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Genetic Instability in *Synechococcus* sp. PCC 7002 under Different Promoters

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Key words: Cyanobacteria, Metal-inducible Promoter, Synthetic Biology, BASIC

Abstract

As a photosynthetic and single-cell microorganism, cyanobacteria is an appealing chassis that convert CO_2 into high-value industrial molecules. For successful and long-term production, strategies for genetic engineering and tightly controlled promoter-gene expression are essential. Genetic tools have yet to be developed for many other potentially suitable strains for industrial applications. In this study we gave an insight into how genetic instability of *Synechococcus* sp. PCC 7002 can occur with the incorporation of inert (eYFP and GFP) and toxic genes (sthA) with varying constitutive and inducible promoters. The stability was investigated with both plasmid-based expression and genome integration. The tri-parental conjugation was validated after 2 weeks of incubation. Genetic instability was seen in terms of radii of the colonies rather than numbers, when repression of the toxic gene lacked e.g. with strong constitutive promoters. It was also hypothesized that *Synechococcus* sp. PCC 7002 did not have any repressive ability for Nickel inducible promoters, hence burden was also seen when sthA was introduced.

Introduction

Cyanobacteria (also known as blue-green algae) are regarded as one of the oldest photosynthetic organisms on Earth, originating approximately three billion years ago (Hedges et al. 2001). Most cyanobacteria are highly resilient to salt concentrations as well as temperatures. They can exist even within waste water, thus removing the obstacles between food and potential biofuel production. Therefore, CO_2 uptaking by cyanobacteria effectively alleviate increasing amounts of atmospheric CO_2 (Ono and Cuello 2007). Due to their ability to produce biofuels and bioactive compounds, it is a promising alternative in biotechnology (Vioque 2007). However, industry choices to

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heterotrophic organisms mostly *E. coli*, cyanobacteria are still lack behind in the world of transformative biotechnology. Heterotrophic organisms require expensive carbon-based sugars for growth whereas cyanobacteria are photoautotrophic (Burja et al. 2001). Moreover, molecular tools and metabolic network of cyanobacteria are not fully identified (Yu et al. 2015). Their prolonged doubling time in comparison to bacteria is another major limiting factor (Heidorn et al. 2011). Despite these gaps as *Synechococcus sp.* PCC 7002 (PCC 7002) and *Synechocystis* sp. PCC 6803 (PCC 6803) have been established as model organism with extensive sequencing (Li et al. 2008). *Synechococcus* sp. PCC 7002 is comparatively a fast growing cyanobacteria capable of sustained growth even under high salinity and high light intensity (Ludwig and Bryant 2012). Therefore, with existing physiological traits and the available genetic tools, it is possible to engineer *Synechococcus* sp. PCC 7002 (Xu et al. 2011).

Genetic instability in cyanobacteria is often neglected and mentioned as negative result in many studies (Jones 2014). Mutants strains regressed to the wild type phenotype in mannitol production (Jacobsen 2014), compromised enzyme activities in isobutanol production (Kusakabe et al. 2013) are scenarios of instability. Another example was- sthA (transhydrogenase enzyme), a valuable component in NADPH synthesis pathway, could not act continuously after a few generations while producing lactic acid (Angermayr et al. 2012).

Though, promoter characterization in cyanobacteria is not as extensive as *E. coli*, a wealth of native promoters and related sequence elements have been demonstrated (Gordon and Pfleger 2019). However, there is lack of in-depth knowledge on the strengths of the inducible promoters when expressed with a toxic/non-toxic gene (Pritam et al. 2023). Ruffing et al. 2016 investigated 24 native constitutive promoters stating that the promoter A2520 (stated as Pstrong in this study) was found to have strong expression, whereby it was previously denoted as a hypothetical protein in PCC 7002. A1930 (Pmedium) was found to have moderate expression and A0255 (Pweak) was found to have low expression levels. The inducible promoters were used to investigate repression of genes in PCC 7002, whereby repression with Nickel and Cobalt inducible promoters were shown in PCC 6803. However, it is unknown on how they would work in PCC 7002. PNrsB was shown to have very low leakiness and a high induction rate29 in PCC 6803. Guerrero *et al.* concluded the metal-includible Pcoa functioned well in PCC 6803 too.Hence, this study aims to compare numerous different inducible and constitutive promoters to in terms of expression of proteins: sthA (a toxic gene), eYFP and GFP (inert genes) in model cyanobacterium strain *Synechococcus* PCC7002. Previously, these promoters were widely studied in strains PCC 6803, but study on different promoters in relation to genetic instability has not been done on PCC 7002. This work has chosen this strain due to its relatively fast replication, high temperature tolerance and ability to grow in salt (Sheng et al. 2011, Ludwig and Bryant 2012). Additionally, we looked into the comparison between genome integration directly and plasmid based integration which also have not investigated before.

