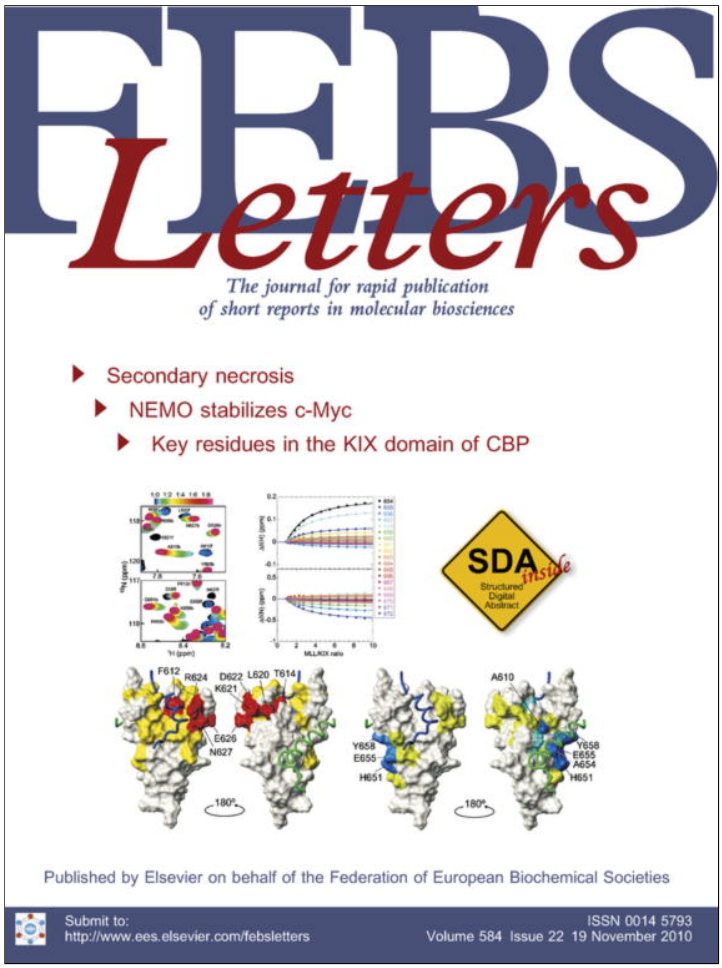

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The proteins involved in sucrose synthesis in the marine cyanobacterium *Synechococcus* sp. PCC 7002 are encoded by two genes transcribed from a gene cluster

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ABSTRACT

It has been reported that higher plants and cyanobacteria synthesize sucrose (Suc) by a similar sequential action of sucrose-phosphate synthase (SPS) and sucrose-phosphate phosphatase (SPP). In the genome of the marine unicellular cyanobacterium *Synechococcus* sp. PCC 7002 there is a sequence that was not annotated as a putative SPP encoding gene (*sppA*), although the sequence was available. In this study, we functionally characterize the *sppA* gene of that strain and demonstrate that it is cotranscribed with *spsA*, the SPS encoding gene. This is the first report on the coordination of Suc synthesis gene expression in an oxygenic-photosynthetic organism.

1. Introduction

Cyanobacteria are oxygenic-photosynthetic prokaryotes worldwide distributed. Particularly, marine strains are the most abundant photosynthetic organisms on Earth. In most oceanic waters, the numerically dominating cyanobacteria are of the genus *Synechococcus* [1].

Salinity is a crucial abiotic factor in aquatic ecosystems. The accumulation of compatible solutes is a universal process for microorganisms tolerate a salt shock or acclimate to low water potentials. During their evolution cyanobacteria have adapted to aquatic habitats with various salt concentrations [2]. The euryhaline strain *Synechococcus* sp. PCC 7002 is capable of growth from freshwater conditions up to over 1 M salt concentrations and synthesises glucosylglycerol (GG), as the main compatible solute, and sucrose (Suc) during salt stress. Also, although at minor extent, this strain accumulates glucosylglycerate that was reported to be salt- and nitrogen-dependent [3,4]. At the fluctuating levels of salinity in coastal waters, the level of GG seems to be determined mainly by different levels of expression of the encoding gene of GG-phosphate synthase (GgpS), the key enzyme involved in its synthesis [3,5]. However, as far as the authors know, Suc biosynthesis proteins were not experimentally investigated in any marine strain.

