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Accelerating Photosynthetic Adaptations to Environmental Responses in Synechococcus sp. PCC 7002

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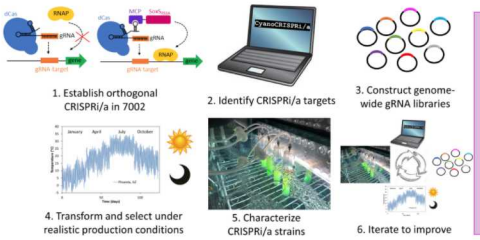
Abstract

Current algal biofuel productivities cannot support sustainable economic algal fuel production targets. Traditional breeding and adaptive evolution approaches require decades of constant pressure for environmental adaptations to develop industrial strain optimizations. On the contrary, recent rational strain designs targeting single genes and pathways have led to ~2-fold improvements in photoautotrophic biomass production under laboratory conditions. Thus, targeting multiple genes or metabolic pathways with a systems level approach characterizing responses to realistic environmental conditions could enable rapid metabolic optimizations during strain development. We have developed high-throughput screens of CRISPR interference and CRISPR activation (CRISPRi/a) in the cyanobacterium Synechococcus sp. PCC 7002. Our goal is to identify genetic modifications that facilitate enhanced growth and biofuel production under realistic environmental production conditions. Our engineered 7002 platform is naturally tolerant to high salt, light, and temperature, but also exhibits relatively fast growth rates. We are modifying previously developed CRISPRi approaches to enable a CRISPRa system in 7002 to finely tune the metabolic flux and regulation of every gene in 7002 to identify genetic modifications that enable improved bioproduction capabilities during simulated outdoor light and temperature.

Project Overview



Toolbox Needs:

- gRNA
- Operon identification
- CRISPRi/a
- *E. coli–7002* shuttle vector for gRNA library

Figure 1. Steps for high-throughput gRNA screening of CRISPRi/a targets in 7002 for improved biomass productivity

gRNA Design

- Protospacer adjacent motif (PAM) sites for 10 different Cas proteins (Table 1) were identified in the Synecchocus sp. 7002 genome and it's 6 native plasmids (pAQ1–7).
- Spy and Sau2 have the highest number of PAMs per kb and therefore the highest gRNA densities (Figure 2).
- PAM densities in the 7002 plasmids are similar to that of the chromosome.

Table 1. List of common Cas proteins and associated protospacer adjacent motif (PAM) sites

Organism	Nickname	PAM
Streptococcus pyogenes	Spy	NGG
Staphylococcus aureus - 1	Sau1	NGRRT
Staphylococcus aureus - 2	Sau2	NGRRN
Neisseria meningitidis	Nme	NNNNGAAT
Campylobacter jejuni	Cje	NNNNRYAC
Streptococcus thermophilus	Sth	NNAGAAW
Treponema denticola	Tde	NAAAAC
Prevotella/Francisella	Cpf1	TTTV
Prevotella/Francisella	Cpf1_m1	TYCV
Prevotella/Francisella	Cpf1_m2	TATV

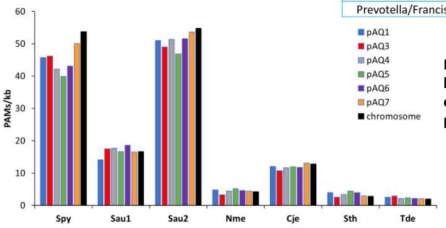


Figure 2. PAM site density per kilobase (kb) for the chromosome and 6 native plasmids from 7002

Development of CRISPRi Tools in 7002

- Spy dCas9 CRISPRi tools from Gordon *et al.* 2016 obtained from Addgene, changed the antibiotic resistance cassette, and genome-integrated into 7002 and two Ypet- expressing strains.
- Inhibition of *cpcB* (phycocyanin production) and *ypet* was confirmed with genome integration of gRNAs targeting these genes (Figure 5), but there was leaky expression from the *tet* promoter.
- Development of CRISPRa system based on 2-hybrid CRISPR-dCas system established in *E. coli* 4 is ongoing (Figure 6). This system uses a single dCas and was shown to have higher activation compared to direct fusion of the transcriptional activator to dCas9.

