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BIOTECHNOLOGY and BIOENGINEERING

Establishing a High Yielding *Streptomyces*-Based Cell-Free Protein Synthesis System

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ABSTRACT: Cell-free protein synthesis (CFPS) has emerged as a powerful platform for applied biotechnology and synthetic biology, with a range of applications in synthesizing proteins, evolving proteins, and prototyping genetic circuits. To expand the current CFPS repertoire, we report here the development and optimization of a *Streptomyces*-based CFPS system for the expression of GC-rich genes. By developing a streamlined crude extract preparation protocol and optimizing reaction conditions, we were able to achieve active enhanced green fluorescent protein (EGFP) yields of greater than 50 μg/mL with batch reactions lasting up to 3 h. By adopting a semi-continuous reaction format, the EGFP yield could be increased to 282 ± 8 μg/mL and the reaction time was extended to 48 h. Notably, our extract preparation procedures were robust to multiple *Streptomyces lividans* and *Streptomyces coelicolor* strains, although expression yields varied. We show that our optimized *Streptomyces lividans* system provides benefits when compared to an *Escherichia coli*-based CFPS system for increasing percent soluble protein expression for four *Streptomyces*-originated high GC-content genes that are involved in biosynthesis of the nonribosomal peptides tambromycin and valinomycin. Looking forward, we believe that our *Streptomyces*-based CFPS system will contribute significantly towards efforts to express complex natural product gene clusters (e.g., nonribosomal peptides and polyketides), providing a new avenue for obtaining and studying natural product biosynthesis pathways.

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Introduction

Crude extract based cell-free systems, which have been used for decades, are of great importance and interest for facilitating the understanding of biological systems and enabling new applications in genetic prototyping and biomanufacturing (Carlson et al., 2012; Hodgman and Jewett, 2012). Because of the absence of cell walls, the reaction environment is open, accessible, and controllable, allowing for direct and easy manipulation, monitoring, sampling, and optimization. Cell-free protein synthesis (CFPS) is one of the most important applications of cell-free systems. CFPS systems help address a growing need for simple, inexpensive, and efficient protein production technologies. So far, they have been widely utilized for manufacturing a wide variety of active protein products that include therapeutic vaccines (Kanter et al., 2007; Yang et al., 2005), antibodies (Min et al., 2016; Stech and Kubick, 2015), virus-like particles (Bundy et al., 2008; Lu et al., 2015), membrane proteins (Henrich et al., 2015; Sachse et al., 2014), metalloproteins (Boyer et al., 2008; Kwon et al., 2013; Li et al., 2016) and proteins harboring non-standard amino acids (Hong et al., 2014, 2015). CFPS has also been applied for the rapid prototyping of biological circuits and metabolic pathways (Garamella et al., 2016; Karim and Jewett, 2016; Sun et al., 2014; Takahashi et al., 2015), biosynthesis of natural products (Goering et al., 2017) as well as designing of paper-based diagnostics (Pardee et al., 2014, 2016a). The ability to freeze-dry CFPS systems is opening the way to novel on demand biomanufacturing applications as well (Dudley et al., 2016; Pardee et al., 2016b; Salehi et al., 2016; Smith et al., 2014).

To produce proteins of interest, CFPS systems harness an ensemble of catalytic components necessary for energy generation and protein synthesis from crude lysates of cells. These activated catalysts act as a chemical factory to synthesize and fold desired protein products upon incubation with essential substrates, which include amino acids, nucleotides, DNA or mRNA template encoding the target protein, energy substrates, cofactors, and salts. In principle,

any organism can be used to provide a source of crude lysate. However, the most commonly used systems are the prokaryotic *Escherichia coli* system (bacterium) (Jewett and Swartz, 2004a; Kim et al., 1996; Kwon and Jewett, 2015), as well as the eukaryotic systems based on crude cell lysates from *Saccharomyces cerevisiae* (fungus) (Gan and Jewett, 2014; Hodgman and Jewett, 2013), wheat germ (plant) (Madin et al., 2000; Takai et al., 2010), tobacco (plant) (Buntru et al., 2015; Komoda et al., 2004), *Spodoptera frugiperda* (insect) (Stech et al., 2014; Tarui et al., 2001), rabbit reticulocytes (mammalian) (Anastasina et al., 2014; Pelham and Jackson, 1976) and Chinese hamster ovary (CHO, mammalian) (Brödel et al., 2014). These CFPS systems are developed for different biological and application-based goals, albeit each one has its own advantages and disadvantages (Zemella et al., 2015). In general, the prokaryotic *E. coli* system is the most robust CFPS platform compared to other eukaryotic systems due to its simple cultivation, fast cell growth, cheap and easy cell extract preparation, and high protein yields; however, *E. coli* extract lacks eukaryotic post-translational modification machinery and can be limited in its ability to express some eukaryotic proteins that need native eukaryotic chaperones for correct folding. By contrast, although the eukaryotic CFPS systems are able to synthesize "difficult-to-express" proteins, their cell extract preparations are usually laborious and expensive and the protein yields can be relatively low (<50 μg/mL).

Given the importance of CFPS in biotechnology and synthetic biology (Carlson et al., 2012; Hodgman and Jewett, 2012), there is an exciting opportunity to explore development of novel cell-free systems from diverse organisms. Indeed, Freemont and colleagues have recently reported the development of two systems (e.g., *Bacillus subtilis*) and shown their utility for studying genetic circuits (Chappell et al., 2013; Kelwick et al., 2016). With a long-term vision of using CFPS for natural product discovery, we have interest in antibiotic producing organisms, such as those in the *Streptomyces* species.