It has been reported in *Synechocystis* sp. PCC 6803, a freshwater unicellular cyanobacterial strain, and in *Nostoc* sp. PCC 7119 and PCC 7120, two closely related filamentous nitrogen-fixing strains, that Suc biosynthesis takes place by the sequential action of Suc-phosphate synthase (SPS, EC 2.4.1.14) and Suc-phosphate phosphatase (SPP, EC 3.1.3.24) [6]. SPS synthesises Suc-6^F-phosphate from UDP-Glc and/or ADP-Glc and fructose-6-P, and it is metabolically associated to SPP, a specific phosphatase, yielding the net synthesis of Suc. In general, SPSs and SPPs are proteins with a modular architecture [6]. Studies on SPSs and SPPs from filamentous nitrogen-fixing strains (like *Nostoc* sp. PCC 7120 and *N. punctiforme*), showed that they define minimal catalytic units: a functional glucosyltransferase and a phosphohydrolase, respectively [6–8]. In *Synechocystis* sp. PCC 6803 and in plants it has been reported the presence of bidomain SPSs [containing a functional glucosyltransferase domain (GTD) and a non-functional phosphohydrolase domain (PHD)] and SPPs (coincidental with a functional PHD domain) [9–11].

Abbreviations: GG, glucosylglycerol; GTD, glucosyltransferase domain; 7002, *Synechococcus* sp. PCC 7002; PHD, phosphohydrolase domain; SPS, sucrose-phosphate synthase; SPP, sucrose-phosphate phosphatase; Suc, sucrose; Syn, *Synechocystis* sp. PCC 6803; *tsp*, transcription start point; U/ADP-Glc, uridine- or adenine-diphosphate glucose

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The occurrence of a putative SPS encoding gene in the *Synechococcus* sp. PCC 7002 genome has been previously reported by our research group [6] and deposited in public data bases (AAR31179.1). In a recent report, several genomes of marine *Synechococcus* (11 strains) and *Probhlorococus* (12 strains) were analysed for Suc synthesis-related genes [1]. In all of them, putative two-domain SPS encoding genes were found; however, putative SPP encoding genes were only retrieved from two *Synechococcus* genomes different to PCC 7002. In this study we report the presence of both SPS and SPP activities in the cyanobacterium *Synechococcus* sp. PCC 7002 and the first functional characterization of their encoding genes (*spsA* and *sppA*) from a marine strain. Additionally, *spsA* and *sppA* are contiguously located and 8-bases overlapped. Such genome organization allowed us to postulate their cotranscription that was experimentally supported. So far, to date this gene structure has not been reported in any other Suc producing organism.

2. Materials and methods

2.1. Bacterial strains and growth

Synechococcus sp. strain PCC 7002 was grown in ASNIII – BG 11 medium supplemented with B12 vitamin. *Escherichia coli* DH5α and BL21(DE3):pLysS (Novagen) strains were grown in Luria Bertani medium supplemented with 50 μg/ml carbenicillin. To study the effect of salt, cells previously cultured at standard conditions up to exponential phase, were collected and seeded either in basal medium or in basal medium supplemented with NaCl (684 mM final concentration). When indicated, chloramphenicol or rifampicin were added up to 30 μg/ml or 50 μg/ml final concentration, respectively.

