Cyanobacteria Biotherapeutic Protein Production:

Phycocyanin-Interferon Fusion Constructs

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ABSTRACT

Cyanobacteria can be utilized for biotherapeutic protein production. Cyanobacteria's ability to use photosynthesis can be exploited as a host organism for biotherapeutic protein production, offering a cheaper alternative to traditional hosts; *E. coli* and yeast. Limitations on steady state protein production in cyanobacteria must first be overcome. By using novel DNA fusion constructs, there is hope for higher yields of desired biotherapeutic proteins. Phycocyanin is an ideal highly-expressed endogenous protein for fusion with foreign proteins to overcome degradation in the cell. Interferon is used as a case study to make phycocyanin-interferon constructs. The central research question asks, how can we increase biotherapeutic protein production in cyanobacteria? And our sub-question answered in this paper is, Can Cyanobacteria produce phycocyanin-interferon fusion constructs? Key results include 45% of phycocyanin present in phycocyanin-interferon transformant. Growth rates of phycocyanin-interferon strain are slowed showing a more gradual slope when compared to the wild-type strain. These DNA fusion constructs are indeed shown to stably accumulate interferon fusion constructs, taking a step towards implementing cyanobacteria as a cell factory for biotherapeutic protein production.

KEYWORDS

Over-expression, Recombinant Protein, *Synechocystis* sp. PCC 6803, Biopharmaceutical proteins, Synthetic Biology.

INTRODUCTION

A new avenue of biotherapeutic protein production is arising through utilization of Cyanobacteria. Health care, pharmaceutical, cosmetic and food industries have applications for various bioproducts (Popper et al. 2014, Daniell et al. 2016, "Theme 5- Novel Bioproducts and Biopharmaceuticals" 2018). Cyanobacteria can use photosynthesis to produce proteins needed for products such as oral vaccines, making them more affordable and accessible (Daniell et al. 2016). Traditionally, *E. coli* and yeast have been the primary hosts for biotherapeutic protein production, which require expensive feedstocks consisting of sugars and ammonia (Buschke et al. 2013). Use of cyanobacteria to produce biotherapeutic proteins is being explored due to the low cost feedstock of sunlight, water, and carbon dioxide (Melis et al. 2023). Cyanobacteria is promising but making the switch from *E. coli* will take more time and research before it is able to produce products at the same level.

Limitations on the expression of foreign proteins have kept cyanobacteria from becoming a suitable host. Overexpression of biotherapeutic proteins in cyanobacteria is difficult due to the cell's recognition and degradation of foreign proteins (Zhang et al. 2021). DNA fusion constructs can overcome this limitation by fusion of the recombinant gene with highly-expressed endogenous ones (Betterle et al. 2020, Zhang et al. 2021). Fusion with endogenous proteins slows down degradation, as the destruction of essential endogenous proteins is not advantageous to the cell. There has been some success using phycocyanin as the endogenous protein in fusion constructs. Phycocyanin is an essential light capturing protein in cyanobacteria. Fusion of a foreign protein to phycocyanin has shown significant success (Betterle et al. 2020). This technology has opened a route for synthesis and accumulation of biotherapeutic proteins in cyanobacteria.

The valuable biotherapeutic protein, Interferon is used as a case study for fusion with phycocyanin in this experiment. Interferon (IFN) is a protein in the class of cytokines which are responsible for antiviral communication in eukaryotic cells (Betterle et al. 2020). IFNs are used to treat various diseases such as Chronic hepatitis, Melanoma, and Multiple sclerosis just to name a few (Castro et al. 2021). IFN is currently produced mainly using *E. coli* as a host cell, which relies on sugar and ammonia for growth (Satta et al. 2023). Cyanobacteria can synthesize

IFN using CO_2, sunlight, and water, making it more sustainable and lower cost than its *E. coli* counterpart (Satta et al. 2023). DNA fusion technology may be used for accumulation of IFN in cyanobacteria. Over-expression is possible, however, there is more that needs to be done before commercialization.

