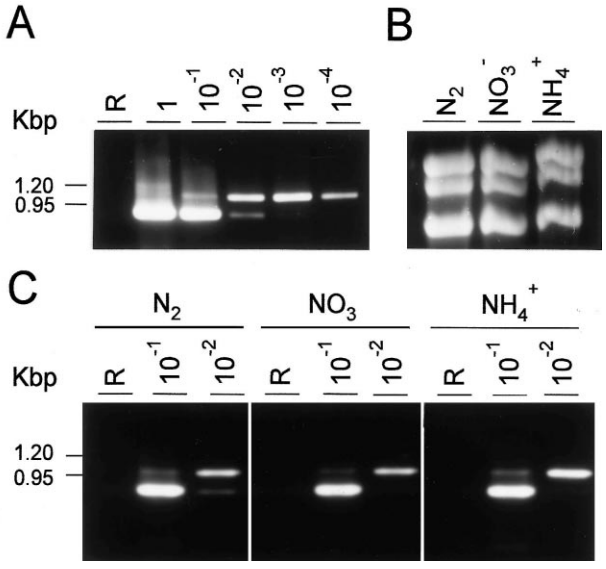

Fig. 2. RT-PCR analysis of the effect of the nitrogen source on *susA* expression. A: Determination of competitive RT-PCR assay conditions using 2.5 μg of RNA isolated from ammonium-grown *Anabaena* cells (1.2-kb PCR product) and serial dilutions of a synthetic competitor (903-bp PCR product). B: Total RNA control in which RNA (5 μg per lane) was visualized by ethidium bromide staining. C: Competitive RT-PCR analysis of *susA* mRNA levels using 2.5 μg of RNA and the indicated dilutions of synthetic competitor. R, control reaction containing maximum concentration of competitor and the corresponding *Anabaena* RNA, with the addition of RNaseA (5 μg) and incubation for 15 min at 37°C.

tured diazotrophically (Table 1), although heterocysts could be observed microscopically (not shown).

4. Discussion

In this report we present the first functional analysis of sucrose metabolism and of the role of SuS in a prokaryotic organism. As deduced from the lack of SuS activity in the *susA* insertional mutant LC30, SusA is the main contributor to SuS activity in *Anabaena* sp. PCC 7119. The previously reported SS-I [9] must be ascribed to a remanent SuS activity of SPS-I (A.C. Cumino, personal communication). The dramatic change in sucrose levels observed, in comparison with those found in the wild type, for the *susA* insertional mutant LC30, which accumulates sucrose, and for the *susA* overexpressing strain LC60, which exhibits undetectable sucrose levels, lead us to conclude that SusA preferentially catalyzes the cleavage of sucrose in vivo. Thus, the previous reported role for SuS in sucrose synthesis during nitrogen fixation [15] should be discarded.

SuS activity, the amount of SusA polypeptide, and *susA* mRNA transcripts are lower in *Anabaena* cells during diazotrophic growth than when grown in the presence of combined nitrogen (Figs. 1 and 2). On the other hand, Inv activity is increased in filaments grown diazotrophically, although, in contrast to what has been previously proposed [15], it is not restricted to heterocysts, since both nitrate- and ammonium-grown cells exhibit this activity. Nonetheless, the higher Inv levels observed in diazotrophically grown cells (Table 1) would be consistent with a putative association of Inv with heterocysts and with a role of Inv in determining sucrose levels in dinitrogen-grown cells. Also, Inv may account for the differences in sucrose levels found between cells grown with nitrate or ammonium.

The simultaneous expression of SPS, SuS and Inv, independently of the culture conditions, suggests that sucrose turnover is very active in *Anabaena* sp. as is the case for various plant tissues [27]. The fact that the increase in sucrose breakdown by SusA in strain LC60 severely affects the diazotrophic performance of *Anabaena* sp. indicates that sucrose is an intermediate in the flux of reduced carbon in the N_2-fixing filament and supports a role for this disaccharide as a carbon carrier molecule [12]. It could also be suggested that down-regulation of *susA* after nitrogen step-down (Figs. 1 and 2) changes carbon fluxes through sucrose in vegetative cells. Sucrose transport to heterocysts and hydrolysis by Inv may complete the pathway.