2.2. Cloning and expression of SPS and SPP genes

Sequences of two *orf*s (named 2157-*orf* and 834-*orf* were retrieved from the *Synechococcus* sp. PCC 7002 genome (http://www.ncbi.nlm.nih.gov) and deposited in GenBank after functional characterization as *spsA* and *sppA*, respectively. Two DNA fragments of 2175 bp and 869 bp were PCR-amplified using the primer pairs sps(F)/sps(R) and spp(F)/spp(R) (Table S1), respectively. Amplification products were ligated into the pRSET-A (Invitrogen, Carlsbad, CA) vector between the restriction sites *Pst*I and *Eco*RI for *spsA*, or *Bam*HI and *Eco*RI for *sppA*, obtaining the recombinant plasmids pR-*spsA* and pR-*sppA*, respectively. The identity of each construct was confirmed by DNA sequencing. *E. coli* BL21(DE3)-pLysS cells were transformed with pR-*spsA* or pR-*sppA*, to produce the recombinant proteins His_6::7002-SPS and His_6::7002-SPP, respectively [12].

2.3. Protein purification and enzyme assays

His_6::7002-SPS and His_6::7002-SPP fusion proteins were purified by Ni-affinity chromatography (Ni-NTA Purification System). Recombinant proteins were eluted from the column with a stepwise imidazole pH 6.0 gradient (50, 100, 150, and 250 mM). Fractions containing the purified recombinant protein were pooled and concentrated in an Amicon (Newtown, PA) ultrafiltration cell. Purified enzymes were stored at −20°C [12]. Partial purification of SPS and SPP from *Synehcoccocus* sp. PCC 7002 cells were carried out from cultures at late exponential phase, as described [13]. Protein extracts were absorbed onto a column of DEAE-Sephacel and the enzymes were eluted with a linear NaCl gradient. Fractions with SPS and SPP activities were pooled and concentrated. SPP was further purified by gel filtration in a Sephadex G-75 column. The recombinant proteins and partially purified SPS and SPP from *Synehcoccocus* sp. PCC 7002 cells were used for product identification according to Porchia and Salerno [13].

2.4. Western immunoblots

Polypeptides were separated by SDS–PAGE on 12% polyacrylamide gels [14] and visualized with Coomassie blue or electroblotted onto a nitrocellulose membrane (HyBond C; Amersham) as described [15]. The membranes were then probed with polyclonal antibodies raised in rabbits against recombinant SPP from *Anabaena* sp. PCC 7120 [8].

2.5. Isolation of RNA, RT-PCR assay and Northern blots

RNA from *Synechococcus* sp. PCC 7002 was isolated using the TRIZOL reagent (Gibco-BRL/Invitrogen). For RT-PCR analysis, total RNA (2.5 μg) treated with DNase (RQ1 Rnase-free Dnase, Promega) was reverse-transcribed using MMLV (Moloney murine leukemia virus) reverse transcriptase (Promega) and specific primers. PCR reactions were run on a Mastercycler® epgradient cycler (Eppendorf) for 20 cycles of 94 °C (1 min), 50 °C (1 min), and 72 °C (1 min), and a single step at 72 °C (5 min). Standarization of RT and PCR reactions were carried out as described [16].

For Northern blots, RNA was separated in a 1.2% agarose-formaldehyde denaturing gel and immobilized in positively charged nylon membranes (0.45 μm, Nytran, Schleicher & Schuell, Keene, NH) by alkaline passive transference. A 480-bp probe (nucleotide 1966 of 2157-*orf* to nucleotide 294 of 834-*orf*) was labeled with [a-^{32}P]dCTP by the random primer extension system

