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Metabolomics-based analysis revealing the alteration of primary carbon metabolism by the genetic manipulation of a hydrogenase HoxH in *Synechocystis* sp. PCC 6803

Hiroko Iijima [a,1], Tomokazu Shirai [b,1], Mami Okamoto [b], Filipe Pinto [c], Paula Tamagnini [c,d], Tomohisa Hasunuma [e], Akihiko Kondo [b,f], Masami Yokota Hirai [b], Takashi Osanai [a,b,*]

[a] School of Agriculture, Department of Agricultural Chemistry, Meiji University, 1-1-1, Higashimita, Tama-ku, Kawasaki, Kanagawa 214-8571, Japan
[b] RIKEN Center for Sustainable Resource Science, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan
[c] i3S/IBMC—Instituto de Investigação e Inovação em Saúde & Instituto de Biologia Molecular e Celular, Universidade do Porto, Rua Alfredo Allen, 208, 4200-135, Porto, Portugal
[d] Faculdade de Ciências, Departamento de Biologia, Universidade do Porto, Rua do Campo Alegre, s/n, 4169-007, Porto, Portugal
[e] Graduate School of Science, Technology and Innovation, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan
[f] Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, 1-1, Rokkodai, Nada, Kobe 657-850, Japan

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ABSTRACT

Cyanobacterial hydrogenases are important owing to the association between hydrogen metabolism and cell physiology and the production of future renewable energy. Many studies have examined hydrogen productivity, transcriptional regulation of hydrogenases, and the biochemistry of hydrogenases; however the relationship between hydrogen and primary carbon metabolism using metabolomic techniques has not been elucidated. Here, we studied the effect of the genetic manipulation of a hydrogenase on primary carbon metabolism in the model unicellular cyanobacterium *Synechocystis* sp. PCC 6803. Metabolomic analysis revealed that the *hoxH* mutant with reduced *hoxH* transcripts exhibited increased sugar phosphates in dark, anaerobic conditions. Organic acids, lactate, succinate, fumarate, and malate increased substantially by the *hoxH* mutation both inside and outside of cells in dark, anaerobic conditions. Transcriptome analysis revealed higher expression of genes encoding the RNA polymerase sigma factor SigE, which is a positive regulator of sugar catabolism, and 6-phosphogluconate dehydrogenase in the *hoxH* mutant than in the wild-type strain. Immunoblotting results showed that sugar catabolic enzymes and SigE proteins increased in the *hoxH* mutant. These results demonstrate the wide alterations of primary metabolism by the genetic manipulation of a hydrogenase subunit in this cyanobacterium.

1. Introduction

Cyanobacteria are a group of photosynthetic bacteria that are of particular interest as cell factories capable of producing biomaterials and bioenergy including hydrogen. Many researchers have considered cyanobacteria as a biological chassis for hydrogen production and have tried to increase hydrogenase activity by genetic engineering [11]. Cyanobacteria possess [NiFe]-type hydrogenases, which have bidirectional catalytic properties with a bias towards H_2 production [14]. The physiological role of hydrogenases in cyanobacteria is obscure, but it is speculated that function as electron valves under anaerobic conditions [2]. The bifunctional hydrogenase consists of five subunits, i.e., HoxEFUYH; HoxEFU constitute a diaphorase moiety and HoxYH constitute a hydrogenase moiety [32,35]. The maturation of a hydrogenase complex of the unicellular non-nitrogen-fixing cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803) starts with the binding of unprocessed HoxH and HoxY [5]. The knockout of *hoxH* results in a complete loss of hydrogenase activity; therefore, HoxH is an important subunit for the bidirectional hydrogenase reaction in *Synechocystis* 6803. Proteomic analysis has revealed that the levels of 17 out of 210 detected proteins, which are involved in photosynthesis and respiration, translation, antioxidant defense, energy metabolism, and transcription, are altered by *hoxYH* knockout [29]. Cyanobacterial hydrogenases have been intensively investigated in view of their potential in applied and basic sciences; however, the metabolomes of hydrogenase mutants in *Synechocystis* 6803 have not been analyzed to date.

The discovery of the closed tricarboxylic acid (TCA) cycle in cyanobacteria revealed the importance of this cycle for primary carbon and energy metabolism [39]. The TCA cycle provides reductants for

* Corresponding author at: 1-1-1 Higashimita, Tama-ku, Kawasaki, Kanagawa 214-8571, Japan.
E-mail address: tosanai@meiji.ac.jp (T. Osanai).
[1] These authors are equally contributed to this work.

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oxidative phosphorylation and four-carbon precursors, such as 2-oxoglutarate (2-OG), succinate, fumarate, malate, and oxaloacetate. The reversal of the TCA cycle (i.e., the reductive TCA cycle) is observed under anaerobic conditions, which is widely conserved among various bacteria as a "metabolite-producing device" [1]. In cyanobacteria, an increase in 2-OG levels is a signal of nitrogen starvation, leading to transcriptional activation mediated by the NtcA and PII signaling proteins [18]. In addition, the levels of succinate, fumarate, and malate increase in response to nitrogen depletion, possibly functioning in carbon storage under nitrogen starvation [25]. Organic acids including succinate, lactate, and acetate, are excreted from *Synechocystis* 6803 cells under dark, anaerobic conditions [27]. Hydrogen and organic acid production in anaerobic conditions depend on the carbon and nitrogen contents of the media, indicating that there is a close relationship between primary carbon and hydrogen metabolism [3,15]. However, the effect of hydrogen metabolism modification on primary carbon metabolism is unclear in this cyanobacterium owing to the lack of metabolomic analyses of hydrogenase mutants.

