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Sucrose in bloom-forming cyanobacteria: loss and gain of genes involved in its biosynthesis

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Summary

Bloom-forming cyanobacteria are widely distributed in freshwater ecosystems. To cope with salinity fluctuations, cyanobacteria synthesize compatible solutes, such as sucrose, to maintain the intracellular osmotic balance. The screening of cyanobacterial genomes revealed that homologues to sucrose metabolism-related genes only occur in few bloom-forming strains, mostly belonging to Nostocales and Stigonematales orders. Remarkably, among Chroococcales and Oscillatoriales strains, homologues were only found in *M. aeruginosa* PCC 7806 and *Leptolyngbya boryana* PCC 6306, suggesting a massive loss of sucrose metabolism in bloom-forming strains of these orders. After a complete functional characterization of sucrose genes in *M. aeruginosa* PCC 7806, we showed that sucrose metabolism depends on the expression of a gene cluster that defines a transcriptional unit, unique among all sucrose-containing cyanobacteria. It was also demonstrated that the expression of the encoding genes of sucrose-related proteins is stimulated by salt. In view of its ancestral origin in cyanobacteria, the fact that most bloom-forming strains lack sucrose metabolism indicates that the genes involved might have been lost during evolution. However, in a particular strain, like *M. aeruginosa* PCC 7806, sucrose synthesis genes were probably regained by horizontal gene transfer, which could be hypothesized as a response to salinity fluctuations.

Introduction

Cyanobacteria are oxygenic-photosynthetic bacteria found worldwide in freshwater, marine and brackish habitats, as well as in terrestrial ecosystems (Whitton and Potts, 2012). In nutrient-rich waters, associated with urban, agricultural and industrial development, some particular strains are able to grow exponentially and produce high biomass concentration. These cell proliferations (also known as blooms) pose a threat to human health when toxic strains (i.e. producers of potent cyanotoxins) are involved (Metcalf and Codd, 2012; Whitton and Potts, 2012). The most common strains associated with blooms belong to either nitrogen-fixing (e.g. *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Lyngbya*, *Nodularia*, *Oscillatoria* and *Trichodesmium*) or non-nitrogen-fixing (e.g. *Microcystis* and *Planktothrix*) genera (Paerl and Paul, 2012). *Microcystis* are cosmopolitan cyanobacteria that contain gas vesicles which provide buoyancy (Whitton and Potts, 2012), and as a consequence tend to accumulate in dense blooms at the water surface. *Microcystis* strains can produce toxins known as microcystins, potent hepatotoxic compounds responsible for human and animal poisoning (Metcalf and Codd, 2012).

In freshwater ecosystems, salinity may increase owing to different processes like long-term droughts, agricultural practices and water management strategies (Paerl and Paul, 2012). The survival of cyanobacteria under these conditions is chiefly explained by the accumulation of compatible solutes that buffer the osmotic potential of the cell in salty or hypertonic environments, and contribute to maintain membrane integrity and protein stability (Hagemann, 2011). This feature seems to be important for cyanobacteria to grow with advantages over other phytoplankton components (Paerl and Paul, 2012). Among cyanobacteria, a correlation was established between the salt tolerance limit and the major compatible solute. While sucrose (Suc) and/or trehalose are the characteristic compounds produced by freshwater strains that tolerate up to 0.7 M NaCl, glucosylglycerol and glucosylglycerate are the main osmolytes in cyanobacteria with moderate salt tolerance (up to 1.7 M NaCl), which include marine strains. Nonetheless, glucosylglycerol is also found in freshwater isolates (Hagemann, 2011; Klähn and Hagemann, 2011).

Suc biosynthesis has been reported in unicellular and filamentous heterocyst-forming cyanobacteria (Salerno and Curatti, 2003). It occurs by the sequential action of

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Suc-phosphate synthase (SPS, EC 2.4.1.14) and Suc-phosphate phosphatase (SPP, EC 3.1.3.24). On the other hand, Suc catabolism can be carried out by Suc synthase (SuS) (which catalyses the reversible cleavage of Suc yielding a sugar nucleotide and fructose), alkaline/neutral-invertase (which irreversibly hydrolyses Suc into glucose and fructose) and amylosucrase (which hydrolyses Suc into hexoses and/or transfers the glucose moiety to a maltooligosaccharide or an α 1,4-glucan) (Kolman *et al*., 2015).

Suc accumulation was first associated with salinity (Hagemann and Erdmann, 1997). Recent studies have enhanced our knowledge on other physiological functions of this disaccharide. In heterocyst-forming strains, Suc plays a key role as an intermediate in the flux of reduced carbon in the filaments (Cumino *et al*., 2007; Marcozzi *et al*., 2009), in glycogen accumulation (Cumino *et al*., 2007; Curatti *et al*., 2008) and in diazotrophic growth (Vargas *et al*., 2011). In the unicellular strain *Synechocystis* sp. PCC 6803, Suc was also proposed to be involved in triggering signalling cascades as part of a general response to salinity changes (Desplats *et al*., 2005).

SPS and SPP have been characterized in cyanobacteria belonging to Chroococcales and Nostocales (Kolman *et al*., 2015). Studies conducted on *Anabaena* strains and *Synechocystis* sp. PCC 6803 have shown that SPS and SPP are proteins with modular architecture (Salerno and Curatti, 2003). Particularly, SPS and SPP from *Nostoc* sp. PCC 7120 (also known as *Anabaena* sp. PCC 7120) define minimal catalytic units, corresponding to a functional glucosyltransferase (GT) and a phosphohydrolase (PH) respectively. The analysis of SPS proteins of different cyanobacterial strains and plants allowed for the identification of (i) unidomainal-type SPS, containing only a GT domain (GTD), and (ii) bidomainal-type SPS, with a GTD fused to a non-functional PH domain (GTD-PHD) (Salerno and Curatti, 2003). On the other hand, phylogenetic analysis based on GTD and PHD sequences pointed towards an ancient origin of Suc metabolism in unicellular strains. This hypothesis is supported by the presence of Suc related sequences in *Gloeobacter violaceus* (Kolman *et al*., 2015), which belongs to a lineage that diverged earliest within the cyanobacterial phylogenetic radiation.