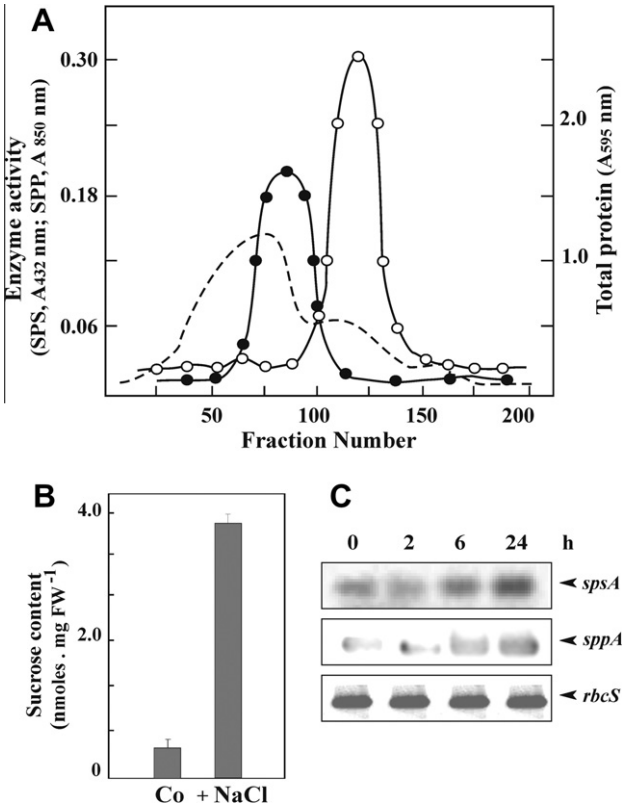

Fig. 1. Occurrence of SPS and SPP in *Synechococcus* sp. PCC 7002 cells. (A) DEAE-Sephacel chromatography of 7002 protein extract. SPS (○), SPP (•) activity and protein (—). (B) Suc content in 7002 cells with (+NaCl) or without (Co, control) 684 mM NaCl. (C) RT-PCR expression analysis of 7002-*spsA* and 7002-*sppA* in salt-treated cells. Amplification of *Synechococcus rbcS* was used as a control to monitor the relative amount of total RNA used in each RT-PCR reaction.

(NEN Life Science Products, Boston, MA). Prehybridization, hybridization and exposure conditions were carried out as described [12].

2.6. Determination of transcription start points (tsps)

Primer extension experiments were performed from total RNA (30 μg), using SuperScript II RNase H reverse transcriptase (Invitrogen) and different oligonucleotides (Table S1). Primers were end-labeled and cDNA products were purified and resolved as described [16].

3. Results

3.1. Functional identification of SPS and SPP in Synechococcus sp. PCC 7002

BLAST sequence similarity searches in the *Synechococcus* sp. PCC 7002 genome revealed two open reading frames (2157-orf and 834-*orf*) with homology to Syn-spsA and Syn-*sppA*, respectively, the genes encoding SPS and SPP in *Synechocystis* sp. PCC 6803 [9,11]. Their deduced amino acid sequences share 63% and 29% identity with Syn-*spsA* and Syn-*sppA*, and corresponded to polypeptides of 81,072 and 31,436 kDa, respectively. Suc had been reported to accumulate in 7002 cells [3]. Thus, the finding of a putative SPP encoding gene (834-orf) that was not annotated led us to investigate whether Suc synthesis could take place through a typical SPS–SPP pathway [10]. The following lines of evidence point to confirm this assumption: (i) SPS and SPP activity were measured in partially purified 7002 protein extracts (Fig. 1A) and the identification of the product were ascertained in each case; (ii) 2157-*orf* and 834-*orf* were identified as functional SPS and SPP encoding genes (7002-*spsA* and 7002-*sppA*) as they conferred SPS and SPP activity to *E. coli* cells (Fig. S1); (iii) 7002-SPP protein and recombinant His_6::7002-SPP have immunological and biochemical properties similar to other characterised SPPs (Fig. 2) [7,8,10,17], and (iv) Suc content increased after 684 mM NaCl addition (Fig. 1B) and transcriptional analysis of 7002-*spsA* and 7002-*sppA*, determined by RT-PCR, likewise showed maximal transcript level after 24 h of salt treatment (Fig. 1C). Additionally, structural analyses revealed that in 7002-SPP, the conserved residues of HAD motifs I, II and III are represented by DLDRTL (residues 10–15), LAYVT (42–46), and KX_{21}GDSGND (180–208), respectively, and the amino acid residues involved in the contact of the enzyme with ligands are conserved [18] (Fig. S2). SPS preparations do not show SPP activity (Fig. S1 and data not shown).