Materials and Methods

For plasmid based cloning, rubidium chloride competent *E. coli* strain DH5α (New England Biolabs, UK) was used whereas, wild-Type PCC 7002 (WT-PCC 7002) was used for the incorporation of all plasmids. Bacteria strain was cultured in Lysogeny Broth (LB) at 37°C at 180 rpm shaking incubator. *Synechocystis* PCC 7002 was grown on A+ culture media sourced from Takara Bio Europe (Stevens et al. 1973), with added 4 mg/l Vitamin B12 at 30°C with 1% CO_2 and a light intensity (warm white light) of 60 $\mu E\ m^{-2}\ s^{-1}$ (Algaetron Photosystem). 200 μg/ml for *E. coli* and 20 μg/ml for *Synechococcus* were the concentration of Erythromycin.

All plasmids were constructed using BASIC assembly, with the protocol detailed in Storch et al. 2015. A schematic representation of the assembly is outlined in Fig. 1.

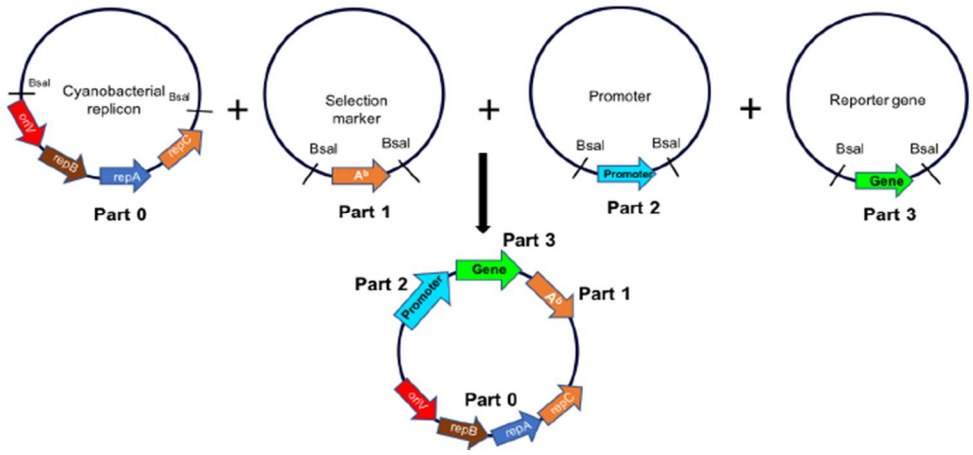

Fig. 1. Overview of the construction of Integrational plasmids. pJET plasmid was the backbone for each component. The backbone contains BsaI restriction sites forming flanks. This plasmid can be pasted on to other backbones in a jigsaw like fashion. Ab: Antibiotic Cassette (figure originally created by the authors).

The promoters used in this study has shown in Table 2. For this reason, Pnrsb and Pcoa were selected to study in PCC7002.

Table 1. Primers designed for the amplification of the gene inserts.

Primer Name	Gene	Primer sequence
IY22_sthA_F	sthA	ATG CCC CAT TCG TAT GAT TAT GAT GC
IY22_sthA_R	sthA	TTA GAA TAA GCG GTT CAG CCC GTT
IY098_GFP_BASIC	GFP	TCTGGTGGGTCTCTGTCCATGCGTAAAGGCGAAGAACTG
IY099_GFP_BASIC	GFP	CGATAGGTCTCCCGAGCCTTATACAGCTCGTCCATACCGTGGG
033_For_BASIC_eYFP	eYFP	TCTGGTGGGTCTCTGTCCATGGTGAGCAAGGGCGAGGAG
034_Rev_BASIC_eYFP	eYFP	CGATAGGTCTCCCGAGCCTTACTTGTACAGCTCGTCCATGCCG

Table 2. (A) Constitutive and (B) Inducible Promoters used in this study. IPTG: Isopropyl_-D-1-thiogalactopyranoside, P_{Nrsb}: Nickel inducible promoter, P_{Coa}: Cobalt inducible promoter, $P_{Clac\ 143}$: Lac operon repressible promoter.

