

# Recombinant protein synthesis and isolation of human interferon alpha-2 in cyanobacteria

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## Keywords

Cyanobacteria, Interferon-alpha2, Phycocyanin, Tobacco Etch Virus

## Abbreviations

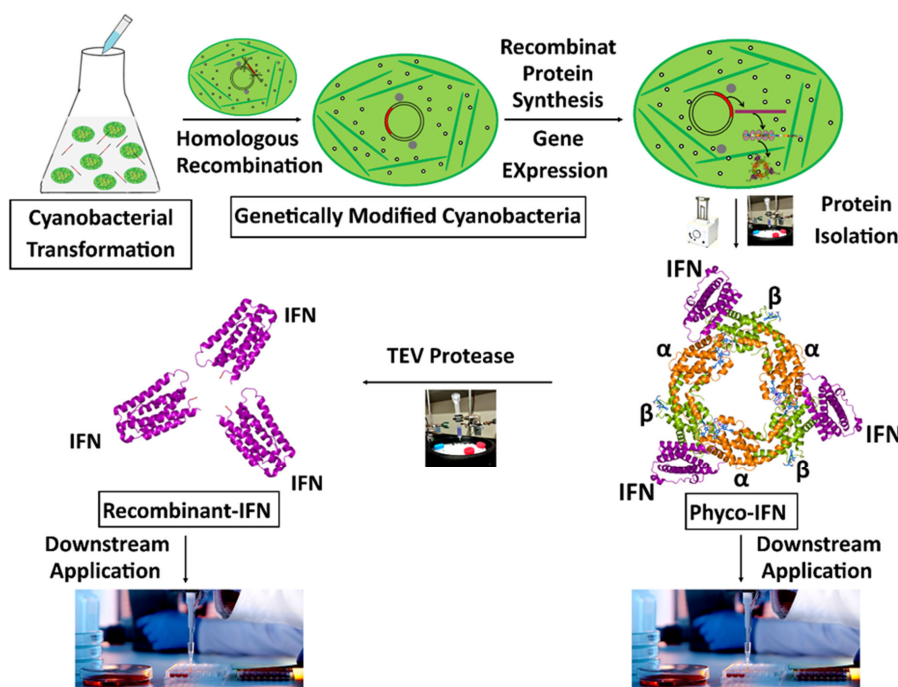
*Synechocystis*: *Synechocystis* sp. PCC 6803; IFN: Interferon; rTEV: Recombinant Tobacco Etch Virus Protease; tev: Tobacco Etch Virus Protease cleaving domain (tev: ENLYFQ/G); S: oligopeptide spacer (S: PAEKWAPGGS); *cpc*: The phycocyanin-encoding operon in cyanobacteria.; *cpcA*: Gene encoding the phycocyanin  $\alpha$ -subunit (sll1578); *cpcB*: Gene encoding the phycocyanin  $\beta$ -subunit (sll1577); *cpcG1*: Gene encoding the phycocyanin-to-allophycocyanin proximal linker protein CpcG1 (slr2051)

## Commentary

Interferons (IFNs) are a class of small immunological proteins that are secreted by infected cells during viral or bacterial infections to combat and prevent infection propagation [1]. They play important roles in triggering signal cascade processes inside the cell that activate other immune cells and limit viral multiplication. More than twenty distinct types of IFNs have been reported from animal research, and they are classified into three categories, Type I, II, and III, based on their functional receptors [2]. Type I interferons (IFN- $\alpha$ , IFN- $\beta$ , IFN- $\kappa$ , IFN- $\epsilon$ , and IFN- $\omega$ ) bind to the native IFN- $\alpha/\beta$  receptor (IFNAR) and activate the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway, producing antiviral proteins within the cell [1,2]. IFN- $\alpha 2$  (IFN) is a subtype of IFN- $\alpha$  and a key immunological protein in cells that helps defend against viral infections. It is a small protein composed of 165 amino acids that comprise a helical secondary structure consisting of 5 alpha helices [3]. It has a considerable market value as an anti-viral medication that prevents infections and boosts natural immunity [4,5]. It also has a high demand among academic professionals for scientific research. However, *in vitro* production of native IFN in mammalian cells for therapeutic purposes is challenging and expensive [4,6]. As a result, biotech companies and academic professionals have lately exploited recombinant-DNA technology to heterologously generate IFNs in a variety of host species, including *Escherichia coli*, yeast, and plants [4,7,8]. The production of active recombinant-IFNs in these heterologous host species presents several challenges due to IFN's susceptibility to heat-stress, protein misfolding, and structural instability (degradation) via the host cell proteasome-mediated recombinant protein removal processes [9,10]. Further, when synthesized in *Escherichia coli* or yeast, there are risks associated with contamination due to the rich media employed, alleviation of which increases costs and renders the entire process less attractive [8]. Photosynthetic organisms have recently been effectively exploited as