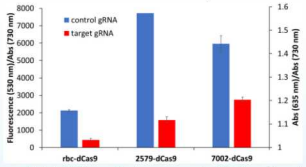


Figure 3. Inhibition of phycocyanin (635 nm) and yellow fluorescence (530 nm) using gRNAs targeting *cpcB* and *ypet* and expression of dCas9 from *tet* promoters, 48h after addition of anhydrotetracycline

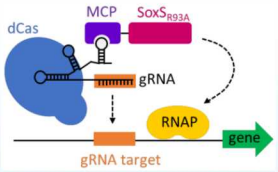


Figure 4. Schematic of 2-hybrid CRISPRa system

Development of E. coli – 7002 Shuttle Vector

- pAQ1 is the smallest native plasmid in 7002.
- 8 overlapping fragments from pAQ1 were transferred into pBISNL2 to test as E. coli- 7002 shuttle vectors – pBIAQ1 (Figure 7), along with the predicted ori.5
- 6 of the 8 putative shuttle vectors produced antibiotic resistant colonies in 7002 after transformation (Table 2).
- Plasmid DNA (pBIAQ1) was isolated from the 7002 transformants and transformed back into E. coli to confirm plasmid maintenance in 7002.
  - Only fragment 2 had more than 1 E. coli transformant (Table 2).
  - The re-transformed plasmid is similar in size to the original vector (Figure 8).

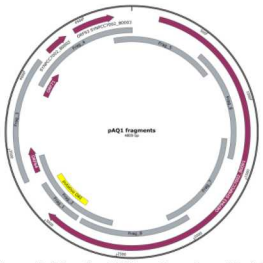


Figure 5. Map for 7002 native plasmid pAQ1 and fragments tested for origin of replication

Table 2. E. coli transformants from 7002 prospective shuttle vectors

Shuttle Vector	# 7002 transformants	# *E. coli* transformants
pBIAQ1-1	0	-
pBIAQ1-2	7	134-300+
pBIAQ1-3	8	3-7
pBIAQ1-4	4	0
pBIAQ1-5	11	0-1
pBIAQ1-6	-	-
pBIAQ1-7	3	0-1
pBIAQ1-8	5	0-1

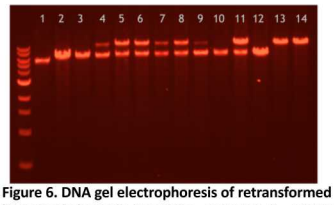
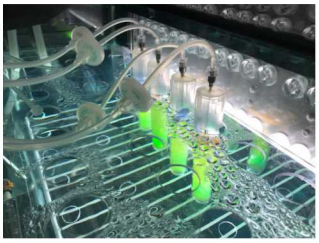
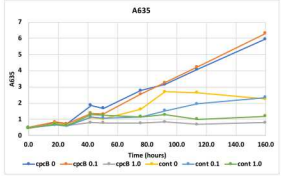
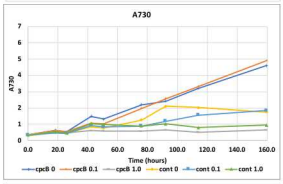


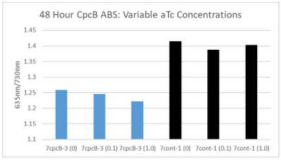
Figure 6. DNA gel electrophoresis of retransformed linearized plasmids. Controls: = pBISNL2, 2 = pBIAQ1-2, and re-transformed shuttle vectors: 3-9 = pBIAQ1-2, 10-12 = pBIAQ1-3, 13 pBIAQ1-7, and 14 = pBIAQ1-8

7002 CRISPRi Environmental Simulation









- Tested gRNAs targeting cpcB under simulated environmental conditions to determine CRISPRi effectiveness in 7002
- Initial test under Summer simulation of 16:8 hr day:night cycle with corresponding trailing temperature changes from 24 – 30ºC
- 7002-dCas strains were transformed with *cpcB* and control gRNA and cultured in A+ media with 0, 0.1, and 1.0 µg/mL anhydrotetracycline (aTc)
- CpcB absorbance at 635 nm was reduced in all gRNA targeting *cpcB* samples when compared to controls
- *cpcB* gRNA targeting strain had leaky expression with improved biomass compared to control strains and slight response to increase aTc

Conclusions and Future Work

- Spy Cas9 will be used for CRISPRi/a due to its high gRNA density in the 7002 genome.
- A gRNA design pipeline was developed for 7002 to account for potential off-target effects.
- CRISPRi was validated and demonstrated to increase biomass for *cpcB* gRNA targeting strains in 7002 under environmental relevant conditions, and the 2-hybrid CRISPRa tool is under construction.
- The 7002 origin of replication was identified for pAQ1 and will be used to construct an E. coli – 7002 shuttle vector for gRNA library expression. Essential ORFs from pAQ1 will be genome integrated, and pAQ1 will be cured from 7002.
- After tool development is complete, the gRNA library will be designed, constructed, and tested in 7002 to identify CRISPRi/a mutants with improved growth under simulated outdoor conditions.

References and Funding

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3. Gordon GC, Cameron JC, and Pfleger BF. Global RNA half lives in cyanobacteria. Gene Expression Omnibus. GSE109174. https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE109174

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