To achieve high yields of Interferon in cyanobacteria, I need to increase the steady state production of the phycocyanin-IFN recombinant construct. First we ask the central question, how can we increase biotherapeutic protein production in cyanobacteria? To answer this question we will use the IFN construct as a case study. If we can optimize IFN construct accumulation, then these methods may be applied for production of other biothherapeutic proteins as well. To begin answering our central question, we must first ask; Can cyanobacteria produce phycocyanin-IFN fusion constructs? We answer this sub-question in this paper and take a step towards implementing cyanobacteria as a host for biotherapeutic protein production.

METHODS

Strain and Growth Conditions

The cyanobacteria strain *Synechocystis* sp. PCC 6803 is used and referred to from here on as wild-type (WT). To ensure changes in growth of our phycocyanin-IFN (IFN) construct are coming from the phycocyanin unit, we use a control transformant with the entire phycocyanin operon deleted called **Δ**cpc. IFN has a chloramphenicol antibiotic resistance cassette and **Δ**cpc has a kanamycin resistance cassette. Visulization of DNA maps are from previous study using same transformants (Figure 1) (Hidalgo Martinez and Melis 2023). Both transformants and WT were first grown on agar plates and then inoculated into a liquid culture. Liquid cultures were grown in 250 mL Erlenmyer flasks with BG11 growth medium with appropriate antibiotic and stir bar for culture mixing. Flasks were placed on magnetic plates in a temperature and light controlled room kept at 25℃.

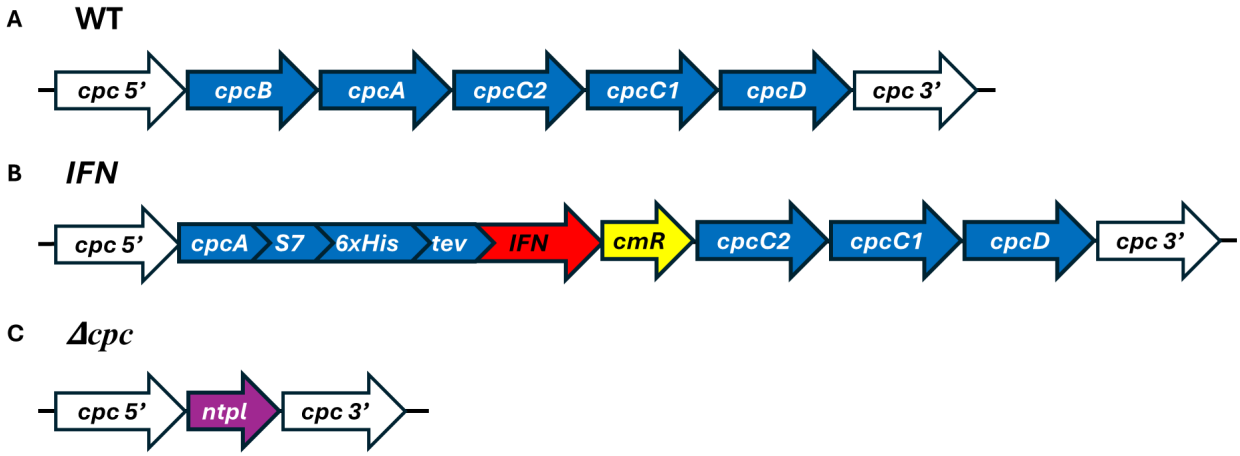

Figure 1. Schematic representation of DNA maps of wild-type and transformants. (A) Endogenous *cpc* operon in *Synechocystis* referred to as WT. (B) Replacement of cpcA gene encoding for **α**-subunit with fusion construct *cpcA*S7*6xHis*tev*IFN* referred to as IFN. Followed by chloramphenicol (cmR) resistance cassette. 6xHistidine (6xHis) tag is used for purification purposes. (C) Entire phycocyanin operon is deleted and replaced by kanamycin resistance cassette (nptI) referred to as **Δ**cpc. (Hidalgo Martinez and Melis 2023).

Absorbance Spectra

Absorbance spectra was taken twice from each liquid culture several days after inoculation (Figure 2a). Whole cells were harvested (~1 mL) under UV sterilized fume hood and a UV-spectrum was taken of WT and transformants. All three spectra were normalized at 682 nm (max peak) for comparison at 620nm where phycocyanin is absorbed. To calculate percent absorbance, **Δ**cpc was set as the base line of 0% absorbance and IFN was calculated relative to WT. An absorbance spectrum of WT and transformants was measured every ~24 hours for seven days. The OD from each culture was taken at 730 nm and plotted against time using Excel.