In this study, we investigated the presence of Suc metabolism-related sequences in genomes of bloom-forming cyanobacteria. Suc genes were retained in Stigonematales and Nostocales strains, probably due to the role of Suc in nitrogen fixation, and lost in most members of Oscillatoriales and Chroococcales. Interestingly, among Chroococcales, *Microcystis aeruginosa* PCC 7806 is currently the only strain with the genetic capability for Suc biosynthesis. Suc metabolism genes in this strain are arranged in a single transcriptional unit that is upregulated by salt stress. We hypothesize that *M. aeruginosa* sp. PCC 7806 might have acquired Suc metabolism genes by lateral gene transfer, contributing to the adaptation of the strain to fluctuations in salinity.

Results

Suc metabolism gene sequences in bloom-forming cyanobacteria

Forty eight out of 191 fully sequenced genomes belong to the genera generally associated with blooms (Paerl, 2014). Among them, only 18 genomes harbour sequences homologous to SPS and SPP encoding genes. Table 1 shows that Suc metabolism-related sequences are ubiquitous among Nostocales and Stigonematales strains. Most Nostocales genomes exhibit sequences coding for putative unidomainal-type SPS (c.a. 420 amino acid residues), which in many cases are accompanied by bidomainal-type SPS (c.a. 730 amino acid residues). Conversely, among Chroococcales and Oscillatoriales, sequences homologous to SPS encoding genes could only be retrieved from the genomes of *M. aeruginosa* PCC 7806 (unicellular strain, hereinafter PCC 7806) and of *Leptolyngbya boryana* PCC 6306 (filamentous nitrogen-fixing strain) respectively. Both strains present unidomainal-type SPS.

Alignments of SPS and SPP deduced amino acid sequences showed that the characteristic motifs of the GTD and PHD modules (Cumino *et al*., 2002) and the critical residues identified after protein crystallization (Fieulaine *et al*., 2005; Chua *et al*., 2008) are conserved (Fig. S1).

The lack of homologous sequences related to Suc metabolism in 30 genomes of bloom-forming cyanobacteria prompted the search for sequences related to the synthesis of other cyanobacterial compatible solutes produced in response to salinity. From most Oscillatoriales genomes, we retrieved homologues to genes coding for proteins related to trehalose synthesis (Table 1), and in some cases also homologues to genes coding for proteins involved in glucosylglycerol synthesis (Table S1). Surprisingly, no sequence related to the synthesis of any described osmolyte could be retrieved from the other 13 genomes of *M. aeruginosa* that lacked Suc-related sequences (Table S1) (Klähn and Hagemann, 2011).

Functional identification of Suc synthesis genes in PCC 7806

For the evolutionary and physiological analyses, we conducted the functional identification of PCC 7806

Table 1. Amino acid deduced sequences homologous to Suc biosynthesis proteins (SPS and SPP) and trehalose metabolism proteins [maltooligosyl trehalose synthase (TreY), maltooligosyltrehalose trehalohydrolase (TreZ) and trehalase (TreH)] in cyanobacterial strains belonging to genera generally associated with blooms.

		SPS			SPP		TreY		TreZ		TreH	
Order	Strain	Size (aa)	ID %[1]	ID %[2]	Size (aa)	ID %[3]	Size (aa)[4]	ID %	Size (aa)[5]	ID %	Size (aa)[6]	ID %
Chroococcales	*Microcystis aeruginosa* PCC 7806	490	56	29	250	47	–	–	–	–	–	–
	M. aeruginosa PCC 7941	–	–	–	–	–	–	–	–	–	–	–
	M. aeruginosa PCC 9432	–	–	–	–	–	–	–	–	–	–	–
	M. aeruginosa PCC 9443	–	–	–	–	–	–	–	–	–	–	–
	M. aeruginosa PCC 9701	–	–	–	–	–	–	–	–	–	–	–
	M. aeruginosa PCC 9717	–	–	–	–	–	–	–	–	–	–	–
	M. aeruginosa PCC 9806	–	–	–	–	–	–	–	–	–	–	–
	M. aeruginosa PCC 9807	–	–	–	–	–	–	–	–	–	–	–
	M. aeruginosa PCC 9808	–	–	–	–	–	–	–	–	–	–	–
	M. aeruginosa PCC 9809	–	–	–	–	–	–	–	–	–	–	–
	M. aeruginosa TAIHU98	–	–	–	–	–	–	–	–	–	–	–
	M. aeruginosa FCY-26	–	–	–	–	–	–	–	–	–	–	–
	M. aeruginosa FCY-28	–	–	–	–	–	–	–	–	–	–	–
	M. aeruginosa NIES-843	–	–	–	–	–	–	–	–	–	–	–
Oscillatoriales	*Leptolyngbya boryana* PCC 6306	422 458	68 58	26 28	247	41	919	60	596	61	–	–
	Leptolyngbya sp. PCC 6406	–	–	–	–	–	–	–	–	–	–	–
	Lyngbya sp. CCY 8106	–	–	–	–	–	–	–	–	–	–	–
	Oscillatoria formosa PCC 6407	–	–	–	–	–	930	61	607	55	–	–
	Oscillatoria sp. PCC 10802	–	–	–	–	–	933	62	606	55	–	–
	Oscillatoria sp. PCC 6506	–	–	–	–	–	930	61	607	55	–	–
	Planktothrix agardhii NIVA-CYA 126/8	–	–	–	–	–	934	57	614	58	–	–
	P. agardhii NIVA-CYA 34	–	–	–	–	–	934	57	606	59	–	–
	P. agardhii NIVA-CYA 56/3	–	–	–	–	–	934	56	606	59	–	–
	Planktothrix NIVA-CYA405	–	–	–	–	–	934	57	606	59	–	–
	Planktothrix NIVA-CYA406	–	–	–	–	–	548	53	606	59	–	–
	P. prolifica NIVA-CYA 540	–	–	–	–	–	934	57	606	59	–	–
	P. rubescens NIVA-CYA 98	–	–	–	–	–	934	57	606	59	–	–
	Planktothrix sp. 585	–	–	–	–	–	531	57	614	58	–	–
	Planktothrix sp. NIVA CYA 15	–	–	–	–	–	934	57	606	59	–	–
	Planktothrix sp. NIVA-CYA407	–	–	–	–	–	934	57	614	59	–	–
	Planktothrix sp. st147	–	–	–	–	–	934	56	614	58	–	–
	Trichodesmium erythraeum IMS101	–	–	–	–	–	–	–	–	–	–	–
Nostocales	*Anabaena cylindrica* PCC 7122	421 733	66 26	25 51	261	39	930	81	614	82	506	80
	Anabaena sp. PCC 7108	422 729	67 27	26 50	248	40	–	–	–	–	–	–
	Anabaena sp. 90	422 728	68 28	25 52	248	48	–	–	–	–	–	–
	A. variabilis ATCC 29413	425 422	96 68	28 26	249	40	922	94	606	93	495	98
	Cylindrospermopsis raciborskii CS-505	419	64	25	264	37	–	–	–	–	–	–
	C. raciborskii CS-506	419	64	25	264	37	–	–	–	–	–	–
	C. raciborskii CS-509	419	64	25	264	37	–	–	–	–	–	–
	Nodularia spumigena CCY9414	421 733	68 27	25 52	249	42	944	62	608	61	–	–
	Nostoc sp. PCC 7107	736	26	50	249 250	39 38	921	86	601	88	505	91
	Nostoc sp. PCC 7120	429 422	100 67	27 25	181	38	922	100	620	100	495	100
	Nostoc sp. PCC 7524	422 734	67 27	25 51	249	41	–	–	–	–	–	–
	Raphidiopsis brookii D9	–	–	–	–	–	–	–	–	–	–	–
Stigonematales	*Fischerella* sp. PCC 9605	424	72	25	250 249	47 41	939	57	610	58	–	–
	Fischerella sp. JSC-11	424	71	26	249	42	942	56	609	59	–	–
	Fischerella sp. PCC 9339	424	71	26	251 254	47 39	944	57	609	59	–	–
	Fischerella sp. PCC 9431	424	72	26	251 249	46 41	944	57	649	57	–	–

