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Altered carbohydrate metabolism in glycogen synthase mutants of *Synechococcus* sp. strain PCC 7002: Cell factories for soluble sugars

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ABSTRACT

Glycogen and compatible solutes are the major polymeric and soluble carbohydrates in cyanobacteria and function as energy reserves and osmoprotectants, respectively. Glycogen synthase null mutants (*glgA-I glgA-II*) were constructed in the cyanobacterium *Synechococcus* sp. strain PCC 7002. Under standard conditions the double mutant produced no glycogen and more soluble sugars. When grown under hypersaline conditions, the *glgA-I glgA-II* mutant accumulated 1.8-fold more soluble sugars (sucrose and glucosylglycer-(ol/ate)) than WT, and these cells spontaneously excreted soluble sugars into the medium at high levels without the need for additional transporters. An average of 27% more soluble sugars was released from the *glgA-I glgA-II* mutant than WT by hypo-osmotic shock. Extracellular vesicles budding from the outer membrane were observed by transmission electron microscopy in *glgA-I glgA-II* cells grown under hypersaline conditions. The *glgA-I glgA-II* mutant serves as a starting point for developing cell factories for photosynthetic production and excretion of sugars.

1. Introduction

Cyanobacteria are photosynthetic prokaryotes that fix atmospheric CO_2 using ATP and reducing power generated from oxygenic photosynthesis. Collectively, cyanobacteria are responsible for the production of one third to one half of global biomass and oxygen in the atmosphere (Rippka et al., 1979; Stanier and Cohen-Bazire, 1977). Glycogen is the major carbohydrate reserve synthesized in most cyanobacteria (Allen, 1984; Nakamura et al., 2005; Preiss, 2006; Shively, 1974). Using α-D-glucose-1-phosphate as the precursor and ADP-glucose as an intermediate, glycogen synthesis is dependent on three enzymes: ADP-glucose pyrophosphorylase (GlgC), glycogen synthase (GlgA) and glycogen branching enzyme (GlgB) (Preiss, 2006). The final product, a homopolyglucan consisting of glucose residues linked through α-1,4-glucosidic linkage and α-1,6-glucosidic linkages at branching points, usually forms granules that occur in the cytoplasm between the thylakoids of cyanobacterial cells (Allen, 1984). Glycogen biosynthesis is tightly regulated in response to different environmental cues. For instance, it has been reported that nitrogen limitation leads to glycogen accumulation in cyanobacteria, while photosynthesis is down-regulated and the antenna system and accessory pigments (*i.e.*, phycobiliproteins (PBP)) are depleted in a response named *chlorosis* (*i.e.*, the cells become yellow–green in coloration) (De Philippis et al., 1992; Sauer et al., 1999; Stevens et al., 1981; Yoo et al., 2007). Cyanobacterial mutants defective in glycogen biosynthesis have altered photosynthesis, respiration, intracellular sugar compositions, and osmotic stress responses (Miao et al., 2003; Suzuki et al., 2010; Suzuki et al., 2007).

In addition to glycogen, cyanobacteria synthesize low molecular weight osmolytes, which are used to balance their intracellular osmotic pressure to that of their environment (Hagemann, 2011; Klähn et al., 2010; Reed et al., 1986a; Reed et al., 1984; Reed and Stewart, 1985). The biosynthetic pathways for osmolytes and glycogen share the same or closely related precursors, and thus the enzymes of these pathways effectively compete for substrates (Fig. 1). In general, high salinity growth conditions result in the accumulation of large amounts of osmolytes (Blumwald and Tel-Or, 1982; Erdmann et al., 1992; Hagemann et al., 1994; Mackay and Norton, 1987; Miao et al., 2003; Warr et al., 1985). A general trend

Abbreviations: PBP, phycobiliproteins; GGol, glucosylglycerol; GGate, glucosylglycerate; GG, glucosylglycerol and glucosylglycerate; Chl a, Chlorophyll a; WT, wild type