	Promoter	Constitutive Strength	Species Tested	Reference
A	P_{A2520}	Strong	PCC 7002	Ruffing et al. (2016)
	P_{1930}	Medium	PCC 7002	Ruffing et al. (2016)
	P_{A0255}	Weak	PCC 7002	Ruffing et al. (2016)
	Promoter	Inducibility	Species Tested	Reference
B	P_{Nrsb}	Nickel	PCC 6803	Englund et al. (2016)
	P_{Coa}	Cobalt	PCC 6803	Guerrero et al. (2012)
	$P_{Clac143}$	IPTG	PCC 6803	Ruffing et al. (2016)

PCC 7002 was transformed with the self-replicating plasmids by tri-parental conjugation using host *E. coli* strain carrying pRL443 and recipient *Synechococcus* strain. This conjugation method was done according to Wolk (1984). After successful transformation, PCC 7002 were grew on A+ media for 3 days. When there was visible growth on plates, cultures were mixed with 500 μl MilliQ water and restreaked on A+ media supplemented with 200 μg/ml Erythromycin. Cultures were then incubated for two weeks until distinct colonies appeared. Table 3 has shown the self-replicating plasmids constructed for this project.

The strains were incubated for sixteen hours in multi-cultivator MC1000, at 38°C with light intensity at 60 μE m-2 s^{-1}, 1% CO_2 concentration. 4 ml wild-type 7002 strain (10^7 cells/ ml) were mixed with 100 ng/ml plasmid at an OD 1 and spread all 4 ml on A+ media supplemented with Vitamin B12 with 20 μg/ml Erythromycin. Plates were incubated for two weeks and 8 random colonies were restreaked on media with same concentration of antibiotic. Table 4 is showing the integrated plasmids used for this study.

Colony number obtained of each mutant strain and their radius were measured by Opencfu software (Geissmann 2013).

Table 3. Self-Replicating plasmids were all assembled by BASIC.

Plasmid Name	Composition of Plasmid
pIY226	pRSF1010-Ery-P_{strong}-GFP
pIY227	pRSF1010-Ery-P_{weak}-GFP
pIY228	pRSF1010-Ery-P_{NRSB}-GFP
pIY241	pRSF1010-Ery-P_{medium}-GFP
pIY272, pIY277, pIY282, pIY287 & pIY292	pRSF1010-Ery-P_{strong}-eYFP(RBS1-RBS5)
pIY273, pIY278, pIY283, pIY288 & pIY293	pRSF1010-Ery-P_{sweak}-eYFP(RBS1-RBS5)
pIY274, pIY279, pIY284, pIY289 & pIY294	pRSF1010-Ery-P_{Nrsb}-eYFP(RBS1-RBS5)
pIY275, pIY280, pIY285, pIY290 & pIY295	pRSF1010-Ery-$P_{clac143}$-eYFP(RBS1-RBS5)
Unnamed	pRSF1010-Ery-P_{coa}-GFP
Unnamed	pRSF1010-Ery-P_{medium}-sthA

Table 4. Integrational Plasmids were all assembled by BASIC.

Plasmid Name	Plasmid Composition
pIY306	pMB1-Amp-GlpK_up-Pstrong-eYFP-termB15-Ery-GlpK_down
pIY307	pMB1-Amp-GlpK_up-Pweak-eYFP-termB15-Ery-GlpK_down
pIY308	pMB1-Amp-GlpK_up-PNrsb-eYFP-termB15-Ery-GlpK_down
pIY309	pMB1-Amp-GlpK_up-Psclac143-eYFP-termB15-Ery-GlpK_down
pIY310	pMB1-Amp-GlpK_up-Pstrong-sthA-termB15-Ery-GlpK_down
pIY311	pMB1-Amp-GlpK_up-Pweak-sthA-termB15-Ery-GlpK_down
pIY312	pMB1-Amp-GlpK_up-PNrsb-sthA-termB15-Ery-GlpK_down
pIY313	pMB1-Amp-GlpK_up-Pclac143-sthA-termB15-Ery-GlpK_down
pIY314	pMB1-Amp-GlpK_up-Pstrong-GFP-termB15-Ery-GlpK_down
pIY315	pMB1-Amp-GlpK_up-Pweak-GFP-termB15-Ery-GlpK_down
pIY316	pMB1-Amp-GlpK_up-PNrsb-GFP-termB15-Ery-GlpK_down

Successful transformation of the strains were validated through Colony PCR. The genomic DNA (gDNA) was extracted by freeze/thaw method. The strains were mixed with 30µl water (Milli Q) followed by boiling at 95°C for three minutes. The cultures were then transferred to the 80°C freezer and again left for three min. The primers used for colony PCR are shown in Table 1. Plasmid extraction was carried out using QIAprep® Spin Miniprep kit (QIAGEN) according to manufacturer's instructions.