The increment in sucrose levels in cells grown in the presence of a combination of NO_3^- and NH_4^+ indicates that factors other than the nature of the nitrogen source regulates

Table 1
Effect of the genetic modification of *susA* expression on sucrose metabolism and *Anabaena* growth rates

	Nitrogen source	*Anabaena* sp. strain		
		PCC 7119	LC30	LC60
SuS activity[a] (nmol Fru mg^{-1} Prot min^{-1})	N_2	12.7 ± 2.4	nd[b]	132 ± 6
	NO_3^-	18.8 ± 3.0	nd	148 ± 20
	NH_4^+	19.6 ± 6.3	nd	ND[c]
SPS activity[d] (nmol Fru mg^{-1} Prot min^{-1}	N^2	1.6 ± 0.2	1.0 ± 0.1	2.0 ± 0.4
	NO_3^-	1.9 ± 0.3	1.4 ± 0.2	1.4 ± 0.3
	NH_4^+	1.6 ± 0.1	1.0 ± 0.1	ND
Inv activity[d] (nmol Fru mg^{-1} Prot min^{-1})	N_2	7.0 ± 0.2	5.9 ± 0.6	5.9 ± 0.7
	NO_3^-	2.8 ± 0.4	4.5 ± 1.6	1.5 ± 0.3
	NH_4^+	5.4 ± 0.5	5.5 ± 0.6	ND
Sucrose[d] (nmol Suc mg^{-1} Chl)	N_2	nd	75 ± 19	nd
	NO_3^-	449 ± 24	3210 ± 49	nd
	NH_4^+	60 ± 7	682 ± 38	ND
μ (d^{-1})[d]	N_2	0.29 ± 0.03	0.25 ± 0.02	0.07 ± 0.01
	NO_3^-	0.35 ± 0.02	0.36 ± 0.01	0.39 ± 0.01
	NH_4^+	0.37 ± 0.02	0.44 ± 0.04	0.43 ± 0.01

[a]Activity assayed in the sucrose cleavage direction. Data are the mean and standard deviation of four independent experiments.
[b]Not detected.
[c]Not determined.
[d]Data are the mean and standard deviation of three independent experiments.

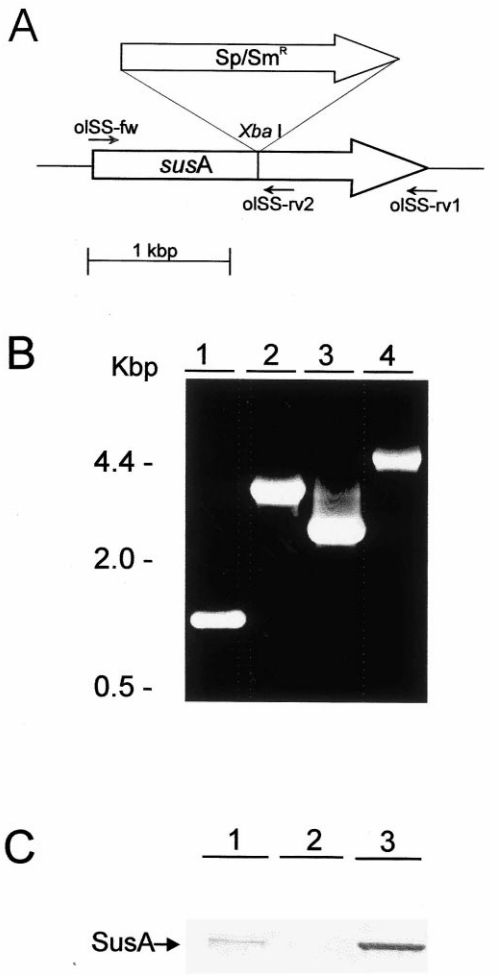

Fig. 3. Genetic modification of *susA* expression in *Anabaena* sp. PCC 7119. A: Integration site of the Sp/Sm^R cartridge used to obtain the insertional mutant LC30. Arrows indicate the direction of transcription. B: PCR analysis of *susA* in LC30 and wild type *Anabaena* sp. PCC 7119. PCR products using total DNA from *Anabaena* sp. PCC 7119 (lanes 1 and 3) or LC30 (lanes 2 and 4) and oligonucleotides olSS-fw and olSS-rv2 (lanes 1 and 2) or olSS-fw and olSS-rv1 (lanes 3 and 4). C: Immunoanalysis of protein extracts (40 μg per lane) from *Anabaena* sp. PCC 7119 (lane 1), LC30 (lane 2) or LC60 (lane 3) grown in nitrate-containing medium, using anti-*Anabaena* SusA antibodies.

sucrose metabolism, taking into account the established NH_4^+ repression of NO_3^- transport and utilization [28]. Both sucrose levels and the enzymes involved in its metabolism appear to be modified according to different environmental and/or nutrient factors. Such a versatility has been long appreciated for higher plants. A compromise between different roles of sucrose metabolism may explain diazotrophic growth sensitivity to salt, in addition to nitrogenase response to Na^+ [29].

Taken together, our results support a role for sucrose in the diazotrophic metabolism of *Anabaena* sp. PCC 7119, as well as the involvement of SusA in the control of carbon fluxes in vegetative cells through the cleavage of sucrose.

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