BLASTP searches were carried out using as query characterized SPS and SPP [([1]) *Nostoc* sp. PCC 7120 SPS-A, ([2]) *Synechocystis* sp. PCC 6803 SPS, and ([3]) *Synechocystis* sp. PCC 6803 SPP], and protein sequences related to trehalose metabolism [*Nostoc* sp. PCC 7120 TreY ([4]) and TreZ ([5]), and TreH ([6])]. Percentages correspond to amino acid identity. aa, amino acid residues.

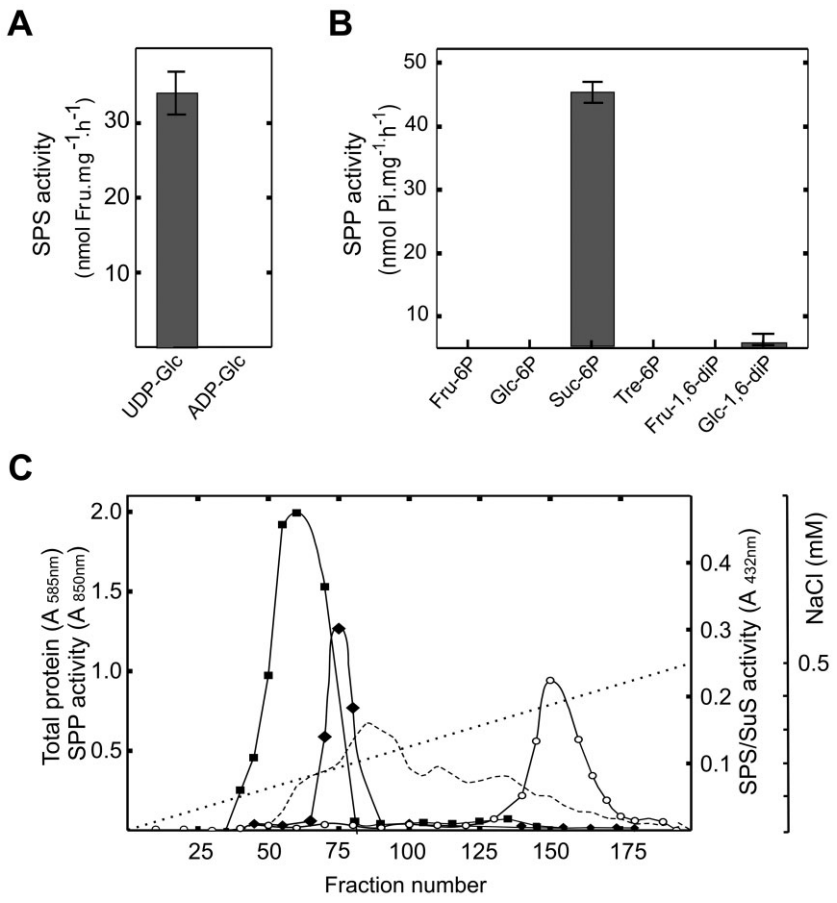

Fig. 1. Functional characterization of SPS and SPP encoding genes from *M. aeruginosa* PCC 7806.
A. SPS activity of the recombinant His_6:: IPF_1564 protein obtained from the expression of the IPF_1564 *orf* in *E. coli* cells. The reaction mixture contained Fru-6P and UDP-Glc (or ADP-Glc) as substrates.
B. Phosphohydrolase (SPP) activity of the recombinant His_6:: IPF_1566 protein obtained from the expression of the IPF_1566 *orf* in *E. coli* cells. Different phosphorylated sugars (Fru-6P, Glc-6P, Suc-6P, Tre-6P, Fru-1,6-diP and Glc-1,6-diP) were assayed as substrate.
C. DEAE-Sephacel chromatography of crude extracts from PCC 7806 cells. SPS (◆) and SPP (■). To complete Suc metabolism enzymes, SuS activity (○) was included. Protein measured as $A_{585\ nm}$ (– – –). The dotted line represents the NaCl gradient.

sequences homologous to SPS and SPP encoding genes. We retrieved two open reading frames (*orf*s IPF_1564 and IPF_1566), 56% and 53% identical to *Nostoc* sp. PCC 7120 SPS-A and SPP encoding genes respectively. These *orf*s were expressed in a heterologous system, and the recombinant His_6-tagged proteins were purified and analysed. The results provided in Fig. 1A and B indicate that His_6:: IPF_1564 is able to catalyse the synthesis of Suc-6P in the presence of fructose and UDP-Glc, and that His_6::IPF_1566 exhibits a specific phosphatase activity, removing the phosphate group from Suc-6P. Consequently, IPF_1564 and IPF_1566 *orf*s correspond to SPS and SPP encoding genes (named *spsA* and *sppA*) respectively. SPS and SPP activity were also measured in partially purified PCC 7806 protein extracts to fully support that this strain is able to synthesize Suc (Fig. 1C).