SDS-PAGE

After liquid cultures reached the end of their growth phase, 50 mL from each culture was harvested in 50 mL falcon tubes and cells precipitated at 5000 rpm for 15 minutes. Supernatant was disposed and pellet was resuspended in 2 mL of water. Cells were lysed by pouring liquid nitrogen into mortar and pestle containing resuspended pellet and the frozen cells were ground until liquid, this process was repeated three times for each pellet. Cellular protein extracts were

stored in 1.5 mL tube in -40℃ freezer. A SDS-PAGE was ran with protein extracts the next day at two different concentrations, 0.40 µg and 0.80 µg. The gel was visualized with Coomassie blue stain for one hour and then rinsed with destaining solution for 20 minutes *x*3, then left on shaker with distilled water overnight. Image was taken the next day.

RESULTS

Absorbance Spectra

Absorbance spectra several days after inoculation showed peaks at 620 nm for WT and IFN transformant. Strain **Δ**cpc, expectedly, has no peak at 620nm. WT absorbance at 620 nm is 0.50 and IFN is 0. after setting **Δ**cpc as the baseline for phycocyanin absorbance. Showing an absorbance (Figure 2a). The OD_{730} over seven days of growth for WT was the most linear growth with the steepest trendline (Figure 2b). Transformants showed more varied growth over the seven days with a moderate upward trend and IFN showing the slowest growth.

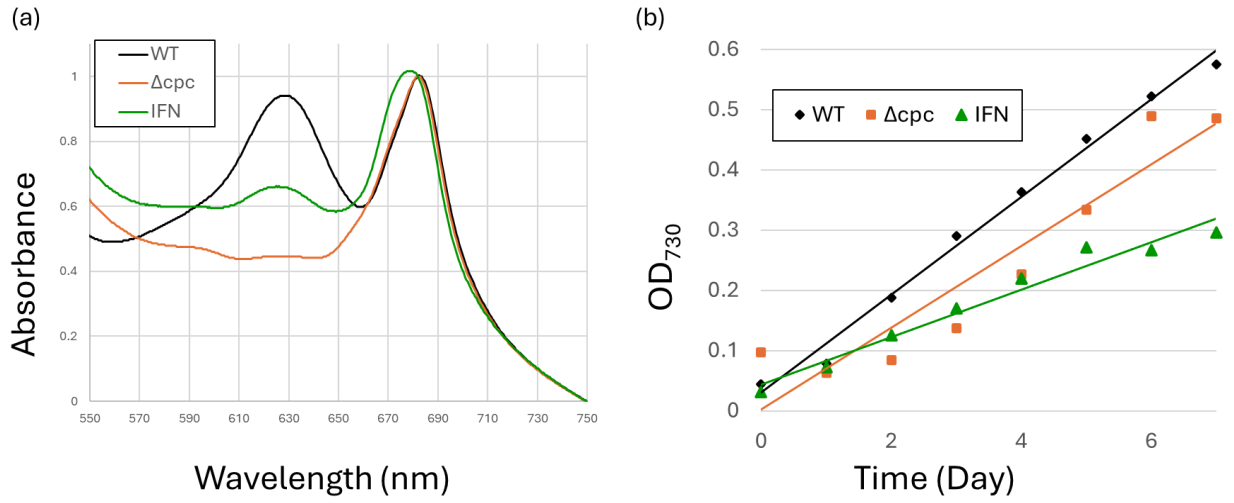

Figure 2. Comparative analysis using 550-750 nm UV Spectrum of Wild-type (WT), Δcpc, and Interferon (IFN). (a) Absorbance was taken of whole cells in BG-11 several days after inoculation. Data was normalized at maximum absorbance which was 682 nm to show differences in peaks at 620 nm. Absorbance of IFN and **Δ**cpc relative to WT are 45% and 0% respectively. (b) Growth curve of WT, **Δ**cpc, and IFN. Whole cell OD at 730 nm measurements were taken every 24 hours for 7 days and plotted as a function of growth time. Slopes of WT, **Δ**cpc, and IFN are 0.89, 0.68 and 0.39 respectively.

