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Metabolic switching of central carbon metabolism in response to nitrate: Application to autofermentative hydrogen production in cyanobacteria

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ABSTRACT

Nitrate removal from culture media is widely used to enhance autofermentative hydrogen production in cyanobacteria during dark anaerobiosis. Here we have performed a systematic inventory of carbon and nitrogen metabolites, redox pools, and excreted product fluxes which show that addition of nitrate to cultures of *Synechococcus* sp. PCC 7002 has no influence on glycogen catabolic rate, but shifts the distribution of excreted products from predominantly lactate and H_2 to predominantly CO_2 and nitrite, while increasing the total consumption of intracellular reducing equivalents (mainly glycogen) by 3-fold. Together with LC–MS derived metabolite pool sizes these data show that glycogen catabolism is redirected from the upper-glycolytic (EMP) pathway to the oxidative pentose phosphate (OPP) pathway upon nitrate addition. This metabolic switch in carbon catabolism is shown to temporally correlate with the pyridine nucleotide redox-poise (NAD(P)H/NAD(P)$^+$) and demonstrates the reductant availability controls H_2 evolution in cyanobacteria.

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1. Introduction

Nitrogen assimilation in cyanobacteria has been well characterized under photosynthetic conditions (Flores et al., 2005). Reduction of a mole of nitrate to ammonia requires eight electrons derived from one NADPH and six Fd_{red} (Fig. 1a). Nitrate is typically imported into cyanobacteria cells via a nitrate permease (*nrtABCD)* which consumes ATP (Omata et al., 1993). The unicellular, marine cyanobacterium examined in the present study, *Synechococcus* sp. strain PCC 7002 (hereafter *Synechococcus* 7002), however, contains a high-affinity nitrate/nitrite permease, *nrtP* (Sakamoto et al., 1999), a major facilitator type protein which relies on ion gradients (H^+ or Na^+) rather than coupling transport directly to ATP hydrolysis as in *nrtABCD*. Nitrate uptake in the strain *Synechocystis* sp. PCC 6803, which uses an ABC-type transporter, was shown to increase upon osmotic up-shift with NaCl, due to the increase in available cellular energy for import (Baebprasert et al., 2011b).

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Investigation of changes in the transcript profiles expressed during photoautotrophic growth following ammonia depletion in the unicellular cyanobacterium *Synechocystis* 6803, were identified to be under control of the global nitrogen regulator NtcA and the group 2 sigma factor SigE (Osanai et al., 2006). Genes for sugar catabolic pathways including glycolysis, the oxidative pentose phosphate (OPP) pathway, and glycogen catabolism were induced by ammonia depletion, and the activities of glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGD), two key enzymes of the OPP pathway, were demonstrated to increase several fold. The consequences of these changes, if any, on the associated proteins or intracellular metabolite fluxes were not described, and thus their metabolic consequences remain unknown in cyanobacteria.

There are two distinct ATP-producing pathways that can be used during dark, anaerobic carbohydrate catabolism within *Synechococcus* 7002, generating reductant as either NAD(P)H or reduced ferredoxin (Fd_{red}). Glucose degradation *via* the Embden-Meyerhof-Parnas (EMP) pathway (denoted "glycolysis") leads to the production of NADH during the oxidation of glyceraldehyde-3-phosphate. Alternatively, catabolic flux through the oxidative pentose phosphate (OPP) pathway generates NAD(P)H in two oxidative reactions catalyzed by glucose-6-phosphate