Phylogenetic analysis of PCC 7806 SPS and SPP

Phylogenetic trees were obtained after multiple alignments of the full-length sequences of PCC 7806 SPS and SPP with homologues from other cyanobacterial strains. As displayed in Fig. 2, SPS sequences group in two clusters, corresponding to unidomainal- (cluster I) and bidomainal-type (cluster II) proteins. Besides, unidomainal-type SPS sequences of filamentous cyanobacteria group in subcluster IA and those of most unicellular strains are found in subcluster IB, including PCC 7806 SPS. The phylogenetic analysis of SPP indicates that sequences from filamentous and unicellular bloom-forming strains also group in two separate clusters (Fig. 3). PCC 7806 SPP is among the subcluster IB sequences that include SPP from unicellular strains.

Suc gene arrangement in PCC 7806 genome

The analysis of Suc gene location in the PCC 7806 genome revealed that *spsA* and *sppA* flank the SuS encoding gene (*susA*), previously characterized (Kolman *et al.*, 2012). The *spsA-susA-sppA* arrangement (Fig. 4A) is a Suc cluster included in an approximately 15 kb island (7806-*Suc* island) with imperfect terminal repeats (IR1 and IR2 of 54 bp in length). Interestingly, the 7806-*Suc* island harbours an *orf* homologous to a transposase (ISMae42). No syntenic region could be detected when a global comparison between the 7806-*Suc* island genomic

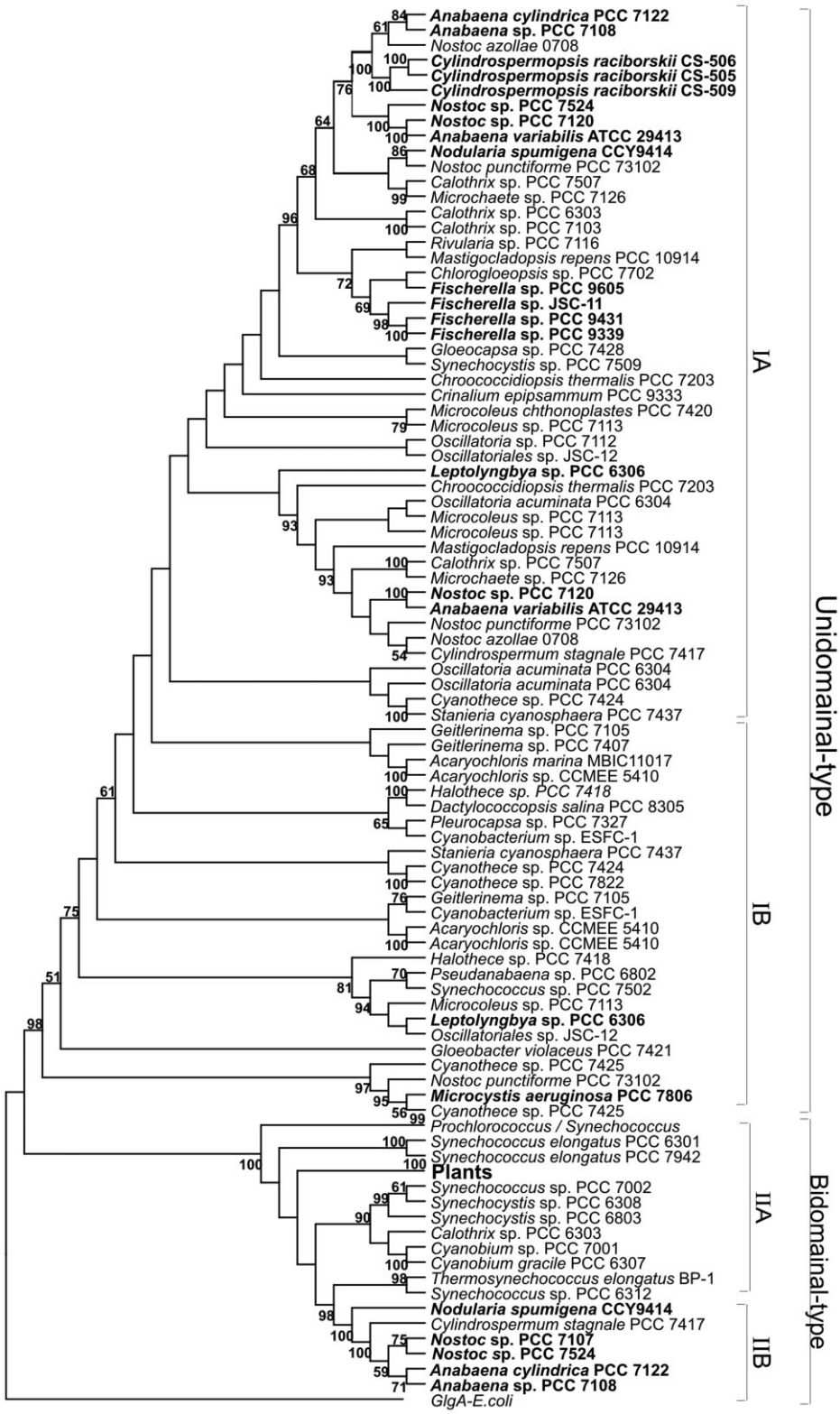

Fig. 2. Phylogenetic analysis of SPS proteins. Representation of consensus maximum likelihood tree obtained from 1000 replicates using the JTT + F + Gamma model after sequence alignments of deduced amino acid SPS sequences using a BLOSSUM 62 matrix. Qualitatively similar tree topologies were observed when using a neighbour-joining algorithm. Bloom-forming cyanobacterial SPS sequences are in bold letters. Evolutionary analyses were conducted in MEGA5. *E. coli* glycogen synthase (*glgA* AAA23870.1) was used as outgroup. Accession numbers of sequences used can be found in Table S2.