SDS-PAGE

Total cellular extracts of each culture were resolved using SDS-PAGE. IFN shows presence of interferon at ~37 kDa and the resistance cassette can be seen at ~22 kDa (Figure 3). Unexpectedly, the **Δ**cpc content shows presence of the phycocyanin subunit between 15 and 20 kDa, perhaps due to contamination. Increasing concentration of sample in the SDS-PAGE lanes shows more intense lines so protein content can be easily distinguished.

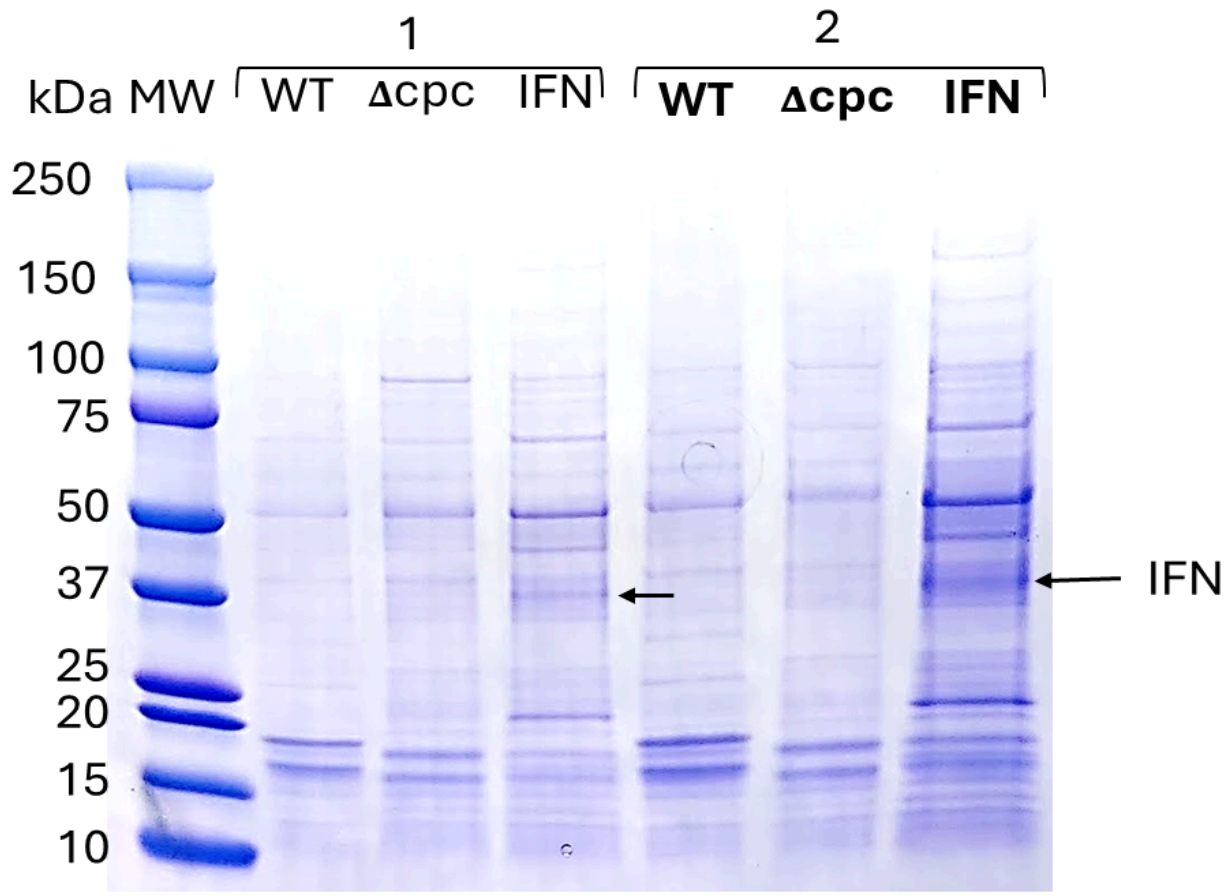

Figure 3. Protein expression analysis of wild-type (WT), Δcpc, and Interferon (IFN). Cells were lysed and a SDS-PAGE was run with total protein extracts at two different concentrations. Bracket 1(left) is at 0.4 µg of protein extract per lane and bracket 2(right) is at 0.8 µg of extract per lane. SDS-PAGE was visualized with a Coomassie blue stain. IFN can be seen at ~37 kDa indicated by black arrows.