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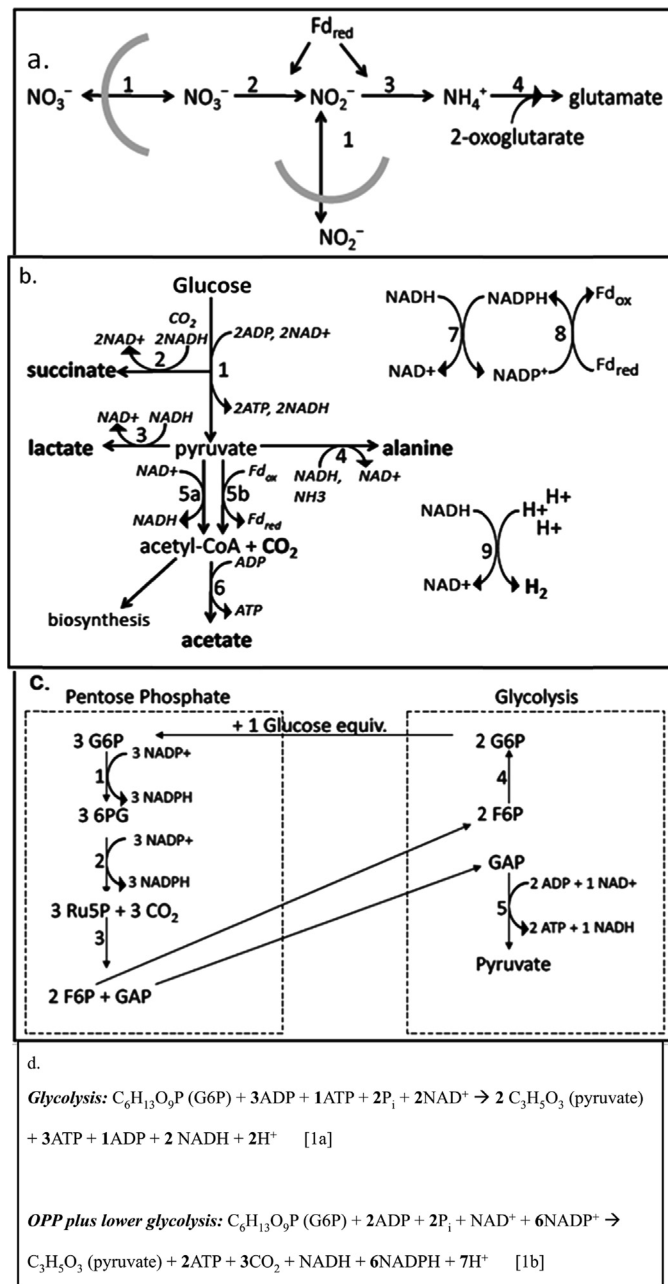

Fig. 1. (a) Nitrate assimilation in *Synechococcus* 7002. 1. Nitrate transporter; 2. Nitrate reductase; 3. Ferredoxin nitrite reductase; 4. Glutamate dehydrogenase (b) Redox poise balance through generating as well as consuming reduced equivalents under dark fermentative carbohydrate catabolism. 1. Glycolytic catabolism (10 steps); 2. Succinate dehydrogenase; 3. Lactate dehydrogenase; 4. Alanine dehydrogenase; 5a. Pyruvate dehydrogenase; 5b. Pyruvate ferredoxin oxidoreductase; 6. Acetyl-CoA synthetase; 7. Transhydrogenase; 8. Ferredoxin-NADP+ reductase; 9. NiFe-hydrogenase (c) Oxidative pentose phosphate (OPP) pathway vs. glycolysis comparison for generating reduced equivalents upon per mole of glucose catabolized. 1. Glucose-6-phosphate 1-dehydrogenase; 2. 6-Phosphogluconate dehydrogenase; 3. Ribulose-phosphate 3-epimerase and transketolase; 4. Glucose-6-phosphate isomerase; 5. Lower-glycolysis. (d) Stoichiometry of glucose-6-phosphate (G6P) catabolism through glycolysis (Eq. 1) and through OPP + lower glycolysis (Eq. 2) yielding pyruvate, ATP, NAD(P)H and CO_2.