We performed transcriptome and metabolome analyses by using microarray analysis, liquid-chromatography/mass spectrometry (LC–MS), and gas-chromatography/mass spectrometry (GC–MS) with the wild-type strain and a *hoxH* mutant. *Synechocystis* 6803 with a hydrogenase mutation exhibited modifications in sugar catabolism and the TCA cycle.

2. Materials and methods

2.1. Bacterial strains and culture growth conditions

The glucose-tolerant (GT) strain of *Synechocystis* sp. PCC 6803 [37] was cultivated in modified BG-11 medium, which was BG-11_0 liquid medium [30] supplemented with 5 mM NH_4Cl and buffered with 20 mM HEPES–KOH, pH 7.8. The GT-I strain was used in this study [10]. Liquid cultures were bubbled with 1% (*v*/v) CO_2 in air and incubated at 30 °C under continuous white light (~50–70 μmol photons m^{-2} s^{-1}). For the *hoxH* mutant, 0.3 μg/mL gentamycin was added during preculture. Cell densities were measured at A_{730} using a Hitachi U-3310 spectrophotometer (Hitachi High-Tech., Tokyo, Japan).

2.2. Generation of the hoxH mutant

The *hoxH* open reading frame (sll1226) was amplified by PCR using KOD Plus Neo Polymerase (Toyobo, Osaka, Japan) and the specific primers 5′-TTCCGCATGCTTACAAGATCCGGCTTTC-3′ and 5′-TTAAGATATCAGCCCGTTGCCGATATT-3′. The amplified DNA fragments were digested with *Sph*I and *Eco*RV (Takara Bio, Shiga, Japan) and cloned into the *Sph*I and *Sma*I site of the pUC119 vector (Takara Bio). The region including the gentamycin resistance cassette was amplified using KOD Plus Neo Polymerase and the primers 5′-TATTCTGGGCCCTTTGCTTCATCGCTCGAG-3′ and 5′-TATCAAGGGC CCATCCAATGTGAGGTTAAC-3′ using a pTGP2031 vector [24] as a template. The amplified DNA was cloned into the *Msc*I site of *hoxH* using DNA Ligation Mix (Takara Bio). Transformation was performed by natural transformation, in which plasmid DNA was mixed with *Synechocystis* cells and spread on a BG-11 plate containing 3 μg/mL gentamycin. After colonies appeared, several colonies were transferred onto a BG-11 plate containing 3 μg/mL gentamycin several times.

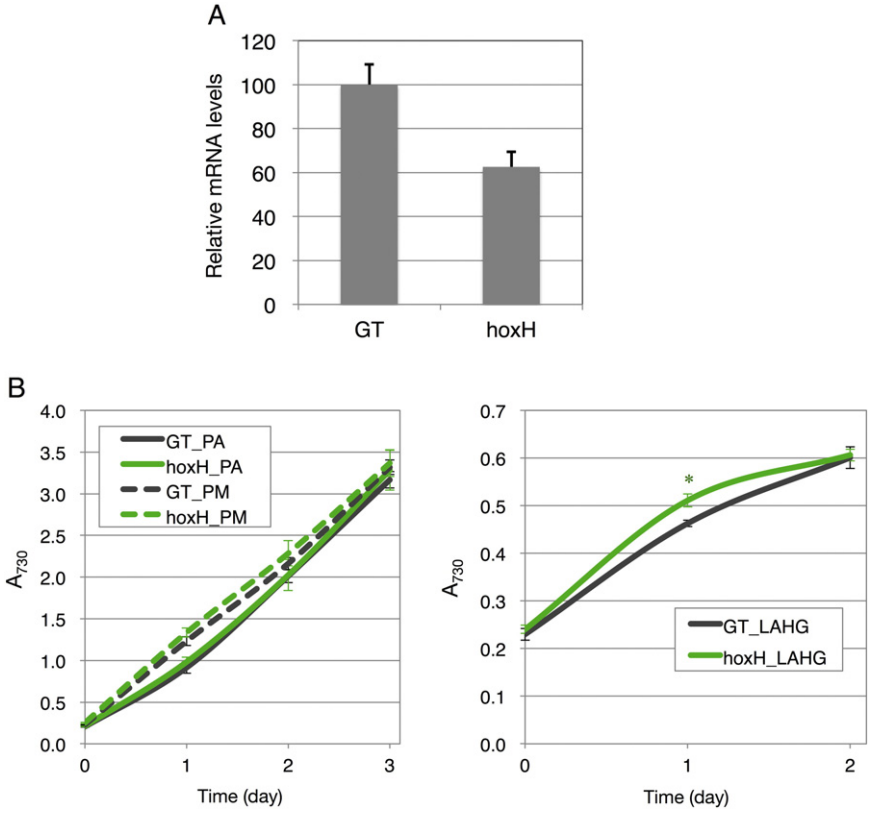

Fig. 1. (A) Transcript levels of *hoxH* in the wild-type strain (GT) and *hoxH* mutant. Data represent means ± SD from biologically independent experiments ($n = 3$). Transcript levels were calibrated relative to those of the corresponding transcripts in GT (set at 100%). (B) Growth curves of GT and *hoxH* mutant strains. Left; cell growth under photoautotrophic (PA) and photomixotrophic (PM, grown with 1 mM glucose under continuous illumination) conditions. Right; cell growth under light-activated heterotrophic conditions (LAHG, grown with 1 mM glucose under continuous dark conditions except for 15 min of light per day). Data represent means ± SD from four independent experiments. Asterisks indicate statistically significant differences between GT and the *hoxH* mutant (Student's *t*-test; $^*P < 0.05$).