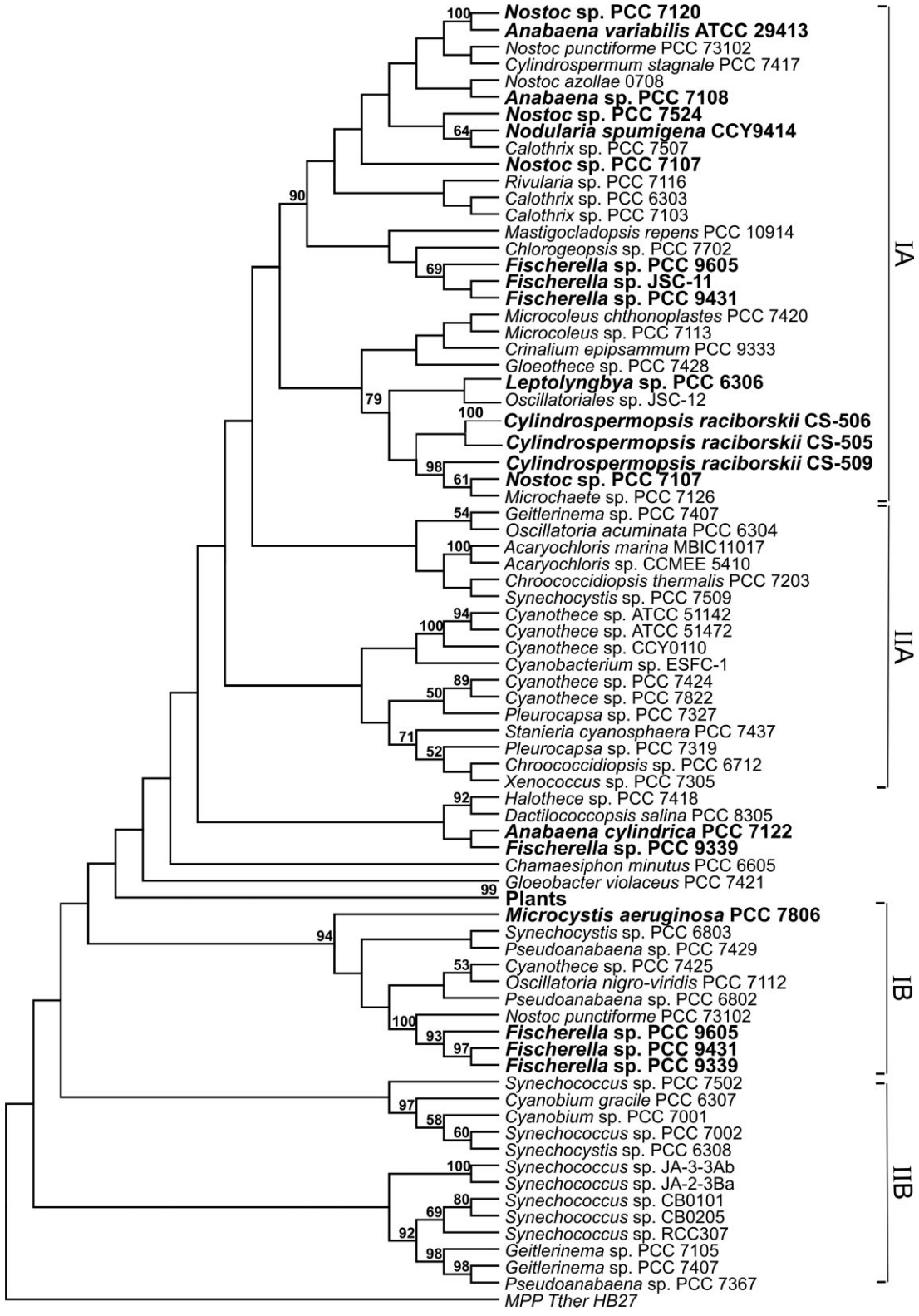

Fig. 3. Phylogenetic analysis of SPP proteins. Representation of consensus maximum likelihood tree obtained from 1000 replicates using the JTT + F + Gamma model after sequence alignments of deduced amino acid SPP sequences using a BLOSSUM 62 matrix. Qualitatively similar tree topologies were observed when using a neighbour-joining algorithm. Evolutionary analyses were conducted in MEGA5. Bloom-forming cyanobacterial SPP sequences are in bold letters. *Thermus thermophilus* Hb27 mannosyl-phosphate phosphatase (MPP YP_004564.1) was used as outgroup. Accession numbers of sequences used can be found in Table S2.