The *Streptomyces* species are Gram-positive bacteria with high GC-content genomes ($>$70% GC) and featured by many complex natural product biosynthetic gene clusters (Bentley et al., 2002). Traditionally, several *Streptomyces* strains have been used for in vivo heterologous protein expression (Anné and van Mellaert, 1993; Binnie et al., 1997; Brawner et al., 1991; Gomez-Escribano and Bibb, 2012). However, an in vitro coupled transcription-translation protein synthesis system from *Streptomyces lividans* 66 was also developed decades ago (Thompson et al., 1984). This system has been used to express and identify the phenoxazinone synthase (one enzyme involved in the actinomycin biosynthesis) gene from three hypothesized gene sequences of *S. antibioticus* (Jones and Hopwood, 1984b), and later the same gene that is silent in *S. lividans* was activated and expressed, showing a particularly interesting application of the CFPS system (Jones and Hopwood, 1984a; Madu and Jones, 1989). The CFPS system was also used to determine the relationships of two genes that are involved in puromycin biosynthesis (Vara et al., 1988) and to investigate the ribosomal resistance mechanisms in response to antibiotics (Calcutt and Cundliffe, 1989; Fish and Cundliffe, 1996). Although the original *Streptomyces* CFPS system had shown utility, the cell extract preparation procedure was time-consuming and laborious (e.g., nuclease treatment of cell extracts, etc.) and yields were low, which perhaps limited adoption and utilization of the system as compared to other CFPS platforms like *E. coli*.

While there has been limited development of *Streptomyces*-based CFPS systems as a protein synthesis platform since their origin in the 1980s, we believe now is a time to revisit this platform for synthetic biology applications. Next generation sequencing and genome mining technologies have revolutionized the discovery of novel natural product biosynthetic gene clusters from micro-organisms, for instance, the *Streptomyces* species (Aigle et al., 2014; Ikeda et al., 2014). Analysis of sequenced genomes from numerous *Streptomyces* species has shown that a single species can carry over 20 natural product gene clusters, of which most are cryptic secondary metabolite gene clusters. To activate these silent pathways and obtain the cryptic metabolites, several heterologous hosts (e.g., *E. coli*, *Streptomyces*, etc.) have been used to express the entire gene clusters (Gomez-Escribano and Bibb, 2014; Li and Neubauer, 2014). However, this strategy often suffers from laborious and time-consuming cloning steps, insoluble expressed proteins, heavy metabolic burdens, and low product yields. With more and more gene clusters being identified from *Streptomyces*, it is necessary to establish a high-throughput method for rapid expression of the biosynthetic pathways. In this context, a robust and high yielding *Streptomyces*-based CFPS system could be a promising platform to complement existing strategies for pathway discovery.

In this work, we aim to establish a robust *Streptomyces*-based cell-free system for the expression of high GC-content genes originated from *Streptomyces*. This is a key step towards the long-term discovery vision described above. Here, we initially developed a CFPS system with the strain *S. lividans* B-12275 by using the codon-optimized enhanced green fluorescent protein (EGFP, 62% GC) as a reporter (Sun et al., 1999). Then, we optimized the system through the cell extract preparation process and cell-free reaction conditions. Under optimal conditions, the EGFP yield reached >50 μg/mL in a batch mode reaction, which was further increased to >280 μg/mL with a semi-continuous cell-free reaction. We also applied our approach to extracts from other *Streptomyces* strains and all of them were capable of synthesizing EGFP in active form. Finally, we expressed four high GC-content genes from different *Streptomyces* strains, showing a significant increase of solubility compared to an *E. coli*-based CFPS system. In the future, we envision that our *Streptomyces* cell-free system will contribute significantly to express complex natural product gene clusters (e.g., nonribosomal peptides and polyketides) from various *Streptomyces* species, enabling the discovery and synthesis of novel natural products.

Materials and Methods

Bacterial Strains and Culture Medium

The *Streptomyces* strains *S. lividans* B-12275, *S. lividans* 66, and *S. coelicolor* ISP-5233 were purchased from the Agricultural Research Service Culture Collection (Peoria, IL). *S. coelicolor* M1152 was kindly provided by Prof. Mervyn Bibb (John Innes Centre, Norwich, UK). The M1152 is a derivative of *S. coelicolor* M145 with four endogenous secondary metabolite gene clusters deleted and one

point mutation in *rpoB* introduced (Gomez-Escribano and Bibb, 2011). All *Streptomyces* strains were grown in the liquid yeast extract-malt extract (YEME) medium consisting of (per liter) 3 g yeast extract, 5 g peptone, 3 g malt extract, 10 g glucose, 340 g sucrose, and 5 mM $MgCl_2$.

Plasmid Construction

The reporter protein used in this study is enhanced green fluorescent protein (EGFP), which has been previously codon optimized for the expression in *Streptomyces* (Sun et al., 1999). The plasmid pIJ8655 harboring the EGFP gene was a generous gift from Prof. Mervyn Bibb (John Innes Centre, Norwich, UK). The EGFP gene (62% GC) was PCR amplified from the template pIJ8655 with a forward primer 5′-ATACATATGGTGAG-CAAGGGCGAGG-3′ (*Nde*I is underlined) and a reverse primer 5′-CCGGTCGACTTACTTGTACAGCTCGTCC-3′ (*Sal*I is underlined). After digestion with *Nde*I and *Sal*I, the EGFP gene was inserted between the T7 promoter and T7 terminator sequences of pJL1 generating the expression vector pJL1-EGFP. The high GC-content genes *tbrP* (72% GC), *tbrQ* (78% GC), and *tbrN* (75% GC), which are involved in the nonribosomal peptide tambromycin biosynthesis of the *Streptomyces* strain F-4474 (Goering et al., 2016), were cloned into the pJL1 vector as well yielding the expression vectors pJL1-TbrP, pJL1-TbrQ, and pJL1-TbrN, respectively. The type II thioesterase gene (TEII, 64% GC), encoded in the nonribosomal peptide valinomycin gene cluster of *S. tsusimaensis* (Li et al., 2015), was also cloned into the vector pJL1. All constructs were confirmed by DNA sequencing.