Results and Discussion

Various constitutive and inducible promoters (shown in Table 2) were incorporated into the self-replicating vectors to assess genetic instability. These promoters allowed an insight into protein expression of sthA (toxic) and eYFP/GFP (inert genes). Colony PCR used to amplify (primers IY_098_GFP_BASIC and IY_099_GFP_BASIC) with the GFP insert which produced a band at 700bp (Fig. 2).

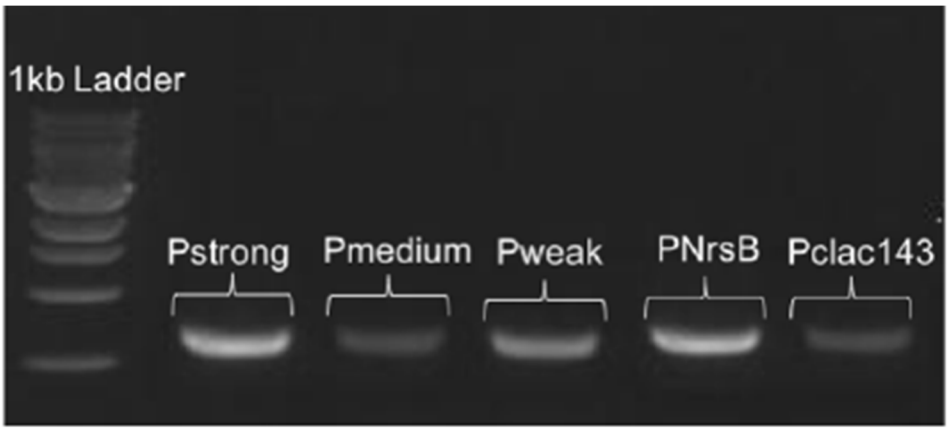

Fig. 2. Verification of the BASIC assembly containing GFP.

Eventually, eYFP and sthA were substituted in place of GFP contained varying ribosome binding strength. This experiment helped to find out the best promoter for expression in PCC 7002 (Fig. 3). Plasmid containing Pcoa-sthA combination did not produce any colony so left out for further experiments.

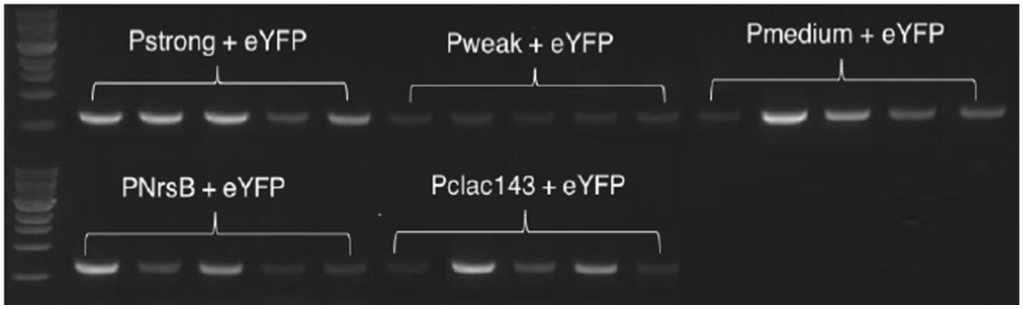

Fig. 3. Validation of the BASIC assembly that contain eYFP, with Ribosome binging site (RBS 1) (far left) to RBS 5 (far right) for each promoter.