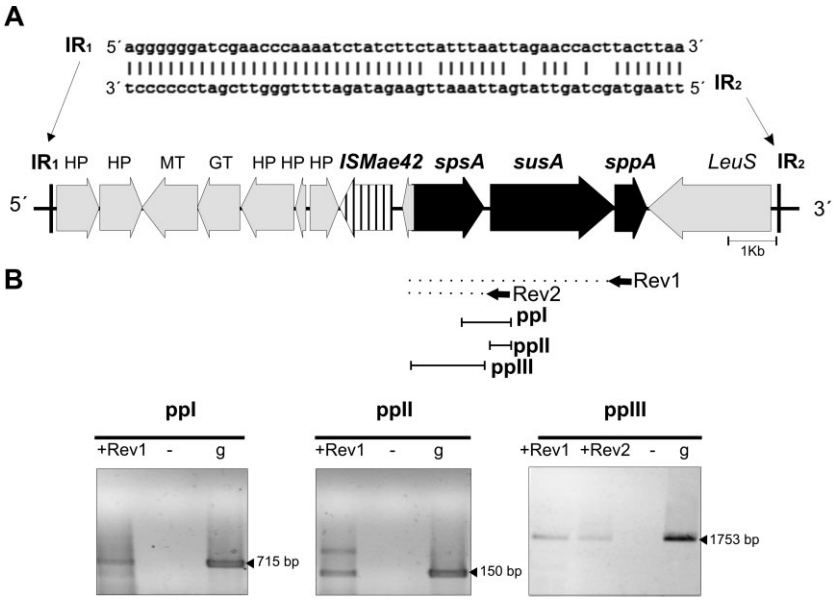

Fig. 4. Organization of Suc genes in the *M. aeruginosa* PCC 7806 genome.
A. 7806-*Suc* island containing: the 7806-*Suc* cluster, black filled [Suc synthesis (*spsA* and *sppA*) and degradation (*susA*) genes]; island terminal inverted repeat sequences (IR1 and IR2); ISMae42, putative transposase sequence (streaked). *orf*s marked in grey are not related to Suc metabolism and were noted as putative genes encoding: GT, glycosyltransferase; MT, mannosyltransferase; HP, hypothetical protein.
B. RT-PCR products from the amplification of total RNA prepared from exponential-phase cultures using oligonucleotide primer Rev1 or Rev2 (initial positions indicated in the above scheme in panel B). The positions of the primers correspond to the ends of the segments indicated in panel B. RNA samples subject to retrotranscription (+) or non-retrotranscribed (–); g, total genomic DNA used as template for amplification.

region and those of the other *M. aeruginosa* sequenced genomes was performed.

The *in silico* analysis of the upstream region of the Suc cluster revealed the presence of a putative promoter located at –87 bp from the start translation codon of *spsA* (Fig. S2), suggesting a possible polycistronic transcription. Accordingly, we performed a reverse transcription polymerase chain reaction (RT-PCR) analysis, from cDNA obtained using two different reverse primers for retrotranscription (Rev1 and Rev2) and PCR amplifications with three primer pairs (ppI to ppIII) (Fig. 4B, Table S3). As shown in Fig. 4B, amplification products indicate the existence of a transcriptional unit.

Suc as a compatible solute in PCC 7806 salt-stressed cells

Despite the fact that Suc synthesis seems to be almost universal among cyanobacteria (Hagemann, 2011; Kolman *et al.*, 2015), as shown above, this is not the case for most *Microcystis* strains. The characterization of Suc biosynthesis pathway in PCC 7806 strain raises the question of the role played by the disaccharide. The effect of salt on Suc and glycogen accumulation was assessed, considering the relevance of Suc synthesis for salt acclimation and its relationship with glycogen in some cyanobacteria (Blumwald and Tel-Or, 1982; Erdmann, 1983; Kolman *et al.*, 2015). NaCl was added to exponential-phase cultures and cells were harvested after 24 h. The treatment resulted in a nearly five- and threefold increases in Suc and glycogen content, respectively, which could be reversed by transferring the cells to BG_{11}–KNO_3 basal medium (Fig. 5A and B). The analysis of *spsA* and *sppA* expression revealed that transcript levels increase 30 min after the onset of salt addition, steadily up to 10 h, and strongly decrease between 10 and 24 h (Fig. 5C).

Discussion

A recent integrated phylogenetic approach for the analysis of compatible solute genes supports a freshwater

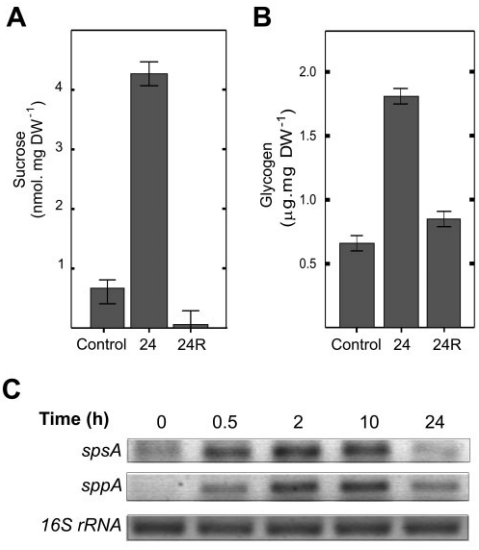

Fig. 5. Effect of NaCl on Suc and glycogen accumulation in *M. aeruginosa* PCC 7806 cells. Determination of Suc (A) and glycogen (B) content. Exponential-phase cultures were added with 180 mM NaCl and cells were harvested after 24 h. Control cells were cultured in BG_{11}–KNO_3 for 24 h. For reversion analysis, 24 h salt-treated cells were transferred to BG_{11}–KNO_3 basal medium for 24 h (24R). (C) Effect of salt on *spsA* and *sppA* transcript levels in PCC 7806 cells analysed by RT-PCR. Total RNA was purified from cells cultured in BG_{11}–KNO_3 (0) or from cells harvested 0.5, 2, 10 and 24 h after salt addition. Amplification products of the 16S *rRNA* gene were used as reaction control (bottom panel).

origin for cyanobacteria and the ancestral emergence of Suc synthesis associated with growth in a low salinity environment (Blank, 2013). This study also proposes that genes coding for trehalose synthesis proteins (the other major compatible solute reported in freshwater strains) may have been acquired by several lineages most likely by lateral gene transfer. Our investigation about the presence of homologues to genes involved in Suc synthesis in genomes of bloom-forming cyanobacteria (Table 1) revealed that they are not ubiquitous, but that they are present in most heterocyst-forming filamentous strains (Nostocales and Stigonematales), often together with homologues to genes related to trehalose synthesis. *Raphidiopsis brokii* D9 is an exception since no sequence related to the synthesis of any compatible solutes described so far could be retrieved. This feature may be ascribed to genome erosion, an evolutionary process that was also proposed for *Raphidiopsis*' lack of nitrogen fixation ability (Stucken *et al*., 2010). Remarkably, Suc physiological function in Nostocales strains seems not to be a mere salt-response molecule. In *Nostoc* strains, the key role of Suc for carbon trafficking and heterotrophic metabolism of heterocysts was fully demonstrated (Curatti *et al*., 2002; Cumino *et al*., 2007; Lopez-Igual *et al*., 2011; Vargas *et al*., 2011), as well as its relationship with glycogen and other polysaccharide accumulation (Curatti *et al*., 2008). Among Oscillatoriales and Chroococcales, only *L. boryana* PCC 6306 and *M. aeruginosa* PCC 7806 retained Suc synthesis genes. Also, *Planktothrix* and *Oscillatoria* strains have the genes to synthesize trehalose as a compatible solute. It may be hypothesized that a massive loss of Suc metabolism genes may have occurred during cyanobacterial radiation, and that in Oscillatoriales trehalose synthesis genes may have been acquired by lateral transfer (Blank, 2013). It could be concluded that the presence of Suc metabolism is not associated with the potential to bloom but rather with the adaptation to certain environmental conditions.