Preparation of Cell Extracts

All *Streptomyces* strains were grown in YEME liquid medium at 30°C in an orbital shaker at 250 rpm. Initial cultivation was performed in 6 mL of YEME in a standard glass culture tube with inoculation from a glycerol stock. After two days incubation, 0.5 mL of the culture was used to inoculate 50 mL fresh YEME medium in a 500 mL baffled flask, followed by 24 h cultivation. Then, the *Streptomyces* cells were grown in 1 L of YEME in a 2.5 L Tunair flask (IBI Scientific, Peosta, IA) with inoculation of 10 mL culture from the last step. After 16 h cultivation (mid-exponential phase), the cells were harvested by centrifugation at 7,000 g and 4°C for 15 min. Cell pellets were then washed twice with cold washing buffer (10 mM HEPES-KOH pH 7.5, 10 mM $MgCl_2$, 1 M NH_4Cl, 5 mM β-mercaptoethanol) and once with S30 buffer (50 mM HEPES-KOH pH 7.5, 10 mM $MgCl_2$, 50 mM NH_4Cl, 5 mM β-mercaptoethanol). After the final wash and centrifugation, the pelleted cells were resuspended in 2.5 mL of S30 buffer plus 10% (v/v) glycerol per gram of wet weight. The smooth suspended cells were disrupted by the EmulsiFlex-C3 homogenizer (Avestin, Ottawa, Canada) with single pass at a pressure of 12,000 psi. The lysate was then centrifuged at 16,000g and 4°C for 30 min. The resultant supernatant was collected and immediately flash frozen in liquid nitrogen and stored at −80°C until use.

Cell-Free Protein Synthesis

Standard CFPS reactions were performed in 1.5 mL microcentrifuge tubes containing the following components: 3 μL S30 buffer (see above), 0.7 μL magnesium acetate (0.1 M), 4 μL synthesis master mix (see below for details), 300 ng plasmid, 0.3 μL T7 RNA polymerase (1 mg/mL) and 5 μL cell extract. The total reaction volume was adjusted to 15 μL with nuclease-free water (Thermo Fisher Scientific, Waltham, MA). The final Mg^{2+} concentration in the reaction was 10 mM (unless otherwise noted). The synthesis master mix contained 200 mM HEPES-KOH pH 8.2, 140 mM ammonium acetate, 280 mM potassium acetate, 7 mM dithiothreitol (DTT), 5 mM ATP, 3.4 mM each of CTP, GTP and UTP, 100 mM phosphoenolpyruvate (PEP), 1.4 mM each of 20 standard amino acids, 7.5% (w/v) polyethylene glycol (PEG) 8000, 0.14 mg/mL folinic acid, and 245 U/mL pyruvate kinase (Sigma P7768). Note that tRNAs are endogenous from the cells without exogenous tRNA supplementation in the reaction. When it was desired to determine the protein yields and analyze the protein products on SDS-PAGE gels, 0.4 μL of L-[^{14}C(U)]-leucine (11.1 GBq mmol^{-1}, PerkinElmer, Waltham, MA) was added to the above mentioned CFPS reactions. All cell extracts used for CFPS were prepared from the *S. lividans* B-12275 strain and the reactions were incubated at 23°C for 3 h unless otherwise noted.

Semi-Continuous CFPS Reactions

Semi-continuous CFPS reactions were carried out in the Pierce 3.5 K MWCO Microdialysis device (Thermo Fisher Scientific, Rockford, IL) with 100 μL reaction mixture that is scaled-up from the standard 15 μL cell-free reaction system. The microdialysis device was placed in a 2 mL microcentrifuge tube, which is filled with 1.4 mL dialysis buffer. The dialysis buffer contained the same composition as in the reaction mixture without the following components of plasmid, T7 RNA polymerase and cell extract. The reactions were run at 23°C for 72 h in the Eppendorf ThermoMixer C (Hauppauge, NY) with a shaking speed at 600 rpm. During the incubation, 2 μL samples were removed at different time points for the EGFP quantification.

Quantification of Synthesized Proteins

The EGFP was used as a reporter protein to measure and optimize protein synthesis activity of the *Streptomyces*-based CFPS system. In order to quantify EGFP, the radioactive ^{14}C-leucine was added to the CFPS reactions as described above. After the reactions, the EGFP yields were determined by measuring the incorporation of ^{14}C-leucine into trichloroacetic acid-precipitable radioactivity with a liquid scintillation counter (MicroBeta2, PerkinElmer, Waltham, MA) as previously reported (Jewett and Swartz, 2004b). Alternatively, the fluorescence of active EGFP was measured using a BioTek Synergy 2 plate reader (Winooski, VT). Two microliters of the CFPS sample were mixed with 48 μL nuclease-free water and placed in a 96-well plate with flat bottom (Costar 3694, Corning Incorporated, Corning, NY). Then, measurements of the EGFP fluorescence were performed with excitation and emission wavelength at 485 and 528 nm, respectively. The fluorescence of

EGFP was converted to concentration (μg/mL) according to a linear standard curve made in house by expressing ^{14}C-leucine-labled EGFP (see Supplementary Fig. S1). The yields of synthesized TbrP, TbrQ, TbrN, and TEII were also quantified by the radioactivity. Total protein yields were measured directly after the CFPS reactions with the 15 μL mixture. For the soluble fraction, 15 μL of the reaction mixture was centrifuged at 12,000g and 4°C for 15 min. Ten microliters of the supernatant were taken out for soluble protein quantification.

Autoradiography Analysis

In order to analyze radiolabeled proteins, 3 μL of each cell-free reaction sample was loaded on a 4–12% NuPAGE SDS-PAGE gel (Invitrogen). After electrophoresis, the gel was stained using SimplyBlue™ SafeStain solution (Invitrogen) and destained in water. Then, the gel was fixed with cellophane films (Bio-Rad), dried overnight in a GelAir Dryer (Bio-Rad) without heating, and exposed for 48 h on a storage phosphor screen (GE Healthcare Biosciences, Pittsburgh, PA). The autoradiogram was scanned using a Phosphorimaging analyzer (Typhoon FLA 7,000, GE Healthcare Biosciences, Pittsburgh, PA) and analyzed with the ImageQuant TL 8.1 software (GE Healthcare Biosciences, Pittsburgh, PA).