heterologous hosts to synthesize a wide range of important bioproducts, including bioactive compounds and biopharmaceutical proteins [11-17]. Photosynthetic cyanobacteria have gained attention in this respect, as potential hosts for the generation of a variety of products due to their autotrophic growth, ease of genetic manipulation, minimal contamination risks, and low-cost growth requirements [18,19]. Importantly, cyanobacteria are readily and stably transformable, and the corresponding culture media comprise simple inorganic nutrients and, therefore, cannot be carriers of zoonotic agents. *Synechocystis* sp. PCC 6803 (*Synechocystis*) is a freshwater cyanobacterium widely used as a model organism for research in photosynthesis [20] and synthetic biology [14]. It has also been used as a potential host for the synthesis of foreign proteins utilizing recombinant-DNA technologies [12,17,21]. In our recent study [17], *Synechocystis* was used as a host to produce a recombinant-IFN protein without many of the pitfalls of recombinant protein degradation mentioned above [12,17,21]. A flow chart of this process is shown in **Figure 1**. In this case, the heterologous IFN (*IFN- $\alpha$ 2*) gene was introduced into the cyanobacterium genomic DNA and was set to express under the strong promoter of the endogenous *cpc* operon [12,17,21]. The *cpc* operon comprises the genes *cpcB*, *cpcA*, *cpcC2*, *cpcC1*, and *cpcD*, which code for different subunits of the abundant phycocyanin protein complex. Phycocyanin assembles as a large multimeric protein complex comprising the light-harvesting phycobilisome, peripherally attached to the outer surface of the thylakoid membrane, above the transmembrane photosystem II (PS II) complex [22,23]. The phycobilisome is made up of core cylinders (containing allophycocyanin), six peripheral rods (containing

phycocyanin), and linkers that connect the peripheral rods to the core cylinders [22,23]. In *Synechocystis*, the peripheral rods of phycocyanin are the most abundant cellular proteins, encoded by the highly expressed *cpcB* and *cpcA* genes, which produce the CpcB ( $\beta$ -subunits) and CpcA ( $\alpha$ -subunits) of phycocyanin. It has been shown that transgenes from eukaryotic organisms and bacteria, when expressed under the control of the *cpc* operon promoter in cyanobacteria, are transcribed and the corresponding mRNA accumulates to substantial quantities, commensurate with the strength of the promoter, as predicted by theory [23]. This, however, does not translate in a high-level accumulation of the corresponding recombinant proteins. For example, a solo expression of the *IFN- $\alpha$ 2* gene, under the control of the *cpc* operon promoter, either in the presence of the *cpcB* and *cpcA* or upon replacing the entire *cpc* operon genes with the *IFN- $\alpha$ 2*, resulted in low-level expression of the transgene [12]. Typically, this low protein level could not be seen in SDS-PAGE Coomassie stains of total protein cell extracts. Sensitive Western blot analysis was required to show extremely low levels. However, fusion constructs of the *IFN- $\alpha$ 2* with the highly expressed *cpcB* gene (*cpcB*\**IFN*) led to stable recombinant-IFN accumulation in the cell, comprising 10-12% of the total cell protein, easily detected and quantified by SDS-PAGE Coomassie stain [12]. Several other subunits of phycocyanin, such as CpcA and CpcG1, have also been used as leading sequences in IFN-fusion constructs to generate notable amounts of IFN in cells [14]. This “fusion constructs” enhancement in recombinant protein expression is not unique to IFN but was previously reported for other plant- and microbial-origin recombinant proteins. Included are the terrestrial plant



**Figure 1.** Schematic representation of recombinant-IFN synthesis and processing in cyanobacteria. The steps include cyanobacterial transformation, cell growth, recombinant CpcB\*IFN fusion construct isolation in phycocyanin ( $(\alpha, \beta^* \text{IFN})_3$  heterohexameric protein form (Phycy-IFN), and isolation following cleaving of the target IFN heterologous protein from the  $(\alpha, \beta^* \text{IFN})_3$  carrier complex upon incubation with a tobacco etch virus (TEV) protease.