For triparental conjugation, the constructs were then transformed into *E. coli.*

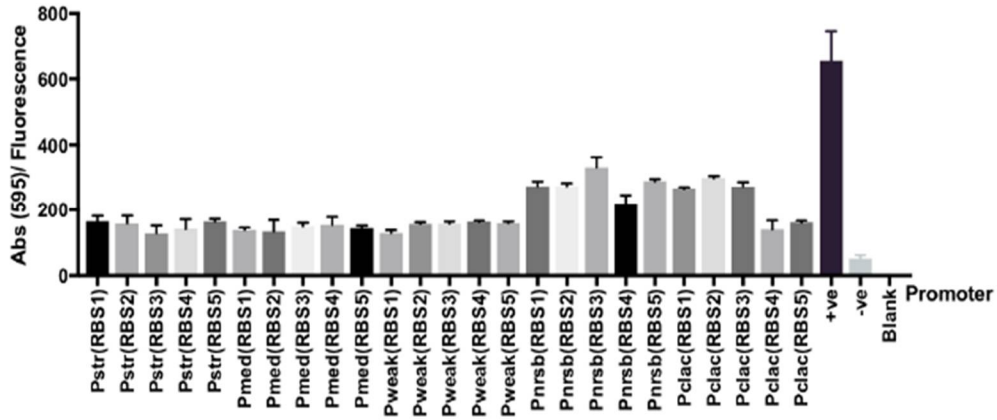

Fig. 4. Promoter activities measured as fluorescence per Abs595. The +ve control is PDF-eYFP and the -ve control is pRSF010-Pclac143-Gm-empty.

According to Englund et al. (2016), to prevent selection pressure to mutate detrimental genes, promoter expression should be switched off in *E. coli* in case of tri-parental conjugation. From Fig. 4 and Fig. 5, a clear difference between promoter expressions can be observed. All promoter absorbance at varying strength of ribosome binding site (RBS) was below 200 nm in *E. coli* strain. From Fig. 5, it was observed that nickel inducible promoter has less leakiness of gene expression in comparison to Plac promoter. Also there was noticeable increase of absorbance threshold when nickel and IPTG was introduced in the culture.

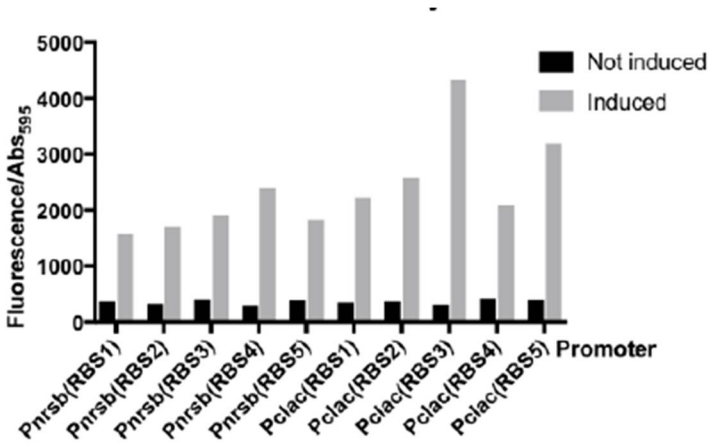

Fig. 5. Fluorescence measurement of promoters upon induction. PNrsB was induced with 2.5 μM nickel and Plac was induced with 1 mM IPTG.