Results and Discussion

Cell-Free Synthesis of EGFP With *Streptomyces*

We began our investigation by trying to directly adopt the protocol from Thompson et al. (1984) that was previously developed for *Streptomyces*-based CFPS, with the exception that we used the strain *S. lividans* B-12275 and the codon-optimized reporter protein EGFP (62% GC), rather than the *S. lividans* 66 strain and plasmids pIJ350 and pBR322 without any reporter protein. Unfortunately, our initial experiments failed. While this could have been due to a variety of issues, we suspected that this could be due to the plasmid we used. We first used the plasmid pIJ8655 harboring the EGFP gene as a template for the cell-free reaction. The pIJ8655 has been used in vivo for EGFP expression in *S. coelicolor* as a reporter (Sun et al., 1999). We suspected that plasmid pIJ8655 was not suitable for protein expression in the cell-free system. Therefore, we cloned the EGFP gene into a cell-free favored expression vector pJL1 that has the T7 promoter and T7 terminator sequences (Li et al., 2016).

With our new expression vector in hand, we first set-out to validate that our cell-free system was competent in transcription, which would be directed by the T7 RNA polymerase. To confirm transcription activity in the crude extract based *Streptomyces*-based cell-free reaction, we chose to use a Spinach aptamer system (Chizzolini et al., 2014). The Spinach aptamer is an RNA structure that displays green fluorescence when bound to the fluorophore 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI) (Paige et al., 2011), allowing one to track mRNA synthesis in real time. To test transcription, we added plasmid DNA containing the Spinach aptamer gene to a cell-free protein synthesis reaction supplemented with DFHBI. Note that the construct used here did not contain EGFP, which if successfully translated would have otherwise convoluted our signal. Upon incubation, we quantified mRNA synthesis by measuring the fluorescence of DFHBI-bound Spinach aptamer mRNA over an 8 h reaction. Our results indicated that the T7 transcription system allows efficient transcription of mRNA for directing protein translation (Supplementary Fig. S2).

Having validated in vitro transcription, we next set-out to test combined transcription and translation. We assembled the cell-free protein synthesis with the pJL1-EGFP template and carried out 15 μL batch reactions for 8 h at 30°C. The course of EGFP synthesis was monitored by online fluorescence measurement (Fig. 1A). Our data indicated that the synthesis of EGFP occurred with a sharply linear increase manner during the first 1 h reaction. Then, the protein synthesis rate declined between 1 and 3 h, followed by

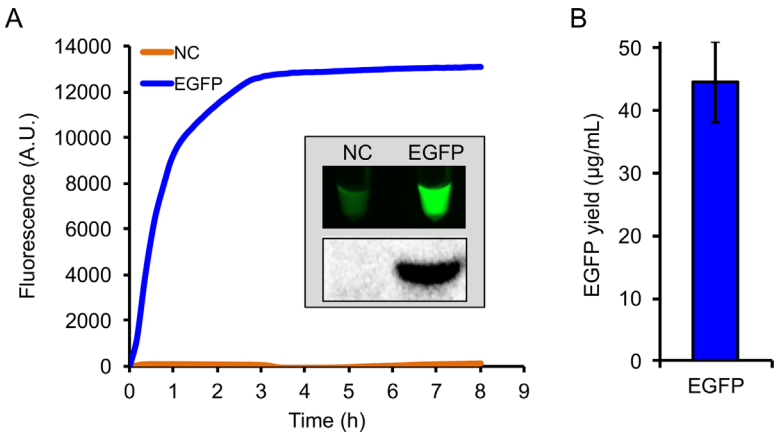

Figure 1. Cell-free protein synthesis of EGFP using *Streptomyces lividans* extract. (**A**) Time course of EGFP synthesis with online fluorescence measurement. Insert *top*: A representative EGFP fluorescence image after the cell-free reaction. Insert *bottom*: A representative autoradiogram of radiolabeled EGFP (The full SDS-PAGE gel and autoradiogram are shown in the Supplementary Fig. S3). NC, negative control without plasmid in the reaction. (**B**) The final yield of EGFP determined by ^{14}C-leucine incorporation. Values show means with error bars representing standard deviations (s.d.) of at least three independent experiments.

termination of the reaction as no obvious increase of the fluorescence. A representative EGFP fluorescence image after the cell-free reaction was shown in Figure 1A (Insert *top*) with bright green color. The synthesis of EGFP was also confirmed by autoradiogram analysis (Fig. 1A, Insert *bottom*). The result indicated that only one protein band with the correct molecular weight (27 kDa) was observed, which is more clearly seen on the complete gel (Supplementary Fig. S3). The final EGFP yield of 44.5 ± 6.5 μg/mL was quantified by monitoring ^{14}C-leucine incorporation (Fig. 1B). To the best of our knowledge, this is the first quantitative report of a total protein yield synthesized by the *Streptomyces*-based CFPS system.

Energy supply in the cell-free system is an important factor affecting the efficiency of protein synthesis (Jewett et al., 2008; Kim and Swartz, 1999; Kim et al., 2007). Thus, we next compared two commonly used secondary energy regeneration systems for CFPS (Hodgman and Jewett, 2013; Jewett and Swartz, 2004a; Lian et al., 2014), which are phosphoenolpyruvate (PEP)-based and creatine phosphate/creatine kinase (CP/CK)-based systems. We found that the protein yield with PEP system was >2-fold higher than that with the CP/CK system (Supplementary Fig. S4A). In addition, the protein synthesis rate of CP/CK system was much slower than the PEP-based system (Supplementary Fig. S4B). Therefore, we selected PEP as an energy source for all following studies.