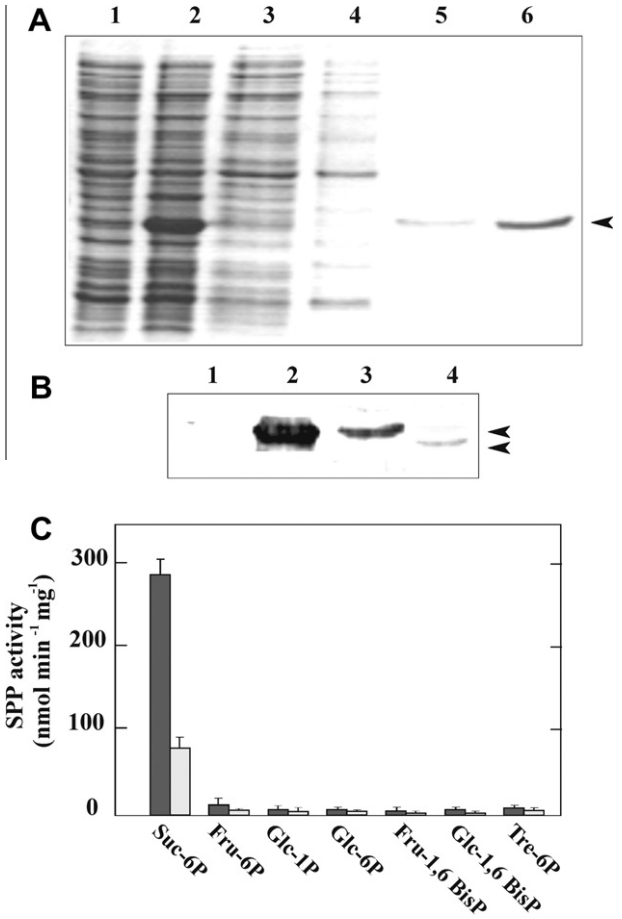

Fig. 2. Characterization of SPP encoding gene (*sppA*) of *Synechococcus* sp. PCC 7002 by heterologous expression in *E. coli*. Proteins were separated by SDS–PAGE in a 12% (w/v) gel. (A) Coomassie Blue staining. Lanes 1 and 2, total soluble extract from *E. coli* (pR-*sppA*) non-induced or induced cells, respectively; lanes 3–5, proteins eluted from the Ni-column with imidazole (pH 6.0). Lane 6, concentrated purified recombinant His_6::7002-SPP. The arrowhead indicates the position of His_6::7002-SPP. (B) Immunoblot revealed with *Anabaena* sp. PCC 7120 SPP antibodies. Lane 1, crude extract from non-transformed *E. coli* cells; lane 2, crude extract from induced cultures; lane 3, His_6::7002-SPP purified by metal-ion affinity chromatography; lane 4, Sephadex G-75 *Synechococcus* SPP fraction. (C) His_6::7002-SPP (dark grey bars) and 7002-SPP (light grey bars) substrate specificity. Different sugar-phosphates were assayed: Suc-6P (1 mM), fructose-6P (Fru-6P, 10 mM), glucose-1P (Glc-1P, 5 mM), glucose-6P (Glc-6P, 5 mM), fructose-1,6-bisphosphate (Fru-1,6 BisP, 1 mM), glucose-1,6-bisphosphate (Glc-1,6 BisP, 1 mM) and trehalose-6P (Tre-6P, 1 mM). Values are the mean ± S.D.; $n = 3$.