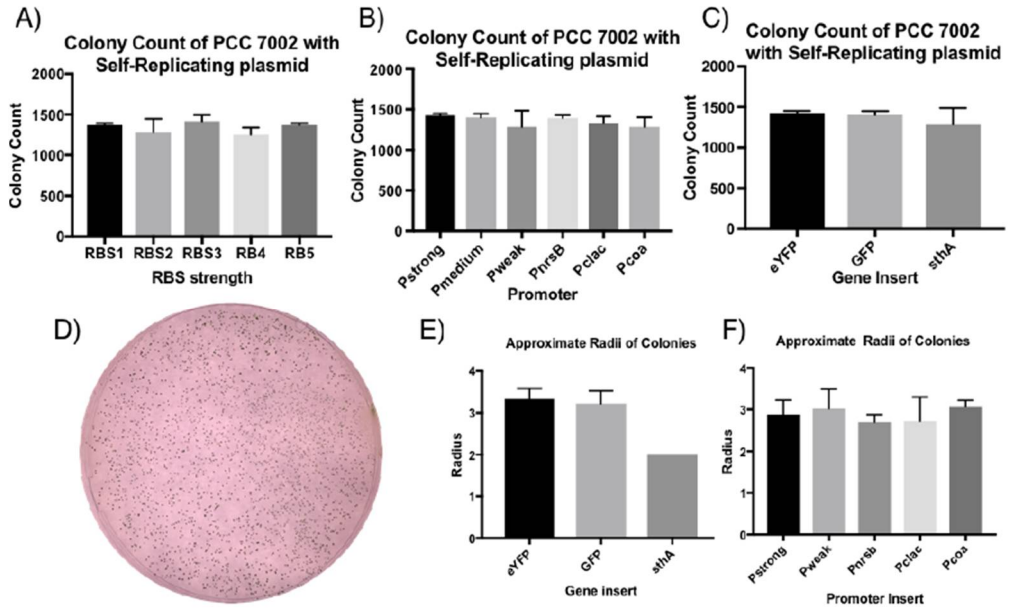

Fig. 6. Analysis of colony count and radii of self-replicating plasmid containing PCC 7002 from OpenCFU software: (A) effects of RBS strength on colony number, (B) effects of different promoters upon colony count, (C) number of colonies in terms of varying genes, (D) image from openCFU E) Effects of genes on radii of colonies, and (F) difference among radii of colonies with different promoters. Error bars are ± 1 standard deviation. Data obtained from triplicates of each promoter-gene combination.

From Fig. 6, it was deemed that RBS3 seemed to be appropriate among five RBS strength by having a slightly greater colony count. Number of colonies do not differ significantly in terms of promoters and genes. However, the radii of sthA differed greatly which was almost half of the radii compared to eYFP and GFP inserts.

BASIC protocol was followed while constructing integrational plasmids. There were GlpK_Up and GlpK_Down regions within the plasmid for the homologous recombination of the plasmid into the genome of PCC 7002 spontaneously. Natural uptake of plasmids provides theoretically more stability of foreign genes without any frame shift mutations (Didelot et al. 2012). All parts of the integrational plasmids were cloned in a pJET vector (Thermo Fisher Scientific and sequence from Snapgene). Therefore, upon any unsuccessful insertions, blunt ended ligation of a gene encoding eco47IR would occur, which is toxic endonuclease to the *E. coli*. The recombinant DNA were validated to see if successful assembly had occurred.

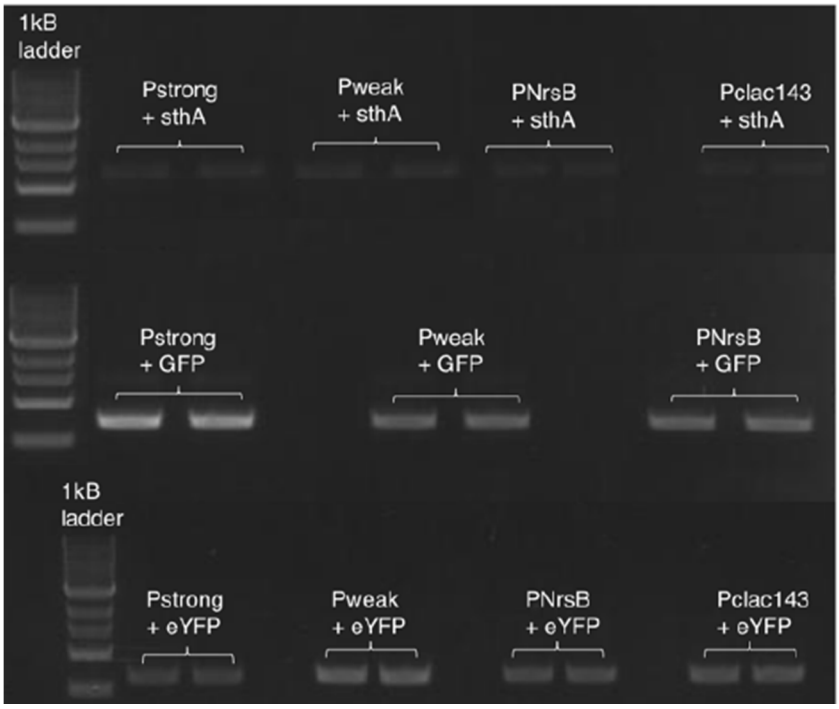

Fig. 7. Colony PCR from transfected *E. coli* for the verification of the BASIC assembly containing sthA, GFP and eYFP with varying promoters on a 1% agarose gel.

After the clear validation of insertion of plasmids into *E. coli*, plasmids were naturally transformed into PCC 7002. At this stage, PCC 7002 strains combined with *E. coli* were incubated at MC1000 with standardized conditions. Triplicates were maintained for each construct. After 16 hrs, 3 ml of the culture spreaded upon A+ media with antibiotic selection pressure. Once the colonies were grown, the plates were then housed into a DIY colony counter, where the plates were analyzed by Open CFU.