Optimization of Cell Extract Preparation

Because CFPS exploits an ensemble of catalytic proteins prepared from the crude lysate of cells, the cell extract (whose composition is sensitive to growth media, lysis method, and processing conditions) is the most critical component of extract-based CFPS reactions. In recent years, systematic optimization of each step in extract preparation for *E. coli* CFPS has led to more robust and productive extracts (Carlson et al., 2012; Kwon and Jewett, 2015). Based on these successes, we next chose to vary extract preparation conditions in search of parameters that might improve reproducibility between extract preps, increase the level of protein synthesized, and allow for potential downstream scalability. Generally, the extract preparation process includes the following major steps: cell cultivation, cell disruption, lysate clarification and some optional steps like run-off reaction and dialysis (see Fig. 2A for a flow chart). Historically, cells used for cell-free transcription and translation experiments have been harvested in mid-exponential phase, where cells are rapidly dividing and are expected to have highly active translation machinery (Hodgman and Jewett, 2013; Kwon and Jewett, 2015). Therefore, we chose to focus on post-growth steps. Specifically, we set-out to optimize cell-lysis and run-off reaction steps.

We began our evaluation of extract process parameters by assessing the impact of total lysis pressure on the extract activity. The lysis efficiency is dependent on the pressure applied to the cell suspension. Therefore, we tested different cell lysis pressures from 8,000 to 18,000 psi. To do so, we first grew the *S. lividans* B-12275 strain in liquid YEME medium to its mid-exponential growth phase (16 h after inoculation). Then, thawed cell suspensions were lysed. Our results indicated that the highest EGFP yield was produced with the pressure at 12,000 psi (Fig. 2B), which is 22% and 15% higher than the pressures at 8,000 and 18,000 psi, respectively. This might be explained by the fact that lower pressure may not completely or

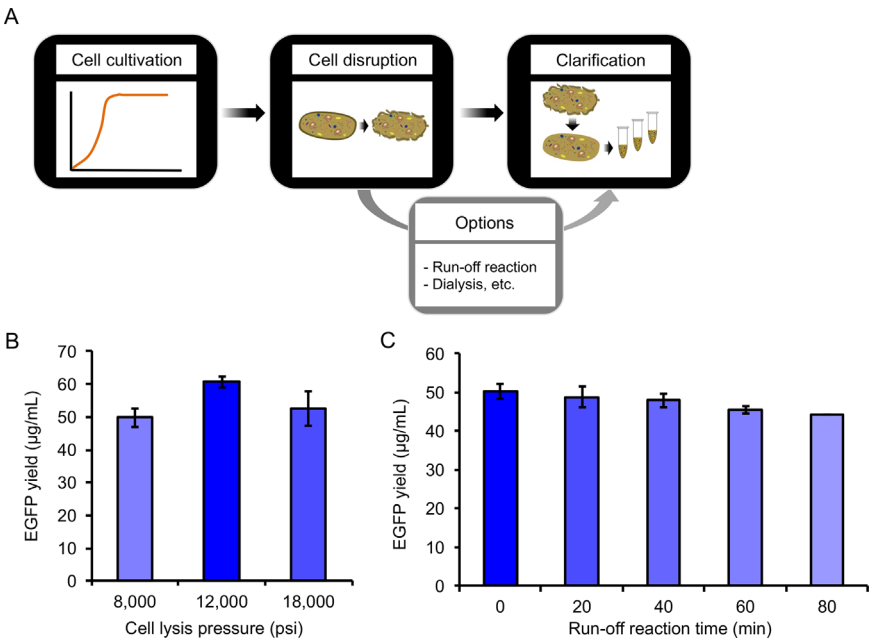

Figure 2. (**A**) A flow-chart schematic for cell extract preparation. Effects of (**B**) cell lysis pressure and (**C**) run-off reaction time on cell-free synthesis of EGFP in a *S. lividans* based CFPS system. Values show means with error bars representing standard deviations (s.d.) of at least three independent experiments.

efficiently break cells (more unlysed cells were observed from the tube bottom after centrifugation compared to higher pressures). By contrast, a higher pressure at 18,000 psi might reduce cell extract activity by inactivating enzymes, in part by heat release associated with larger pressure drops.

After we defined a reproducible cell lysis strategy to generate highly active extracts, we decided to investigate a post-lysis extract preparation step. Specifically, we evaluated the effect of the run-off reaction, which consists of supernatant incubation at 30°C with 250 rpm agitation for a specified time after the first centrifugation. The run-off reaction is hypothesized to liberate ribosomes from endogenous mRNAs which are then degraded by ribonucleases (RNases) (Jermutus et al., 1998). This step is considered to be beneficial for reducing background gene expression and increasing target protein production. However, a recent study suggested that impact of run-off reaction on CFPS activity significantly depends on different strain sources (e.g., K- and B-type of *E. coli*) (Kwon and Jewett, 2015). That means run-off reaction could either notably increase or decrease the cell extract activity. We therefore evaluated the effect of the run-off reaction (pre-incubation at 30°C) on the *Streptomyces*-based CFPS system. We found that the *Streptomyces*-based system was relatively insensitive to the run-off reaction, with EGFP yields only slightly decreasing with up to 80 min pre-incubation as compared to the one without run-off reaction (Fig. 2C). This finding is promising because (i) the time of cell extract preparation is notably reduced (no pre-incubation needed); (ii) the components of cell extract are robust and stable (no obvious activity was lost even after 80 min pre-incubation); and (iii) more importantly, no background expression was observed when we did not perform the run-off reaction (no observable protein bands on the autoradiogram, see Supplementary Fig. S3). Since pre-incubation is not necessary, we chose to not include the run-off reaction step from our *Streptomyces* cell extract preparation procedure.