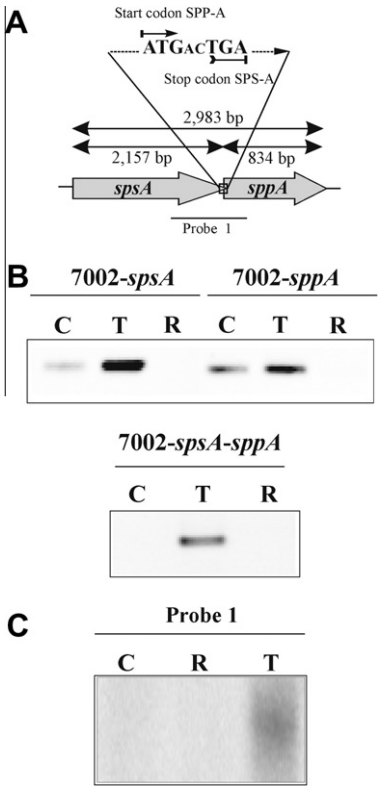

Fig. 3. Expression of SPS and SPP encoding genes from *Synechococcus* sp. PCC 7002 (7002-*spsA* and 7002-sppA). (A) Schematic representation of 7002-spsA and 7002-sppA location in the *Synechococcus* sp. 7002 genome. Gene overlapping and position of the probe used in Northern blots are depicted. (B) RT-PCR from total RNA prepared from 7002 cells treated with 684 mM NaCl and 30 μg/ml choramphenicol for 24 h (lane T) or from cells treated with 684 mM NaCl and 50 μg/ml rifampicin for 24 h (lane R). As a control, RT-PCR was performed with RNA from cells cultured without salt addition (lane C). Amplification of 7002-*spsA* and 7002-*sppA* (top), and of a region including a portion of *spsA* and *sppA* sequence that include the overlapping region (7002-*spsA*-*sppA*) (bottom). (C) Northern blot analysis using probe 1 showed in (A). References of lanes are identical to (B).

3.2. Expression of Synechococcus sp. PCC 7002 spsA and sppA

The sequences of 7002-*spsA* and 7002-*sppA* are eight nucleotides overlapped (Fig. 3A). To analyse the presence of a possible polycistronic transcript, total RNA from NaCl-treated 7002-cells was amplified by RT-PCR with different primer pair combinations (Table S1). To detect higher size mRNA accumulation, we added chloramphenicol together with NaCl. It has been reported that chloramphenicol addition to a bacterial culture when the newly synthesised mRNA is being traversed by ribosomes, protects mRNA from degradation [19,20]. Actually, the salt treatment in the presence of 30 μg/ml chloramphenicol allowed to detect transcripts that include a region of 7002-spsA and 7002-sppA genes (7002-spsA-sppA) (Fig. 3B, bottom), pointing to the occurrence of a bicistronic messenger. As expected, the addition of rifampicin abolished transcription. Northern blot analysis suggests that long transcripts may be initially synthesised, and a subsequent degradation into smaller size products may occur, as indicated by the presence of smears in the blot (Fig. 3C).

We also investigated the promoter region of 7002-*spsA-sppA* by determining the RNA 5′-ends that correspond to *tsp*s, mapped by primer extension of RNA obtained from 7002 cultures. Two RNA sizes (RNA_I and RNA_{II}) were observed, starting at the −62 (putative tsp_I) and −94 (putative tsp_{II}) nucleotides upstream of the translation initiation site (Fig. 4). When the upstream sequence of the two putative promoters was analysed, we identified two −10 boxes similar to that of *E. coli* σ70 promoter and a (GGAtC-N19-CGTT) motif (Fig. 4B). The consensus sequence GGAAC(N16/17)CGTT has been found in the binding site of an alternative sigma factor involved in responses to saline and osmotic stress in *Rhizobium etli rpo*E4, in several *rpoE*4-regulated genes identified by transcriptome analysis, and in other genes that control the transcription of stress-regulated genes in Gram negative eubacteria [21].

3.3. Analysis of PHD domains of 7002-SPS and 7002-SPP

The secondary and tertiary structures of 7002-SPP is coincidental with that of Syn-SPP, corresponding to PDB1s2oAo (certain

