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Redistribution of metabolic fluxes in *Chlorella protothecoides* by variation of media nitrogen concentration

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ABSTRACT

In this study, the Elementary Metabolite Unit (EMU) algorithm was employed to calculate intracellular fluxes for *Chlorella protothecoides* using previously generated growth and mass spec data. While the flux through glycolysis remained relatively constant, the pentose phosphate pathway (PPP) flux increased from 3% to 20% of the glucose uptake during nitrogen-limited growth. The TCA cycle flux decreased from 94% to 38% during nitrogen-limited growth while the flux of acetyl-CoA into lipids increased from 58% to 109% of the glucose uptake, increasing total lipid accumulation. Phosphoenolpyruvate carboxylase (PEPCase) activity was higher during nitrogen-sufficient growth. The glyoxylate shunt was found to be partially active in both cases, indicating the nutrient nature has an impact on flux distribution. It was found that the total NADPH supply within the cell remained almost constant under both conditions. In summary, algal cells substantially reorganize their metabolism during the switch from carbon-limited (nitrogen-sufficient) to nitrogen-limited (carbon-sufficient) growth.

1. Introduction

Over the last 50 years, algae have been utilized to produce a wide range of valuable commercial products (Rosenberg et al., 2011). However, recently, there has been particular interest in algae derived products as nutraceuticals and biofuels (Schmidt et al., 2010). Compared to other fuel sources, biofuels are renewable, non-toxic, and considered environmentally friendly (Miao and Wu, 2006). Due to the opportunities in algae biofuel production and the increased need for alternative energy, considerable attempts are being undertaken to optimize production of lipids and other biofuel precursors in microalgae. Commonly used microalgae strains for commercial applications include *Chlorella*, *Chamydomonas*, *Haematococcus* and *Dunaliella* (Rosenberg et al., 2011). *Chlorella protothecoides*, in particular, has been shown to produce high amounts of lipid precursors to biofuels in comparison to other strains (Miao and Wu, 2006). When algae cells are cultivated under nitrogen-limited conditions, the rate of lipid production has shown to increase compared to cultivation under nitrogen-sufficient growth. This is believed to be a survival mechanism as the accumulated lipids serve as energy storage when the cell is under stress (Rosenberg et al., 2008). Nitrogen limitation can increase production of lipids, but decreases the growth rate. *C. protothecoides* grown heterotrophically have yielded as much as 53% of its dry weight in lipids, typically stored as oil droplets in the cells, while photoautotrophic growth resulted in only 14% of its dry weight as lipids (Miao and Wu, 2006). The high production capability makes *C. protothecoides* a good candidate for an alternative fuel source. In an attempt to address this issue, growth studies using 13-C labeling have been performed to better understand metabolite flows within the cell in various growth conditions (Xiong et al., 2010).

Metabolic flux analysis (MFA) is a powerful tool that can provide details about all metabolic fluxes within a microorganism using stoichiometric constraints and radiolabeled tracers (Wiechert, 2001). The accuracy and methods of MFA have advanced significantly and is today based on stable-isotope labeling experiments and analysis of MS data for distributions of cellular metabolites (Antoniewicz et al., 2007b). At steady state, the resulting labeling pattern of each metabolic intermediate is fully and uniquely determined by the intracellular fluxes of the cell. Mathematical models relating network fluxes and mass isotopomer abundance from MS data in metabolic intermediates can facilitate this analysis (Antoniewicz et al., 2007b). Few studies have provided comprehensive and accurate flux models for algae cells. In particular, MFA using a skeletal representation of central metabolism, based on GC–MS-derived amino acid labeling patterns (Xiong et al., 2010), has revealed the re-routing of flux through the pentose phosphate pathway upon nitrogen starvation. However, we wanted to explore the role of glycine as a dual carbon and nitrogen source. Furthermore, a model that considered additional

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sources of NADPH, such as the chloroplastic transhydrogenase (Chopowick and Israelstam, 1971) that can alter the predicted fluxes through central metabolism (Bonarius et al., 1998), would be worthwhile. These differences warranted a further analysis of the generated GC–MS labeling data in order to obtain a more complete picture of the flux distribution and metabolic response of central metabolism to shift in nutrient limitation.

Hence, we have re-evaluated the reaction network of this *C. protothecoides* using previously generated amino acid labeling data and an expanded metabolic model in order to elucidate the driving forces behind the changes in metabolism during nitrogen-rich and nitrogen-limited growth conditions. This knowledge will enable researchers to further optimize growth and lipid production by understanding how the intracellular metabolism changes as a result of MFA analysis. After decomposing the network using the Elementary Metabolite Units (EMU) algorithm, fluxes were estimated by minimizing the variance-weighted sum of squares of deviation from experimentally observed labeling distributions (Antoniewicz et al., 2007a). This analysis indicated an increased dependence on the pentose phosphate pathway for generating NADPH following the shift from glucose to nitrogen-limited growth in order to meet the demands for increased lipid production, in conjunction with a reduced TCA cycle and glyoxylate shunt flux. Interestingly, the glyoxylate shunt was found to be partially active in both growth conditions to enable glycine incorporation into central metabolism. We also found that the total intracellular NADPH production remained relatively constant during both growth conditions.