Optimization of Cell-Free Reaction Conditions

As a means to further increase protein expression yields, we next optimized several well-known cell-free reaction parameters including magnesium ion concentration, template plasmid concentration and reaction temperature. We started with magnesium concentration, which is known to be a fundamentally important physicochemical salt used in cell-free systems that influences the functional activity of the translation apparatus (Jewett et al., 2008; Klein et al., 2004; Liiv and O'Connor, 2006; Yamamoto et al., 2010). Previous reports have shown that the optimal Mg^{2+} concentrations in different CFPS systems are also different, for example, *E. coli* (12 mM) (Jewett and Swartz, 2004a), yeast (7 mM) (Hodgman and Jewett, 2013), *Bacillus subtilis* (15 mM) (Zaghloul and Doi, 1987), PURE system (9 mM) (Shimizu et al., 2001), wheat germ extract (2.5 mM) (Marcu and Dudock, 1974) and tobacco BY-2 extract (1.44 mM) (Buntru et al., 2014). Although the early study reported that 12 mM of Mg^{2+} was optimal for the *Streptomyces* cell-free system (Thompson et al., 1984), we decided to re-examine the magnesium ion concentration. As shown in Figure 3A, the optimal Mg^{2+} concentration was 10 mM and the EGFP yield was increased >20% compared to the cell-free reaction with 12 mM Mg^{2+}.

We next sought to investigate the effect of DNA template concentration (300–900 ng of plasmid per 15 μL reaction) on the

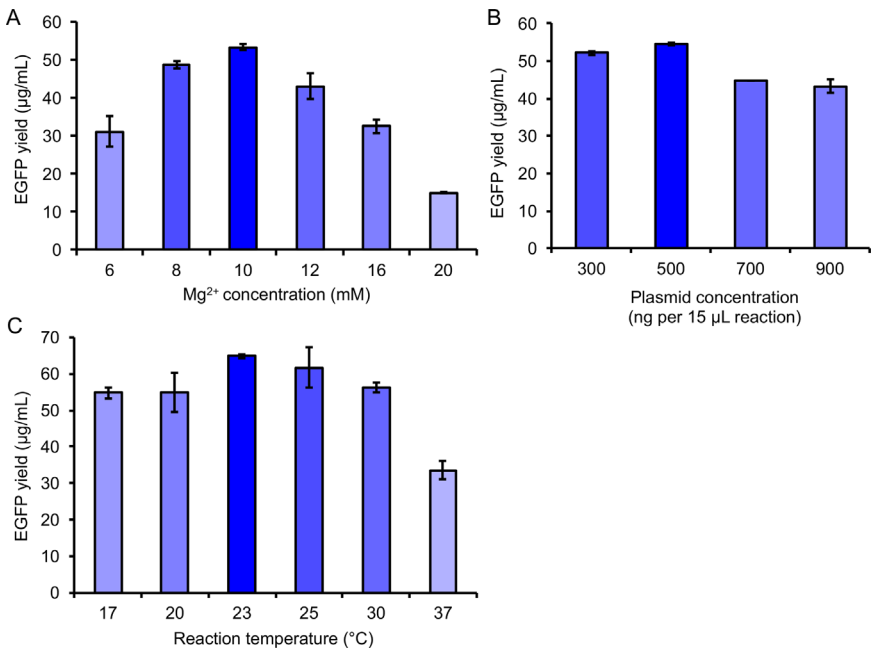

Figure 3. CFPS reaction optimization. The physicochemical environment of the CFPS reaction was optimized by altering (**A**) Mg^{2+} concentration, (**B**) plasmid concentration and (**C**) reaction temperature for cell-free synthesis of EGFP. Values show means with error bars representing standard deviations (s.d.) of at least three independent experiments.

EGFP synthesis. The results indicated that the EGFP yields reached at 52.2 ± 0.5 and 54.6 ± 0.3 μg/mL with 300 and 500 ng of plasmid in the reaction, respectively (Fig. 3B). However, further increases (>700 ng) in the reaction slightly reduced the protein yields, perhaps as a result of consuming nucleotide substrates.

Cell-free reaction temperature is another key factor needs to be optimized, because it affects enzyme activities and protein folding. We therefore compared the EGFP yields at different reaction temperatures from 17 to 37°C. The data suggested that lower temperatures favored protein synthesis expression yields with 23°C maximizing protein expression in our experiments (Fig. 3C). Our finding is similar to other cell-free systems like yeast at 21°C (Hodgman and Jewett, 2013) and tobacco BY-2 extract at 25°C (Buntru et al., 2014).

Cell-Free Synthesis of EGFP With Different *Streptomyces* Strains

After a systematic optimization of the *Streptomyces*-based CFPS system, we were curious to know if the extract preparation and CFPS synthesis methods might work with other *Streptomyces* strains and organisms, since there might be some benefits being able to use different *Streptomyces* species to achieve our long-term goals of natural product pathway synthesis and discovery. We compared extracts from the following strains: *S. lividans* 66, *S. coelicolor* ISP-5233, and *S. coelicolor* M1152. *S. lividans* and *S. coelicolor* are widely used as heterologous hosts to express proteins and secondary metabolite gene clusters (Anné and van Mellaert, 1993; Gomez-Escribano and Bibb, 2012). We chose the engineered strain *S. coelicolor* M1152 especially because its four native gene clusters were deleted, showing higher expression of heterologous gene clusters in vivo (Gomez-Escribano and Bibb, 2011). Figure 4 showed the expression of EGFP with four strains. We determined the EGFP yields after a 3 h CFPS reaction carried out at 23°C. Not surprisingly, the observed yields were different (Fig. 4A). Despite differences in expression yields (noting previous cell-free protein synthesis work has shown the importance of strain background on expression titers [Hong et al., 2015; Kwon and Jewett, 2015]), our method was general in the sense that EGFP was synthesized in all cases. In general, *S. lividans* strains were more productive than the two *S. coelicolor* strains. Interestingly, between the two *S. lividans* strains, the strain 66 produced less EGFP than the strain B-12275 after 3 h reaction, however, both strains synthesized the same level of EGFP after 6 h (Fig. 4B). It is clear that CFPS systems derived from extracts from the strain 66 has a slower protein synthesis rate than those from strain B-12275. Since the entire genome information and versatile genetic manipulation tools are available in a variety of *Streptomyces* organisms, a potentially promising future direction is to modify the genome to maximize protein production as has been done in *E. coli* based CFPS (Calhoun and Swartz, 2006; Goerke et al., 2008; Hong et al., 2015; Jiang et al., 2002; Knapp and Swartz, 2007; Michel-Reydellet et al., 2004).