isoprene synthase [24],  $\beta$ -phellandrene synthase [25], the bacterial tetanus toxin fragment C (10), as well as the 2, C-methylerythritol 4-phosphate pathway enzymes isopentenyl diphosphate isomerase and geranyl diphosphate synthase, all of which could be seen in SDS-PAGE Coomassie stain only when in a fusion construct configuration with a stably-expressed endogenous cyanobacterial gene, regardless of the promoter used (please see also below). Further to the high-level production of IFN as a fusion construct in cyanobacteria, the technology was developed to isolate the active fusion construct from the crude cellular extracts. Modified fusion construct proteins were designed with a His-tag between the *cpcB* and *IFN* genes (*cpcB\*6xHis\*IFN*), which was used to effectively isolate the corresponding CpcB\*6xHis\*IFN protein from crude cellular extracts through differential Co-column affinity chromatography [21]. Moreover, generating the natural IFN, in pure form, from the CpcB\*6xHis\*IFN fusion protein required a further modification to introduce a protein cleaving site designed to release the natural form of IFN from the fusion construct. This was accomplished upon introduction of the tobacco etch virus cleaving domain “tev” (tev: ENLYFQ/G) just prior to the IFN in the fusion construct, generating the CpcB\*6xHis\*tev\*IFN variant. Initial rates of cleaving this variant upon incubation with recombinant TEV protease (rTEV) were slow, attributed to the tev cleaving site being inaccessible to the rTEV protease, apparently due to the tertiary configuration of the CpcB\*6xHis\*tev\*IFN variant. We surmised that the tev cleaving site was obstructed in the fusion protein complex. To alleviate this issue, a spacer “S” was designed (S: PAEKWAPGGS), comprising two proline constituent amino acids, which was included between CpcB and IFN, just prior to the tev cleaving site, making the CpcB\*6xHis\*S\*tev\*IFN variant, serving to both extend the tev cleaving site further away from the leading protein complex, and to change the relative orientation of the trailing IFN relative to the leading CpcB protein, thus making it more accessible to rTEV protease [17]. In this respect, it is known that proline amino acids in a peptide cause a bend, or turn, in the protein secondary structure, thus exposing parts that may otherwise be obstructed. Thus, addition of a spacer “S” to the fusion protein (CpcB\*His\*S\*tev\*IFN) oriented the recombinant-IFN in a way that the tev cleaving site was more accessible to the rTEV protease. Inclusion of such spacer significantly increased the rTEV catalytic efficiency, reaching more than 90% separation of the IFN from the fusion construct.

In summary, *Synechocystis* (cyanobacteria) can be used in large scale cultivations to produce a variety of biopharmaceutical proteins, including IFNs, because of its remarkable ability to integrate foreign genes into the genome and synthesize and accumulate the corresponding transgenic products without a need to provide organic nutrients, in a process driven by sunlight. Moreover, producing recombinant IFNs as part of a phycocyanin protein complex stabilizes the heterologous protein and prevents its degradation by the cellular proteasome, which is designed to remove, among other, exogenous proteins. It was concluded that cells may tolerate the presence of otherwise unstable recombinant proteins, when fused to a native protein, which is required for growth and survival, thus enabling their accumulation in functional form. Importantly, the fusion constructs technology can be generalized to increase the concentration of normally unstable recombinant proteins and or enzymes, while retaining their catalytic activities. Functionality of the fusion CpcB\*IFN was tested from bioactivity analyses, assessed from

the relative antiviral proliferation properties of the cyanobacterial recombinant CpcB\*IFN protein, and favorably compared with that of commercially available natural interferon [12]. Similarly, the activity of other CpcB\*Fusion enzymes was shown, including, among other, the terrestrial plant isoprene synthase [24], and  $\beta$ -phellandrene synthase [25], conferring upon transformant cyanobacteria the ability to produce the corresponding heterologous isoprene and  $\beta$ -phellandrene bioactive compounds. Current efforts are focusing on the structure, amino acid composition, and tertiary configuration of would be target proteins to develop a better understanding on how suitable they may be for fusion overexpression. This basic research effort seeks to assess the role of alpha-helices, versus beta-sheets, in target proteins and how these may affect overexpression and activity in CpcB\*Fusion configurations. These novel insights on fusion constructs will find significant and direct application in cyanobacterial cell factories for the photosynthetic production of useful recombinant proteins, enzymes, and bioactive compounds.

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