Except for PCC 7806, *Microcystis* strains lack sequences homologous to those known to participate in osmolyte biosynthesis. PCC 7806 synthesizes Suc in response to salinity, which is in line with a rise in the transcription level of the genes involved. This strain also accumulates glycogen under the same treatment, which could be ascribed to a higher ADP-Glc supply from Suc cleavage by SuS (Cumino *et al*., 2007; Curatti *et al*., 2008), an enzyme that has already been shown to be activated by salt (Kolman *et al*., 2012).

The coordinated transcription of the 7806-*Suc* island genes could be advantageous in terms of the ability to activate both Suc synthesis and breakdown, which could generate a 'Suc cycling' in response to salinity. This mechanism, still not fully elucidated, has been reported in plant tissues, and more recently in *Nostoc* sp. PCC 7120 (G.L. Salerno, pers. comm., 2015). Such a cycling was suggested to allow the net flux through the involved pathway to respond to external factors with a high degree of sensitivity, modulating rates of synthesis and degradation, controlling respiration and sugar accumulation, maintaining osmotic potential, and promoting sugar signalling (Roby *et al*., 2002). The inconsistency derived from comparing the 16S rRNA phylogenetic tree (Shih *et al*., 2012) with those of Suc genes (Figs 2 and 3), and the presence of a putative transposase gene, indicative of a mobile genetic element (Lin *et al*., 2011) in the 7806-*Suc* island, support the re-acquisition of Suc genes by horizontal transfer during the evolutionary history of the strain. In the context of aquatic ecosystems, where massive cell lysis events can take place, lateral gene transfer via natural transformation is a likely scenario (Lorenz and Wackernagel, 1994). This mechanism has been reported for PCC 7806 cells that exhibit pilus-like structures, necessary for DNA internalization, and the machinery for the integration of exogenous DNA fragments (Nakasugi and Neilan, 2005). Recently, the horizontal transfer of a Suc transcriptional unit from methanotroph bacteria to cyanobacteria has been reported in seven *Synechococcus* strains (Perez-Cenci and Salerno, 2014).

The re-acquisition of the complete set of genes to metabolize Suc could have conferred some environmental advantage to PCC 7806 cells. Even though *Microcystis* strains have freshwater habitat origins (Table S4), strain PCC 7806 was isolated from the Braakman water reservoir (The Netherlands), which has a history of ocean water intrusion (Guljamow *et al*., 2007), and as such bears euryhaline communities of algae (Nienhuis, 2008). We speculate that this habitat could have been important to genetically fix Suc metabolism genes in the PCC 7806 strain.

Experimental procedures

Biological material and culture conditions

The *M. aeruginosa* PCC 7806 cells were cultured in $BG11_0$ medium supplemented with 2 mM $NaNO_3$ and 10 mM $NaHCO_3$ under white fluorescent light (30 $\mu E\ m^{-2}s^{-1}$), at 20°C with orbital shaking. To study the effect of salt, cells previously cultured in BG11-KNO_3 (where $NaNO_3$ was replaced by KNO_3) up to exponential phase were collected and grown either in basal medium or in basal medium supplemented with NaCl (180 mM final concentration). After the salt treatment, cells were harvested, washed and stored at –80°C until use.

Escherichia coli DH5α and BL21(λDE3):pLysS strains (Novagen) were grown in Luria–Bertani liquid or agar medium with 30 $\mu g.ml^{-1}$ chloramphenicol and 50 $\mu g.ml^{-1}$ carbenicillin at 37°C, and used for cloning and recombinant protein production respectively.

Isolation and manipulation of nucleic acids

DNA experimental procedures were performed according to standard protocols (Sambrook and Russell, 1989). Isolation and purification of RNA were carried out using the TRIZOL reagent (Gibco–BRL/Invitrogen). RNA quality was visualized after electrophoresis in 1% agarose gels and stained with ethidium bromide.

Amplification, cloning and identification of SPS and SPP encoding genes

Homologous sequences to *Nostoc* sp. PCC 7120 *spsA* and *Synechocystis* sp. PCC 6803 *sppA* were retrieved from PCC 7806 genome. DNA fragments were PCR-amplified using the primer pairs described in Table S3. The PCR products were ligated into the expression vector pRSET-A (Invitrogen) between the restriction sites *Bam*HI and *Kpn*I obtaining the recombinant plasmids pR-*Ma-sps* and pR-*Ma-spp*. The identity of each construct was confirmed by DNA sequencing. The *E. coli* BL21(DE3)pLysS cells were transformed with pR-*Ma-sps* or pR-*Ma-spp* to produce the recombinant proteins His_6::IPF_1564 and His_6:: IPF_1566. Sequences functionally characterized in this study have been deposited in the GenBank database as the encoding genes of PCC 7806 *spsA* (Accession Number JQ742077) and *sppA* (Accession Number JQ742078).

RT-PCR

For RT-PCR analysis, total RNA (1 μg) treated with DNAse (RQI Rnase-free Dnase, Promega) was reverse transcribed using MMLV (Moloney murine leukaemia virus) reverse transcriptase (Promega) and specific reverse primers (Table S3). The PCR reactions were run on a Mastercycler Epgradient (Eppendorf) for 24 cycles of 94°C (1 min), 65°C (30 s) and 72°C (45 s), and a single step at 72°C (5 min). Standardization reactions were carried out as described (Cumino *et al*., 2007). As a control of the relative amount of total RNA used in each RT-PCR reaction, aliquots of the same RNA were reverse-transcribed in parallel and subjected to 18 cycles of PCR with *16S-RNA* specific primers (Sevilla *et al*., 2010).