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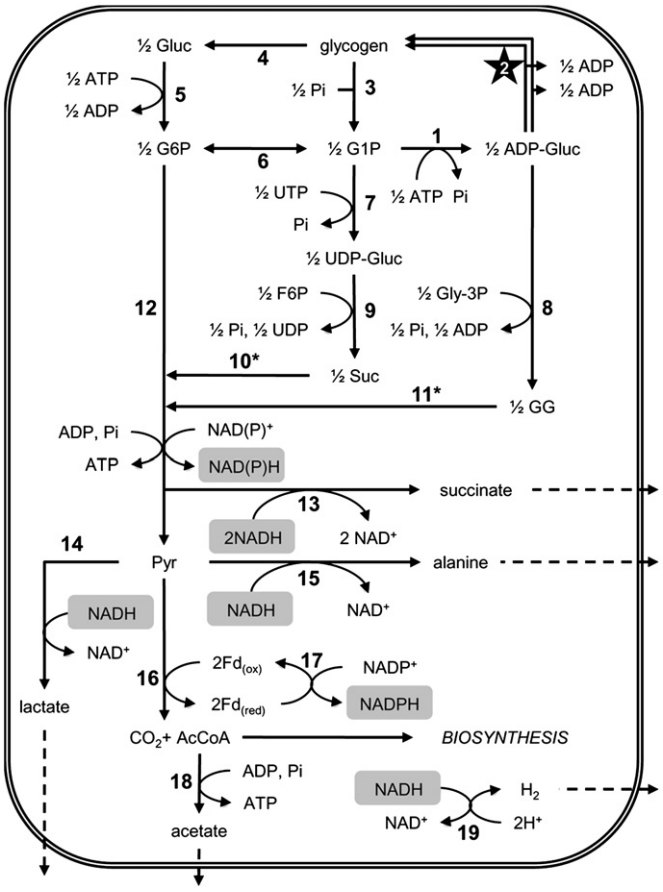

Fig. 1. Pathway model of carbon storage and auto-fermentation in *Synechococcus* sp. strain PCC 7002. The star indicates the glycogen synthase genes (*glgA-I* and *glgA-II*) that were replaced by an antibiotic resistance cassette. A complete legend of compound abbreviations and enzyme identifications is given in Table S1. Asterisk denotes steps where enzymes are unknown.

was found between the intrinsic halotolerance of cyanobacteria and the nature of the main osmolyte employed: sucrose and trehalose are produced by weakly halotolerant cyanobacteria, glucosylglycer-(ol/ate) is produced by moderately halotolerant and halophilic cyanobacteria, and betaines are synthesized in extremely halophilic cyanobacteria (Hagemann, 2011). For example, the freshwater cyanobacterium *Synechocystis* sp. strain PCC 6803 and marine/euryhaline *Synechococcus* sp. strain PCC 7002 both synthesize sucrose and glucosylglycer-(ol/ate) as the main osmolytes (Klähn et al., 2010; Reed and Stewart, 1985). Additionally, cyanobacteria liberate considerable amounts of osmo-protective compounds to the medium when subjected to a hyposaline shock, and the amount of osmolytes released is proportional to the osmolarity change imposed (Fulda et al., 1990; Reed et al., 1986b; Tel-Or et al., 1986; Warr et al., 1985).