Enhanced Synthesis of EGFP With Semi-Continuous Reactions

Protein yields synthesized by the CFPS systems are generally limited in the batch reaction mode, partly because of substrate depletion (e.g., energy components and amino acids) and toxic byproduct accumulation (e.g., inorganic phosphate) (Jewett and Swartz, 2004c; Kim and Choi, 1996; Kim and Swartz, 2000; Liu et al., 2015; Schoborg et al., 2014). Substrate shortage in the reaction could be alleviated by fed-batch feeding of the components and this strategy could improve the protein yield and extend the reaction duration (Jewett and Swartz, 2004c; Kim and Swartz, 2000). However, accumulated byproducts like inorganic phosphate cannot be removed from the reaction, which ultimately poison the reaction (Schoborg et al., 2014). In order to replenish substrates and remove detrimental byproducts simultaneously, a semi-continuous cell-free reaction format has been employed, suggesting that the exchange of small molecules through a dialysis membrane could prolong the protein synthesis and lead to a higher protein yield (Kim and Choi, 1996; Liu et al., 2015; Schoborg et al., 2014).

We, therefore, utilized the semi-continuous method to enhance protein synthesis with our *Streptomyces* system. The semi-continuous cell-free reactions were performed in a commercial microdialysis device with a 3.5 kDa molecular weight cut-off (MWCO) membrane (Fig. 5). The membrane allows small molecules to passively diffuse between the reaction (100 μL) and the dialysis buffer (1.4 mL). Large molecules like template DNA,

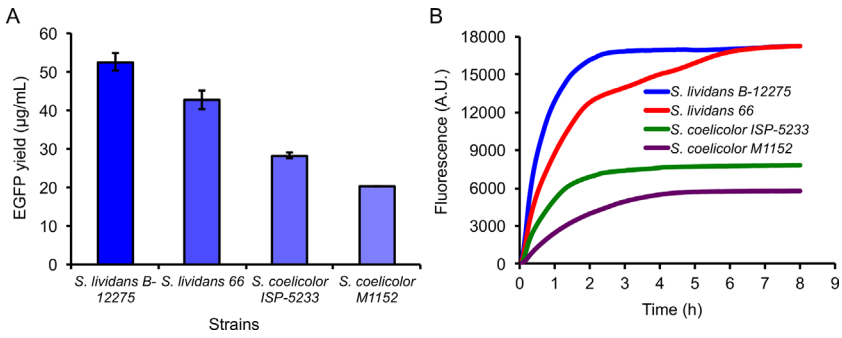

Figure 4. Cell-free synthesis of EGFP with different *Streptomyces* strains. (**A**) The EGFP yields determined after 3 h reaction. (**B**) Online EGFP fluorescence monitored for 8 h. Values show means with error bars representing standard deviations (s.d.) of at least three independent experiments.

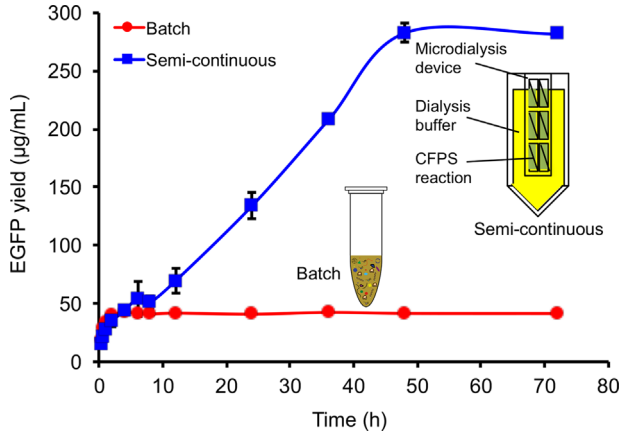

Figure 5. Semi-continuous cell-free reaction improves EGFP yields. A schematic of the experimental set-up for the semi-continuous exchange reactions and the test tubes is shown in the figure. Reactions were sampled at the appropriate time points to measure active EGFP yield. Values show means with error bars representing standard deviations (s.d.) of at least three independent experiments.

ribosomes and other enzymes from the cell extract are unable to diffuse across the membrane (3.5 kDa MWCO) and thus remain in the reaction chamber. We compared the EGFP yields of semi-continuous and batch reactions (both at a 100 μL reaction volume) over 72 h. The results demonstrated that semi-continuous reactions showed a linear EGFP synthesis rate of 5.83 μg/mL/h for up to 48 h at maximum production, compared to only 3 h in batch reactions (Fig. 5). The prolonged protein synthesis duration achieved a maximum yield of 282 ± 8 μg/mL EGFP after 48 h, a ~7-fold improvement over the batch control reactions. The stability of the CFPS system is also highlighted by this approach. Namely, our data indicate that the *Streptomyces* cell extract is robust and active for up to two days. The semi-continuous reaction duration of our *Streptomyces* cell-free system (48 h) is longer than values reported other in vitro systems (<20 h) (Kim and Choi, 1996; Liu et al., 2015; Schoborg et al., 2014).

Cell-Free Synthesis of *Streptomyces*-Originated Proteins

Upon demonstration of a high yielding *Streptomyces*-based CFPS system with a model protein, we sought to demonstrate the applicability of the *Streptomyces* cell-free system for the expression of high GC-content genes. To this end, three *Streptomyces*-originated genes *tbrP* (72% GC), *tbrQ* (78% GC), and *tbrN* (75% GC), which are involved in the nonribosomal peptide tambromycin biosynthesis of the *Streptomyces* strain F-4474 (Goering et al., 2016), were cloned into the cell-free favored expression vector pJL1. In addition, the type II thioesterase (TEII, 64% GC) gene, which is from the valinomycin biosynthetic gene cluster of *S. tsusimaensis* (Li et al., 2015), was cloned for expression.