To analyse the presence of a polycistronic mRNA, 1 μg of total RNA was mixed with 40 ρmol of oligonucleotide Rev1 (*rev-SPPMae*) or Rev2 (*rev-SPS-SuS IG*). Extension reactions were carried out at 47°C for 1 h in a final volume of 32 μl containing 0.25 mM each deoxynucleoside triphosphate, 100 U of reverse transcriptase (Superscript II; Invitrogen) and the buffer recommended by the provider. To control the presence of contaminating DNA, samples containing 1 μg of RNA were processed without the reverse transcriptase (-RT control). Polymerase chain reaction was carried out with 2 μl of a retrotranscription mixture as the template and the following oligonucleotide primer pairs: *fwd-SPS-SuS IG*/*rev-SPS-SuS IG* (for the fragment ppI), *fwd-SuSMae*/*rev-SPS-SuS IG* (fragment ppII) and *fwd-SPSMae*/*rev-SPSMae* (fragment ppIII). Samples containing DNA as the template and the same oligonucleotides were run in parallel as controls. Polymerase chain reaction was performed by standard procedures, and the PCR products were resolved by electrophoresis in 1% agarose gels.

Protein extracts and purification

Protein extracts from PCC 7806 cells were carried out from cultures at exponential phase as described (Porchia and Salerno, 1996). Extracts were desalted through Sephadex G-50 columns before enzyme activity assays (Cumino *et al*., 2001).

His_6::IPF_1564 and His_6:: IPF_1566 fusion proteins were purified by Ni^{2+} affinity chromatography (Ni-NTA Purification System). Recombinant proteins were eluted from the column with a stepwise imidazole pH 7.0 solution (50, 100 and 150 mM). Fractions with SPS or SPP activity were pooled and concentrated in an Amicon (Newtown, PA) ultrafiltration cell. Purified enzymes were stored at –20°C (Kolman *et al*., 2012). The SPS and SPP partial purification from late-exponential phase PCC 7806 cells was performed as described (Porchia and Salerno, 1996). Clarified extracts were loaded onto a DEAE-Sephacel column and proteins were eluted with a linear NaCl gradient (0–0.5 M).

Enzyme assays

Crude extracts prepared from PCC 7806 cells were desalted through Sephadex G-50 columns before enzyme activity assays (Cumino *et al*., 2001). Fractions with SPS and SPP activity were pooled and concentrated. SPS activity was assayed in the presence of 10 mM Fru-6P, 10 mM UDP-Glc (or ADP-Glc), 10 mM $MgCl_2$ and 100 mM Hepes-NaOH pH 7.5, and Suc-6P formation was quantified (Porchia and Salerno, 1996). The SPP activity was assayed by measuring phosphate formation, in a mixture reaction containing 100 mM Hepes-NaOH (pH 6.5), 10 mM $MgCl_2$ and 1 mM Suc-6P, at 30°C. For substrate specificity determination, enzyme activity was assayed in the presence of different sugar-phosphate (fructose-6P, glucose-6P, trehalose-6P, fructose-1,6P or glucose-1,6P), replacing Suc-6P in the reaction mixture. SuS activity was assayed as described (Kolman *et al*., 2012).

Sequence analysis

Deduced amino acid sequences were retrieved from the Joint Genome Institute (http://img.jgi.doe.gov/) by BLASTP searches using as query *Nostoc* sp. PCC 7120 SPS-A, and *Synechocystis* sp. PCC 6803 SPS and SPP. To identify enzymes related to trehalose synthesis, BLASTP searches were carried out using the deduced amino acid sequences of *Nostoc* sp. PCC 7120 TreY, TreZ and TreH. Sequence alignments were generated with CLUSTALW (Thompson *et al*., 1994) and dendrograms were obtained using the neighbour-joining (1000 replicates) and maximum parsimony method from the MEGA5 software (Tamura *et al*., 2011).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Sequence alignment analysis of *M. aeruginosa* SPSs (A) and SPPs (B). Deduced amino acid sequences of GTDs (I and II, characteristic motifs of SPS proteins) and PHDs (III-V, characteristic motifs of SPP proteins). *Microcystis aeruginosa* PCC 7806 (M.aer-7806) is in bold letters. Residues involved in the catalytic mechanism are marked with asterisk (*). *Nostoc* sp. PCC 7120 (Nos-7120), *Anabaena cylindrica* (A.cyl-7122), *Anabaena* sp. PCC 7108 (Ana-7108), *Anabaena variabilis* ATCC 29413 (A.var-29413), *Cylindrospermopsis raciborskii* CS505 (C.rac-CS505), *Cylindrospermopsis raciborskii* CS506 (C.rac-CS506), *Cylindrospermopsis raciborskii* CS509 (C.rac-CS509), *Fischerella* sp. JSC11 (Fis-JSC11), *Fischerella* sp. PCC 9339 (Fisc-9339), *Fischerella* sp. PCC 9431 (Fisc-9431), *Fischerella* sp. PCC 9605 (Fisc-9605), *Leptolyngbya* sp. PCC 6306 (Lepto-6306), *Nodularia spumigena* CCY 9414 (N.spu-9414), *Nostoc punctiforme* PCC 73102 (N.punc-73102), *Nostoc* sp. PCC 7107 (Nos-7107), *Nostoc* sp. PCC 7524 (Nos-7524).

Fig. S2. Prediction of the transcription start site. The region located at the 500 bp upstream of the translation start codon of PCC 7806 *spsA* gene was analysed to predict the putative −35 and −10 boxes (grey-shaded) and the putative transcription start site (in bold letter) using the NNPP server (http://www.fruitfly.org/seq_tools/promoter.html).

Table S1. Accession numbers of deduced amino acid sequences homologous to proteins involved in trehalose, glucosylglycerol and glucosylgerate biosynthesis. BLASTP searches were carried out using as query deduced amino acid sequences of proteins related to trehalose metabolism [*Nostoc* sp. PCC 7120 maltooligosyl trehalose synthase (TreY), maltooligosyltrehalose trehalohydrolase (TreZ) and trehalase (TreH)], glucosylglycerol synthesis [*Synechocystis* sp. PCC 6803 glucosylglycerol-phosphate synthase (GgpS) and glucosylglycerol-phosphate phosphatase (GgpP) from] and glucosylglycerate synthesis [*Persephonella marina* glucosyl 3-phosphoglycerate synthase (GpgS) and glucosyl 3-phosphoglycerate phosphatase (GpgP)].

Table S2. Accession numbers of SPS and SPP deduced amino acid sequences (A and B, respectively) used in phylogeny reconstruction.

Table S3. Primers used in this study.

Table S4. Original habitats of strains belonging to genera generally associated with blooms.