Although there was no real overall difference in colony counts, it did show all strains seemed to vary, Pweak + eYFP varying the most. Anoticeably lower number of colonies was observed on Plac expressing eYFP gene and Pweak expressing GFP inert protein

(500 cfu). The highest number of colonies were found on Pstrong promoter plate with low range of variation. Additionally, second highest colony was formed on Pnrsb plates expressing toxic sthA gene.

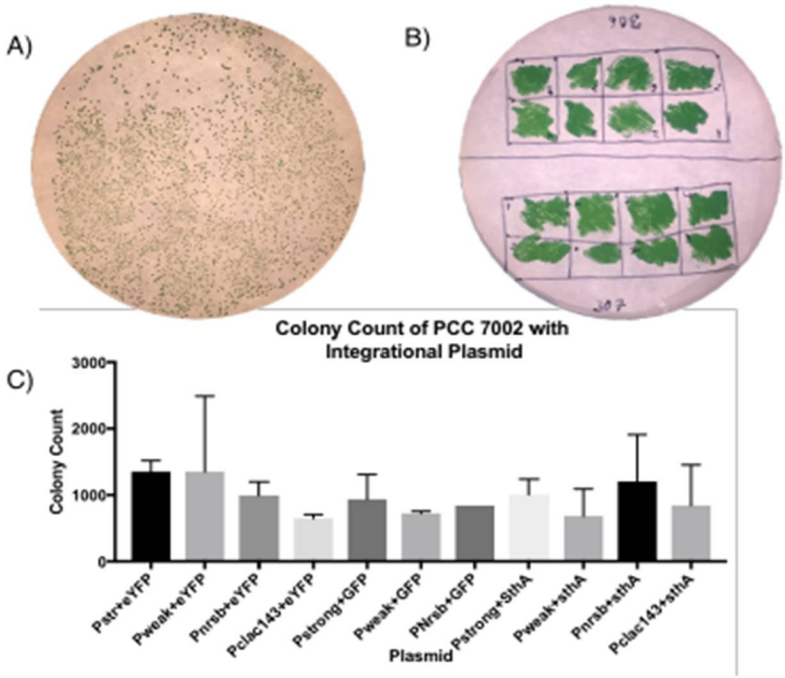

Fig. 8. (A) Colony count from OpenCFU, (B) shows the restreaks of eight random colonies, and (C) differences in colony counts with different combination of plasmids. Although there was no real overall difference in colony counts, it did show all strains seemed to vary, Pweak + eYFP varying the most. Pictures of each plate were taken as shown above (A = Pstrong + eYFP). Error bars are ± 1 standard deviation.

Cyanobacteria have the combined capacity of oxygenic photosynthesis and carbon fixation with engineered pathways for metabolism which in turn results in various bioproducts (Angermayr et al. 2012). However, researchers often overlook the issues of unexpected drop in production in commercial cultivation of these chemicals (Jones 2014). This study aimed to also address this challenge called genetic instability, by recombining toxic and inert genes with varying constitutive and inducible promoters in self-replicating and integrational plasmids. Additionally, DNA uptake ability of the mutant cells, impact of synthetic parts on colony formation were also emphasized.

This will provided evidence on how inducible promoters act in PCC 7002. Ruffing et al. 2016 investigated twenty-four native constitutive promoters, and found the hypothetical promoter of PCC 7002 A2520 (Pstrong hereafter) which was found to have strong expression. A1930 (Pmedium) was found to have moderate expression and A0255 (Pweak) was found to have low expression levels. The inducible promoters nickel and cobalt inducible promoters were extensively studied in PCC 6803 for their tight

regulation of gene expression. Pnrsb and Pcoa are Inducible promoters allowing gene expression to be turned on or off in response to an external stimulus, such as the addition of a specific metal ion (Zess et al. 2016).