Expression of the four proteins (TbrP 42 kDa, TbrQ 39 kDa, TbrN 64 kDa, and TEII 28 kDa) was carried out with the optimized batch reaction system. As a comparison, we also expressed these genes in the *E. coli*-based CFPS system as described previously (Li et al., 2016). In order to eliminate the effect of temperature on the protein expression and folding, the CFPS reactions with *E. coli* system were also performed at 23°C for 3 h in batch. The results indicated that, in both cell-free systems, all four proteins were successfully expressed with correct molecular weights as confirmed by the autoradiogram analysis (see Supplementary Fig. S5). Total and soluble protein yields were also quantified by determining radioactive ^{14}C-leucine incorporation. As can be seen from Table I, the soluble yields of TbrP were comparable in the *Streptomyces* and *E. coli* systems. However, the percent solubility of TbrP in the *Streptomyces* system was increased over 7 times as compared to the *E. coli* system. While the soluble yields were lower in the *Streptomyces* cell-free reaction for the proteins TbrQ and TbrN, the percent solubility of both proteins was still notably higher as compared to the *E. coli* system. The TEII protein was previously expressed in vivo in *E. coli* cells, but was almost completely insoluble (Li et al., 2015). Surprisingly, TEII expression in the *Streptomyces* cell-free system was 100% soluble, which is also higher than the *E. coli* cell-free expression system.

While the percent soluble expression of high GC-encoded genes was increased in the *S. lividans* platform, the *E. coli* based platform had higher protein titers (Table I). Given that the *E. coli*-based CFPS system has been developed, enhanced, and improved for more than 20 years to achieve its current productivity, we are optimistic that general yields can be improved in the *Streptomyces*-based platform in the future. As a step towards increasing yields, we here sought to leverage the semi-continuous reaction set-up above (see Fig. 5) to improve expression titers of the high GC-encoded genes. We chose the lower expressed proteins TbrQ and TEII (Table I) as targets. Our results indicated that the soluble yield of TbrQ in the semi-continuous reaction (100 μL, 48 h) was 16.1 ± 0.5 μg/mL, which is >2 times higher than that of the 100 μL batch reaction (Supplementary Fig. S6A). For the soluble yield of TEII, a 23% increase was also observed in the semi-continuous reaction compared to the batch reaction of the same reaction volume (Supplementary Fig. S6B). Although improvement of the soluble yield varies based on different proteins, the semi-continuous CFPS reaction could be a potential way in the future to synthesize sufficient protein for characterization and biosynthetic discovery applications. We additionally plan to improve overall yields through bioprocess engineering, physicochemical optimization, and strain engineering strategies.

Table I. Comparison of cell-free protein synthesis of high GC-content genes with *Streptomyces*- and *E. coli*-based platforms.

	S. lividans		*E. coli*[a]	
Protein	Yield[b] (μg/mL)	Solubility(%)	Yield[b] (μg/mL)	Solubility(%)
TbrP	17.0 ± 0.9	70.4	23.9 ± 0.8	9.6
TbrQ	11.9 ± 0.3	90.3	125.6 ± 5.5	76.7
TbrN	18.6 ± 0.5	86.9	110.9 ± 12.6	65.1
TEII	13.3 ± 1.4	100	336.3 ± 3.8	90.0

[a]CFPS with *E. coli* system was performed at 23°C.
[b]Soluble yield.

Our results suggest that the *Streptomyces*-based CFPS system may be beneficial for the soluble expression of high GC-content genes (Table I). The reasons might be (i) the protein translation rate is slower allowing enough time for correct protein folding; (ii) some essential factors (e.g., protein chaperones) that help protein folding and keep protein stable are only available in *Streptomyces* cell extracts; and/or (iii) *Streptomyces*-originated proteins favor a similar physicochemical environment from the cell extract that is close to their native strains. While these four proteins are solubly expressed, currently we are not able to test their activities because they are involved in complex natural product biosynthetic pathways and their substrate formats (free molecules or intermediates loaded on other protein modules, etc.) and catalytic mechanisms have not been characterized (Goering et al., 2016; Li et al., 2015). However, our *Streptomyces* cell-free system shows promise for soluble expression of high GC-content genes.

Conclusions

The goal of this work was to expand the current CFPS repertoire by developing and optimizing a *Streptomyces*-based CFPS system. In this study, we achieved this goal. Using EGFP as a model protein, we initially optimized the cell extract preparation process and cell-free protein synthesis reaction conditions. Our extract preparation procedure is streamlined and simplified in that it only contains three main steps, i.e., cell cultivation, cell disruption, and clarification. Other optional steps like pre-incubation (run-off reaction) are not necessary to increase the activity of the cell extract and were thus removed from our procedure. After developing a streamlined extract preparation procedure, we optimized the reaction conditions to achieve more than 50 μg/mL of EGFP in a 3 h batch reaction. This EGFP yield was significantly increased up to ~280 μg/mL with a semi-continuous cell-free reaction after 48 h, which provided fresh reaction substrates and removed potentially inhibitory byproducts. In addition, we applied the cell-free system to other *Streptomyces* strains. We found that all cell extracts of the tested strains were active to produce EGFP. Thus, building off past work (Thompson et al., 1984) we established a robust and high yielding *Streptomyces* cell-free protein synthesis platform.

We applied our system to the expression of four *Streptomyces*-originated high GC-content genes. The solubility of all expressed *Streptomyces* proteins was dramatically increased (e.g., >7 times) compared to their expression in an *E. coli*-based cell-free system, demonstrating our *Streptomyces*-based CFPS system is beneficial for the expression of GC-rich genes. While this work establishes a high yielding CFPS from *Streptomyces,* the stated goal, we anticipate that future efforts will demonstrate the feasibility of synthesizing, discovering, and studying complete natural product biosynthesis pathways in vitro, promising to bring the benefits of tunability, scalability, and rapid single-pot reactions to the study of natural product biosynthesis, with DNA as the user-supplied input.

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