Development of *Anabaena* 33047, a fast-growing N_2-fixing cyanobacterium, as a carbon neutral bioproduction platform

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Abstract

Cyanobacteria, oxygenic photosynthetic prokaryotes, are emerging as promising platforms for cost effective conversion of sunlight and CO_2 into high value end products. Recent discovery of fast-growing cyanobacterial strains, with growth rates comparable to their heterotrophic counterparts, have alleviated one of the long-standing bottlenecks in the commercialization of these organisms for bio-production. *Anabaena* sp. ATCC 33047, a heterocystous cyanobacterium, stands out in this category for its ability to utilize atmospheric N_2 in addition to CO_2 and generate biomass at unprecedented rates. Developing such a strain as a chassis can eliminate the need for fixed nitrogen in production systems, a significant step towards cost reduction. Our work aimed to develop *Anabaena* 33047 into such a bioproduction platform.

Anabaena 33047 exhibits rapid growth (~3.8h doubling time) utilizing high light, CO_2 and N_2 [1]. Under controlled culture conditions, this strain can fix up to 3.0 g CO_2 L^{-1} day^{-1}, the highest conversion rate of atmospheric CO_2 known for cyanobacteria. Much of this fixed carbon (~47%) is secreted into the medium in the form of exopolysaccharides (EPS) with production rates as high as 1.4 g L^{-1} [2]. Cyanobacterial EPS exhibits unique physical and chemical properties and harbors bioactive potential which can be explored for a variety of applications [3]. However, this strain was previously known to be recalcitrant to genetic manipulation and hence, despite its many appealing traits, remained largely unexplored. During this project, we have successfully developed a genetic manipulation system that has enabled targeted genome modifications.

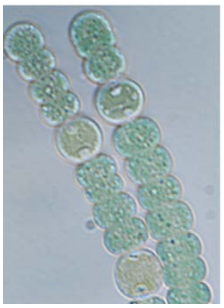

Figure 1. ***Anabaena*** **33047** image showing high frequency of heterocysts.

Genome-scale metabolic models (GSM) facilitate comprehensive understanding of the metabolism of non-model organisms and metabolic engineering for over production of target chemicals [4]. In the course of this project, we developed a genome scale metabolic model, *i*AnC892 [5] for *Anabaena* 33047. This model will help expand our understanding of its metabolism and identify genetic interventions to divert carbon and nitrogen flux towards products of interest. The model was constructed by retrieving annotations from multiple databases: KEGG [6], MetaCyc [7] and ModelSEED [8] and a recently published model for the closely related strain *Anabaena* 7120 [9]. *i*AnC892 contains 953 unique reactions representing the annotation of 892 genes. The diazotrophic life cycle of *Anabaena* 33047 is captured by accounting for both vegetative and heterocyst cell types. This is achieved by creating super-compartments that reflect the metabolic differences and interactions between these two cell types (Figure 2). The model provided insight into importance of light dependent electron transport in the heterocyst and pointed pathway combinations that can supply reducing equivalents and ATP in the appropriate ratio for optimal N_2 fixation [5]. The model was used alongside the strain design algorithm, OptForce [10], to identify genetic interventions that would lead to overproduction of nitrogen-rich compounds [5].

To validate the existing metabolic models and to have a better understanding of the metabolism of *Anabaena* sp. ATCC 33047 under mixotrophic, heterotrophic, autotrophic and diazotrophic growth conditions, we performed a series of stable-isotope tracing experiments and quantified metabolic fluxes using state-of-the-art tools for ^{13}C-metabolic flux analysis (^{13}C-MFA). Our results validated the core

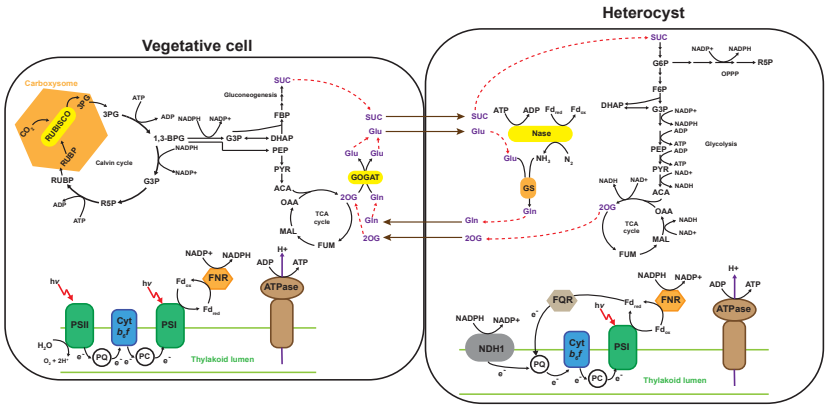

Figure 2: Two cell model of *Anabaena* 33047. The genome scale metabolic model, *i*AnC915, has two super-compartments, the vegetative cell and the heterocyst, in-order to capture the diazotrophic and heterocyst forming lifestyle of this cyanobacterium.

metabolic network model assumptions for this organism. To assess metabolite exchange between vegetative cells and heterocysts during diazotrophic growth, we attempted to obtain independent metabolomics and stable-isotope labeling data from vegetative cells and heterocysts. While we were able to successfully separate the two cell types, we observed significant leakage of metabolites from the cells, suggesting that further refinement of our protocol is needed to obtain more reliable metabolomics data.

Using our newly developed engineering strategy and based on our model predictions, we generated a Δ*nblA* strain of *Anabaena* 33047. NblA is involved in the degradation of phycobilisomes (PBS), light harvesting antenna proteins. The Δ*nblA* mutant exhibited resistance to PBS degradation and retained high amounts of these antenna proteins in its heterocysts. Quantitative analysis of PBS in individual heterocysts of the mutant and the WT (using a Fluorescence Kinetic Microscope - FKM) revealed ~ 8-fold higher amounts of PBS in the heterocysts of the mutant (Figure 3) [1]. Intriguingly, the Δ*nblA* mutant displayed ~ 2.5 folds higher rates of nitrogen fixation compared to the WT. Spectroscopic analysis revealed altered PSI kinetics in the mutant, with increased cyclic electron flow around PSI, a route that contributes to ATP generation and nitrogenase activity in heterocysts. Thus the Δ*nblA* mutant of *Anabaena* 33047 offers an improved platform for bioproduction. Overall, this project has laid the foundation for developing the non-model cyanobacterium *Anabaena* 33047 into a highly efficient chassis for bioconversion of solar energy and atmospheric CO_2 and N_2 into high value bio products.

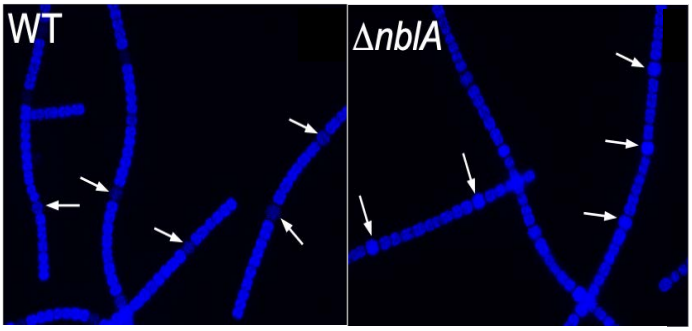

Figure 3. FKM analysis of WT and Δ*nblA* heterocysts. Bright signal from PBS in heterocysts of the Δ*nblA* mutant compared to the WT (arrows).

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