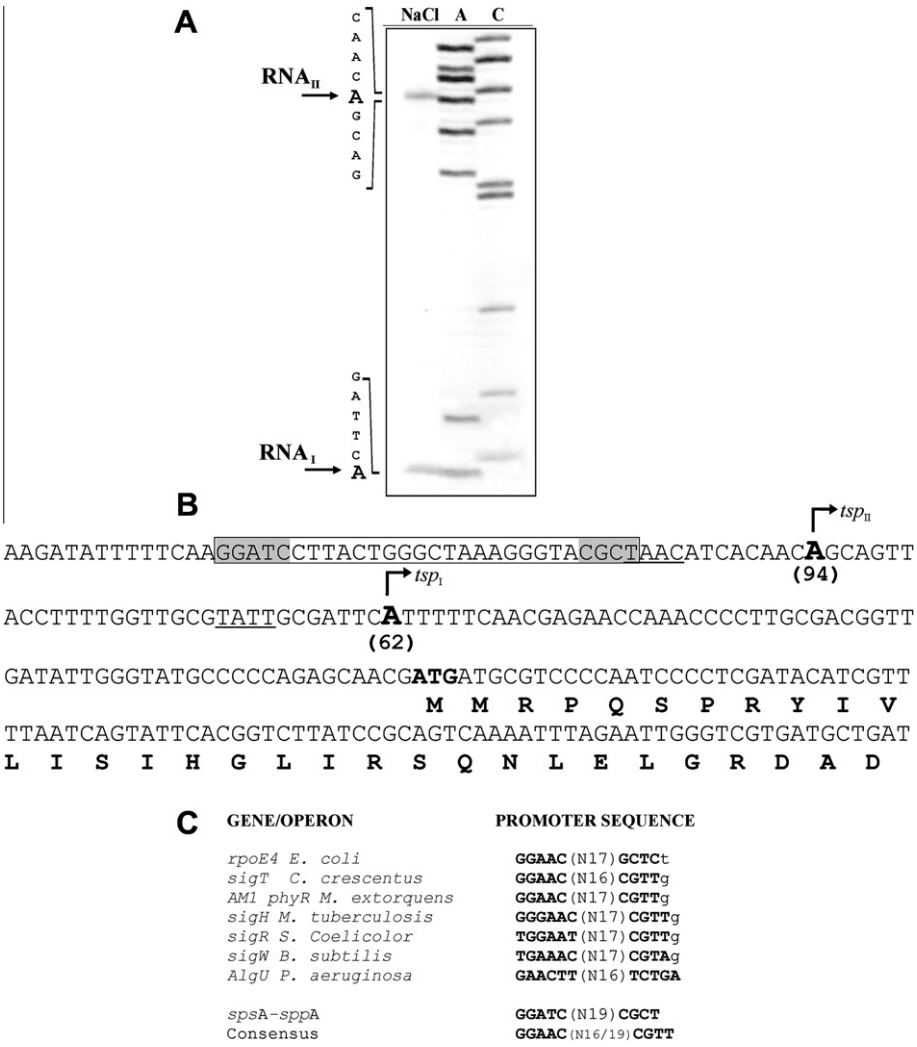

Fig. 4. Origins of gene transcription and analysis of the putative promoter regions. (A) Primer extension mapping of the transcription start points (putative *tsps*) of *spsA-sppA* carried out with total RNA (30 μg) from 7002 cells. The sequencing ladders presented (lanes A and C) were generated with the same primers used in the primer extension reactions. Arrowhead points to the extension product identifying putative *tsps*. Similar results were obtained using TSP-5 or TSP-6 (Table S1). (B) Nucleotide sequence of 7002-*spsA-sppA* upstream region. The putative *tsps* (arrows) and start translation codon are in bold. Numbers in parentheses indicate the relative position from the translation start. A putative consensus sequence identified here resembles the sequences of promoters recognized by the ECF sigma factors (extracytoplasmic function subfamily or_E-related sigma factors) is boxed. The −10 box regions are underlined. (C) Alignment of *Synechococcus* 7002-*spsA-spp*A promoter with promoter consensus motifs (boldface) of different genes (correspond mainly to alternative sigma factors of group 4) whose products act in response to several stress conditions (*E. coli rpo*E4, *C. crescentus sig*T, *M. extorquens AM1 phy*R, *M. tuberculosis sig*H, *S. coelicolor sig*R, *B. subtilis sig*W, and *P. aeruginosa alg*U).

grade, score above 65 and $E < 10^{-6}$) [18] (Fig. S2 and not shown), both SPP active proteins [11, this paper]. All of the residues implicated in Suc, Suc-6P, glucose and phosphate binding site are conserved. However, the Lys152 residue that was reported to be replaced in plant SPPs by Gln, Met, Ile or Val [18], in the case of 7002-SPP is substituted by Glu (Fig. S2).