The model cyanobacterium *Synechococcus* sp. strain PCC 7002 synthesizes glycogen as its major carbohydrate reserve during photoautotrophic growth; however, the physiological role of the glycogen synthases of this strain have not been studied to date (Stevens et al., 1981). This euryhaline cyanobacterium can tolerate a wide range of salinities, ranging from a few millimolar to more than 2.0 M NaCl (Batterton and Baalen, 1971) by synthesizing glucosylglycerol (GGol) and sucrose as its major and minor osmolytes, respectively. In addition, it can synthesize glucosylglycerate (GGate) in lower amounts (henceforth, GGol and GGate will together be referred as GG) (Klähn et al., 2010; Kollman et al., 1979; Reed and Stewart, 1985). Two genes *spsA* and *sppA*, encoding sucrose phosphate synthase and sucrose phosphate phosphatase respectively, which are involved in sucrose synthesis, were identified in the genome of *Synechococcus* sp. strain PCC 7002 (Cumino et al., 2010). GGol phosphate synthase, GGate phosphate synthase and corresponding genes, *ggpS* and *gpgS* respectively, were described in the *Synechococcus* sp. strain PCC 7002 genome, while a GGol phosphate phosphatase (*ggpP*) similar to the one described in *Synechocystis* sp. strain PCC 6803 and a GGate phosphate phosphatase (*gpgP*) similar to the one described in the bacterium *Persephonella marina* are also identified (Costa et al., 2007; Engelbrecht et al., 1999; Hagemann et al., 1997; Klähn et al., 2010).(Fig. 1and Table S1).

Under dark anoxic conditions, *Synechococcus* sp. strain PCC 7002 metabolism can switch its metabolic growth mode to autofermentation, during which endogenous photosynthetically derived carbohydrates are catabolized to produce ATP and reductant in the form of NAD(P)H for cell maintenance. The production and excretion of H_2, lactate, acetate, alanine and succinate allows for the regeneration of $NAD(P)^+$ to maintain cellular redox poise and to continue ATP production (McNeely et al., 2011; McNeely et al., 2010).

In the current study, the two glycogen synthase genes, *glgA-I* and *glgA-II*, in *Synechococcus* sp. strain PCC 7002 were deleted, and three fully segregated null mutants, *glgA-I*, *glgA-II* and *glgA-I glgA-II* were constructed. We hypothesized that glycogen biosynthesis would be abolished in the *glgA-I glgA-II* mutant and that soluble sugars (*i.e.*, glucose, sucrose, and GG) would accumulate in higher amounts. Additionally, these mutants provided an opportunity to see how cells cope with the anticipated stresses that should occur when glycogen synthesis (induced by N-limitation) or degradation (induced by dark anoxia) is disrupted. For example, a defect in glycogen biosynthesis should affect the physiological response of cells to N-limitation and might lead to increased production of soluble sugars. Similarly, because sucrose and GG function as major cellular osmolytes, their synthesis and accumulation might be further stimulated in the *glgA-I glgA-II* mutant under hypersaline conditions. To test these hypotheses, we characterized the carbohydrate and pigment composition, as well as photosynthetic oxygen evolution and respiratory oxygen consumption properties, of the three mutant strains grown under three different conditions; standard (0.3 M NaCl, 12 mM nitrate as N-source), N-limitation (0.3 M NaCl and 1.2 mM nitrate) and hypersaline (1 M NaCl, 12 mM nitrate), as well as under dark anoxic auto-fermentation conditions (in otherwise standard conditions). In addition, the expression profiles of relevant genes were analyzed under similar conditions. Our data indicate that, the *glgA-I glgA-II* mutant is incapable of synthesizing glycogen and as a result accumulates and excretes large amounts of soluble sugars under certain conditions. This makes this mutant strain useful for future biotechnological applications.

2. Materials and methods

2.1. Deletion of the glgA-I and glgA-II genes

DNA sequences of the *glgA-I* (SYNPCC7002_*A1532*) and *glgA-II* (SYNPCC7002_*A2125*) genes were retrieved from Cyanobase (〈http://genome.kazusa.or.jp/cyanobase/SYNPCC7002〉). Standard molecular biology methods were used to delete these genes by replacing the coding sequences with antibiotic resistance cassettes. A detailed description of the procedure and primer sequences used is available in Supplemental information (SI).

2.2. Strains and cultivation conditions

The WT, *glgA-I*, *glgA-II* and *glgA-I glgA-II* strains were grown in liquid A^+ medium (Stevens et al., 1973) at 38 °C, were illuminated by cool-white fluorescent lamps with an irradiance of 250 μmol photons $m^{-2}\,s^{-1}$, and were sparged with 1% (v/v) CO_2 in air. As defined by