dehydrogenase and 6-phosphogluconate dehydrogenase, resulting in ribulose-5-phosphate, which is (2 mol) further converted to sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate. The glyceraldehyde-3-phosphate is further oxidized by entering lower-glycolysis (Fig. 1c). The net yields per glucose from these competing pathways are two moles of NADH in the case of glycolytic catabolism (Fig. 1b), and yields six moles NADPH plus 1 mole NADH when the OPP and lower-glycolysis pathways are employed (Fig. 1c). These stoichiometries are summarized in Fig. 1d. When a mole of glucose is completely oxidized through the OPP pathway, it yields 12 moles of NADPH (Zhang et al., 2007), but this complete oxidation does not happen under *in vivo* conditions. Thus, the incomplete oxidation of a mole of glucose using OPP and lower-glycolysis produces 1 pyruvate, 3 CO_2 and seven moles of NAD(P)H. The pyruvate generated by either pathway may be further oxidized by pyruvate:ferredoxin oxidoreductase (PFOR) or the pyruvate dehydrogenase complex (PDC), generating Fd_{red} or NADH, respectively (Fig. 1b), producing CO_2 and acetyl coenzyme A (acetyl-CoA). Including the oxidation of pyruvate to acetyl-CoA, the maximum potential H_2 yield via glycolysis-only degradation is four moles of H_2, known as the Thauer limit (Thauer et al., 1977), compared to eight in the case of OPP coupled to lower-glycolysis. Dark anaerobic cyanobacteria produce much less than 4 moles of H_2 per mole of carbohydrate catabolized; the observed yield remains less than one H_2 per glucose equivalent (Das and Veziroglu, 2008). The cyanobacterium *Synechococcus* sp. 7002 which we examine herein excretes other reduced products during dark anaerobiosis, including lactate, alanine, and succinate; only a small fraction of the total available reductant is excreted as hydrogen, equivalent to 0.23 moles H_2 per glucose equivalent or roughly 1/48 of maximum theoretical yield (McNeely et al., 2010). Although observations of strain-specific H_2 evolution rates and yields have been reported for numerous cyanobacteria, there exists a critical lack of understanding concerning the regulation of metabolic production and utilization of reductant for H_2 production in these organisms. Until recently it had been accepted that NiFe-hydrogenases found in cyanobacteria are reduced exclusively by NAD(P)H, based on *in vitro* studies of similar hydrogenases (Lauterbach et al., 2013). However, it was shown recently that both flavodoxin and ferredoxin can directly reduce the bidirectional NiFe-hydrogenase of *Synechocystis* sp. PCC 6803 *in vitro* (Gutekunst et al., 2014). This study also reported that a merodiploid ferredoxin-NADP reductase mutant of *Synechocystis* sp. PCC 6803 produced more hydrogen photolytically compared to native strain, consistent with elimination of a competing pathway. This study also showed that reduced ferredoxin generated *in vitro* by pyruvate:flavodoxin/ferredoxin oxidoreductase (PFOR) donates electrons to the NiFe-hydrogenase under fermentative conditions, in agreement with an earlier *in vivo* study (McNeely et al., 2011).

Removal of nitrate from the fermentation medium under dark anoxic conditions, inhibition of nitrate reductase after growth in replete medium, and genetically removing the nitrate/nitrite reductases have all been shown to increase hydrogenase-mediated fermentative H_2 evolution in several cyanobacteria. A 4-fold increase in the rate of hydrogen production was also observed in *Arthrospira maxima* when nitrate was removed from the fermentation medium (Ananyev et al., 2008). Inhibition of the nitrate reductase in *Synechocystis* 6803 resulted in a similar 4-fold increase in the observed H_2 yield (Gutthann et al., 2007). A strain of *Synechocystis* 6803 lacking both nitrate and nitrite reductases showed a 140-fold increase in H_2 production compared to WT in nitrate-replete media (Baebprasert et al., 2011a). These studies indicate that nitrate competes with the reduction of protons for H_2 under fermentative conditions in these organisms. As a result, the observed redistribution of excreted metabolites was predicted to be correlated with shifts in the intracellular redox poise (herein defined as the ratio of reduced to oxidized pyridine nucleotides, $NAD[P]H/NAD[P]^+$, as well as the ratio of the reduced to oxidized electron carrier ferredoxin (Fd_{red}/Fd_{ox}). Redox control of the redistribution of metabolites might occur via mass action or regulatory influences, although the precise mechanism of control is not investigated in this study.

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