2. Materials and methods

2.1. Description of the model

A one-compartment metabolic model for *C. protothecoides*, strain 0710, originally obtained from the Culture Collection of Alga at the University of Texas in Austin, grown heterotrophically on glucose and glycine was constructed consisting of glycolysis, pentose phosphate pathway, TCA cycle, glyoxylate shunt, and all of the amino acid biosynthetic pathways. Supplementary Tables S2 and S3 provides the complete list of reactions and metabolites included in the metabolic model of *C. protothecoides*. The primary carbon source, glucose, is metabolized via the central carbon metabolic pathways, ultimately producing the precursors for biomass production. The supplied nitrogen source is glycine, which is also a carbon source. Sulfur is incorporated into the two amino acids, cysteine and methionine. Phosphate is used for ATP synthesis and energy production. Incorporated oxygen is assumed to be used exclusively for oxidative phosphorylation.

The model also includes cofactor and energy components, which address the overall energy demand for biosynthetic processes. A general ATP hydrolysis reaction is included to account for all ATP requirements over the quantifiable growth-associated maintenance (GAM) of 15.58 mmol/gdw during nitrogen-sufficient growth and 20.36 mmol/gdw during nitrogen-limited growth. This difference arises from changes in biomass composition in the form of increased lipid content and decreased protein content upon nitrogen starvation. Quantifiable ATP costs include biosynthesis and polymerization of macromolecules such as proteins, DNA, RNA, lipids, and carbohydrates. Additional costs covered by the ATP hydrolysis reaction include protein activation, futile cycles, non-growth associated maintenance, and unquantifiable GAM costs.

The reversible transfer of hydride between NAD and NADH and NADP and NADPH has also been included in the model to account for the activities of the mitochondrial and chloroplastic nicotinamide nucleotide transhydrogenases (Krawetz and Israelstam, 1978) and the presence of isozymes using different cofactors. The lack of compartmentalization prevents the distinction between the various compartmental cofactor pools. Since intercompartment cofactor shuttling does not generate or consume NADH or NADPH, information about total NADH, NADPH, and ATP production within the cell can be reliably extracted from the obtained flux distribution. Furthermore, relaxation of cofactor constraints using transhydrogenase and an ATP sink prevents any bias arising from incorrectly included cofactor balances (Bonarius et al., 1998).

The non-oxidative pentose phosphate pathway consists of three reactions following the bisubstrate ping-pong mechanism (Nilsson et al., 1997; Jia et al., 1997), with three possible group donors and three possible acceptors. In order to capture all possible carbon transitions accurately, they are modeled as half-reactions (Kleijn et al., 2005).

Synthesis of glutamate is modeled using the GS:GOGAT system. Aspartate, alanine, and serine are synthesized by transamination reactions with glutamate as the amino group donor. Glycine is taken up from the medium and converted to glyoxylate by a glyoxylate aminotransferase enzyme. The synthesis of the remaining amino acids is assumed to be the same as in bacteria. The synthesis of lipids is described in terms of acetyl-CoA and energy terms NADPH and ATP (Stephanopoulos et al., 1998). The lipid profile of *C. protothecoides* has been described previously (Xiong et al., 2010). Using this information, a stoichiometric equation for synthesis of 1 g lipids has been calculated.

The defined biomass equations describe the formation of 1 g biomass in terms of macromolecules (DNA, RNA, proteins, lipids, and carbohydrates). Lipids are assumed to be exclusively diacylglycerols (DAGs) based on the previously described biomass equation for *C. protothecoides* (Xiong et al., 2010). The composition of fatty acids and amino acids used in the model for this strain have been previously defined (Xiong et al., 2010). Carbohydrates are modeled in terms of hexose monomer units (molecular weightt 162 g/mole). DNA and RNA are modeled in terms of nucleotide monophosphates (NMPs and dNMPs), the precursor and energy requirements for which have been defined previously (Stephanopoulos et al., 1998).

2.2. Raw data

The experimental data used in our model was kindly obtained from a previous study assessing the impact of nitrogen starvation on *C. protothecoides* (Xiong et al., 2010). Glucose and glycine were used as the carbon and nitrogen source respectively. 10% of the supplied glucose was labeled with U-13 C. The growth rates and the glucose consumption rates were measured, and the amino acid mass spectra was obtained using GC–MS analysis. Using this data, a comprehensive model describing the metabolic network of *C. protothecoides* under nitrogen-sufficient and nitrogen-limited conditions was constructed in the current study.

2.3. Mass spectrometry fragment selection

To improve accuracy during metabolic flux analysis, error related to the GC–MS data is minimized. Amino acid fragments were selected for analysis based on accuracy and precision criteria described in a previous study (Antoniewicz et al., 2007a). Supplementary Table S1 provides details about which fragments were used in the flux model and which ones were rejected. Fragments were excluded from the analysis for two reasons: their absolute intensity was low, or the data resulted in negative fractions after correction for natural abundance.