The “one-pot” assembly process of vector cloning named BASIC, allowed a quick and standardized way with flanking sequences that enable them to be assembled with combining compatible overhangs (Storch et al. 2015). Cobalt native inducible promoters were tested in Syn6803 but with limited success (Cavet et al. 2021). Similar result was observed in this study in self-replicating plasmids incorporated with sthA self-replicating plasmids. The reason for the lack of success was unknown, therefore after many attempts, the assembly procedure had to terminate and the next stages were prioritized. There have been affirming report on successful BASIC protocol of 4-5 parts of DNA parts with double antibiotic selection pressure, almost 99.7% expression (Bartasun et al. 2019). This was finely aligned with our current attempt of constructing 4 parts plasmid assembly. With two-step preparation protocol, set up of integrational plasmids found out more difficult prone to complications and scar formation (Kieleczawa 2006). Though regardless of BASIC's temperament in this study, it was overall a smooth process which was further complimented by the overall modular component layout of the plasmids.

Additional another plasmid construct was made with gene insert- pyruvate dehydrogenase complex (PDC) which acts as a ‘mild’ toxic insert. This was not assessed because of the lack of variability in the promoter, e.g. only Pcoa-PDC was successful.

Firstly, 25 plasmids were constructed containing five different RBS strengths in PCC 7002. This was primarily done to investigate the most suitable RBS for the different promoter. The interaction between the promoter and RBS determines translation efficiency. To choose a suitable RBS, consider using a strong promoter with a weak RBS for moderate expression, or a weak promoter with a strong RBS for high expression (Zhu et al. 2024). The ‘leakiness’ of the promoters was then investigated (Englund et al. 2016), where fluorescence expression of the constitutive promoters fluoresced slightly more than expected. The possible reason of this low expression of genes without inducers can be- widely used promoters from *E. coli* do no repressed completely in cyanobacteria (Huang and Lindbald 2013). However PnrsB seemed to work the highest which aligns with recent works of Lazar and Tabor 2021. Transformation with transhydrogenase enzyme results with a quantification of the size differences, in terms of radii size of the colonies. The probable reason behind due to excess NADPH or NADH which imbalances caused by the engineered enzyme (Gerdes et al. 2006). However, it is important to note that no conclusions can be made from the self-replicating plasmids in terms of promoter leakiness despite being conducted in triplicates.

In case of the integrational plasmids, experiment was performed divided in 2 parts and plasmids were assembled in four parts by BASIC final assembly. The integrational plasmids were successfully transformed into PCC 7002, where partial or full segregation occurred. Integrative plasmid pattern is defined by the linearization and subsequent

insertion into host chromosomal DNA which was a successful attempt in several studies (de la Cruz and Davies 2000, Mann et al. 2005, Encinas et al. 2014). Restreaking of colonies with antibiotic selection pressure, again proved the successful integration.

After two weeks of initial transformation, it was apparent that Pstrong-sthA, the constitutive promoter (Pstrong) was always on, as expected, thereby no repression was taken place. The number of colonies on a plate is dependent on the ability of the promoter to maintain a tight regulation and minimise leaky expression leading to metabolic burden. Compared with PnrsB, the expression of sthA is similar to Pstrong, in regards to being always on. The bleaching, therefore, indicates that toxicity/burden has occurred presenting a phenotypic response rather than genotypic. Ni^{2+} inducible promoter of nrsBACD operon maintains homeostasis and expression was 350 fold increase compared to other native inducible promoters (Peca et al. 2007) However, sthA cassette was not visible or remained intact upon amplification after first colony PCR. This could potentially be explained due to the lack for repression strength in the constitutive promoters leading to instability, expression of a toxic gene. Eventually, the inserted sthA gene was removed yet keeping the antibiotic cassette, consequent of antibiotic selection pressures.

Synthetic part, especially promoter analysis for the development of engineered cyanobacteria is still far behind than other model organisms. Fully repressed and highly induced promoter library can certainly reduce genetically instable behavior which till now is omitted from various scientific reports. No concrete evidence of toxicity was shown by the self-replicating plasmids in terms of colony count. The integrated plasmid further gave a clear phenotypical and molecular evidence of instability when introduced with a hypothetical strong constitutive promoter and the nickel inducible prompter to express sthA. In future, qPCR needs to be done to check for quantitative status. For a more in-depth analysis of the genetic instability in PCC 7002, constructing plasmids with light-driven native constitutive promoter Pcpc or PpsbA *which have evidence to have strongcontrol of gene expression in Synechocystis 6803 and 6714.* Thus expanding the toolbox of synthetic biology parts and systems for PCC 7002, including robust promoters, modular vectors, and other genetic elements will be used in developing and validating these tools for use in various cyanobacterial species beyond the model strains.

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