Modeling of the SPP-like domain (PHD) of 7002-SPS on the Syn-SPP structure [18] predicted that 115 out of 244 residues would occupy identical positions. Most of the key residues in the substrate binding site are conserved in 7002-SPS. However, Asp9, which has a key role in the nucleophilic attack that initiates Suc-6P hydrolysis, and Asn189 of Syn-SPP, which is involved in Suc-6-phosphate binding, are replaced by Tyr and Ala, respectively.

4. Discussion

Although Suc synthesis is likely to be a general feature of marine cyanobacteria to challenge saline environments together with GG accumulation [4], its biosynthetic pathway has not been yet described in any marine strain. Suc occurrence in 7002 was reported several years ago [3], but only a bidomain SPS-like protein was inferred from the analysis of the 7002 genome [4,6]. Our results demonstrate that Suc is synthesised in 7002 through the concomitant action of two independent proteins (7002-SPS and 7002-SPP) involved in the typical two-step pathway (Fig. 1). It seems not to be the case for most picoplanktonic *Synechococcus*, like *Synechococcus* sp. WH7803, where sequences homologous to enconding genes of two-domains SPSs can be found. In those strains, it could be speculated that SPS may be a bifunctional protein or that a phosphatase different to SPP may dephosphorylate Suc-6-phosphate. Also, in *Synechococcus* WH8102 Suc could not be detected even though there is a putative *sps* sequence in their genomes [4]. Further functional characterization of Suc genes in marine cyanobacterial strains is needed to clarify this issue.

In most cyanobacterial genomes SPS and SPP genes are located separately, frequently in different regions of the chromosome. In prokaryotes, about 50% of the genes are organized in operons, being one of their major structural and regulatory features [22]. Proteins with tendency to operon participation seem to be involved in relatively static complexes and possible linear pathways [23]. However, this seems not to be the rule for Suc genes in cyanobacteria so far. The driving forces behind operon formation and the balance between individually regulated genes versus genes in operons are still not elucidated [23]. The identification of 7002-*spsA* and 7002- *sppA* uncovered their cluster arrangement (Fig. 3A), a rare feature in cyanobacteria, rising the question about their origin [6]. We wonder if this indicates that it is an extant arrangement of ancestral genes or they might have been acquired by lateral gene transfer during evolution. A similar organization is found in *Synechococcus* sp. PCC 7001 (*Cyanobium* sp. PCC 7001) genome, where a putative two-domain SPS gene is 19-bp overlapped with a putative SPP encoding gene. From the genome of the proteobacteria *Magnetococcus* sp. MC-1, *Mariprofundus ferroxidans* PV-1, *Thiomicrospira crunogena* XCL-2 and *Desulfobacterium autotrophicum* HRM2 it can be retrieved clusters of two homologs to putative bidomainal SPS and putative SPP encoding genes. It has been suggested that proteobacteria are likely to have acquired those genes through lateral gene transfer from cyanobacteria and plants [6]. Whether their protein products are Suc-related proteins remains to be demonstrated. In *Agrobacterium tumefaciens*, a soil proteobacteria, homologs to genes coding for SPS and SPP are arranged in an operon structure, but it was shown that they are involved in the synthesis of mannosylfructose [12].

The addition of chloramphenicol to salt-treated cells allowed stabilization of 7002-*spsA*-*spp*A transcripts relative to controls without the antibiotic (Fig. 3B, C). Additionally, our experiments report two different transcriptional start sites (Fig. 4) that differ by 32 bases in length. Both transcripts are particularly evident after a salt treatment. A transcriptional regulation can be suggested for *spsA*-*sppA* expression from the presence of a consensus motif characteristic of promoters of stress-regulated genes [21], specially osmotic- and salt-activated genes.

In summary, the regulation of the SPS and SPP genes involved in Suc biosynthesis in the marine cyanobacterium *Synechoccoccus* sp. PCC 7002 can be coordinated by their cluster organization, which is the first report of such coordination in an oxygenic-photosynthetic organism.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.10.040.

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