DISCUSSION

Wild-type cyanobacteria containing the endogenous phycocyanin operon had an absorbance of 0.94 and the IFN transformant has an absorbance of 0.63. The absorbance of IFN relative to WT is 45% after setting the transformant **Δ**cpc as the baseline of 0% absorbance (Figure 2a). During the initial growth phase WT was observed to grow the fastest with a slope of 0.081 in the first seven days. Transformants IFN and **Δ**cpc had a slope of 0.039 and 0.068 in the first seven days, respectively (Figure 2b). Interferon is observed to be present at ~37 kDa in IFN (Figure 3). We observed lines between 15 and 20 kDa, corresponding to cpcA and cpcB subunits of the cpc operon, to be present for both WT and **Δ**cpc (Figure 3).

Comparative Analysis

Pigment content

Absorbance at 620 nm shows evidence of phycocyanin-IFN construct. IFN has 45% of the absorbance relative to WT absorbance at 620 nm where phycocyanin is absorbed (Figure 2a). This means cyanobacteria can accumulate close to half of the phycocyanin that is expected without the fusion construct. This result is promising and supports the conclusion that cyanobacteria are able to stabely accumulate phycocyanin-IFN constructs. The relative absorbance in this study is in line with previous studies on accumulation of phycocyanin constructs (Hidalgo Martinez and Melis 2023). Growth rates between WT and transformants can also give insight to how well cyanobacteria can act as cell factories for biotherpeutic protein production. Slower growth rates for stain containing fusion constructs are expected as shown in previous studies (Zhang et al. 2021, Hidalgo Martinez and Melis 2023). Our results follow this trend with IFN having a slope of 0.039 compared to WT rate of 0.081. Comparative analysis of cellular pigments and growth rates between WT and transformants shows evidence of our phycocyanin-IFN construct and suggests cyanobacteria are able to survive while maintaining some level of protein production.

Cellular Metabolites

Analysis of total cellular metabolite extract reveal presence of IFN further suggesting cyanobacteria can produce IFN using phycocyanin as a leader fusion protein. We observed presence of IFN is low compared to other proteins in the cell (Figure 3). Low concentration does not mean our construct is not good. For accumulation of biotherapeutic protein to be deemed commercially viable, it needs to be able overcome steady state levels of 0.1% and other studies have optimized these methods to yield up to 10-20% of total cellular protein (Melis et al. 2023) Complete purification of our protein is needed to determine this. However, our results show promise to meet this condition (Figure 3).

Conclusion

Stable accumulation of Biotherapeutic proteins in cyanobacteria is possible through the use of DNA fusion constructs. This is important as free foreign proteins are readily degraded by the cellular proteasome (Satta et al. 2023). This case study using phycocyanin-IFN constructs showed low steady state levels of IFN production. Comparative analysis of pigments and protein content between wild-type and transformants revealed that cyanobacteria are able to produce IFN but the process slows cell growth. This addresses our subquestion, “Can cyanobacteria produce phycocyanin-IFN fusion constructs?” And takes us a step closer to answering the central research question, “How can we increase biotherapeutic protein production in cyanobacteria?” The key result from this current study is the presence of IFN in our transformant. Optimization of this method has shown higher levels of expression and high IFN yields after protein purification (Betterle et al. 2020). A complete analysis of phycocyanin-IFN construct production shows cyanobacteria are able to produce this construct and suggests that using phycocyanin as a fusion construct can increase production of foreign proteins in cyanobacteria.

Limitations and Future Directions

The use of cyanobacteria in synthetic biology is becoming increasingly significant. The potential for it to become a cell factory for biotherapeutic protein accumulation is great however, these method must be optimized before commercial application. The use of phycocyanin as a fusion protein for stable production of other exogenous proteins in addition to interferon is possible (Hidalgo Martinez and Melis 2023). Future studies will focus on the growth of these transformants for consistent yields of greater than 10-15% biomass. Purification of these proteins and how they accumulate in the cell may vary with different proteins. This is a major limitation on the ability of DNA fusion constructs to overcome cyanobacteria's ability to steadily accumulate a variety of biotherapeutic proteins. Efforts will continue and if these limitations are overcome, cyanobacteria will become a sustainable alternative for the biotherapeutic industry.

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