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Research paper

Synthesis of 2.3 mg/ml of protein with an all *Escherichia coli* cell-free transcription–translation system

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ABSTRACT

Cell-free protein synthesis is becoming a useful technique for synthetic biology. As more applications are developed, the demand for novel and more powerful *in vitro* expression systems is increasing. In this work, an all *Escherichia coli* cell-free system, that uses the endogenous transcription and translation molecular machineries, is optimized to synthesize up to 2.3 mg/ml of a reporter protein in batch mode reactions. A new metabolism based on maltose allows recycling of inorganic phosphate through its incorporation into newly available glucose molecules, which are processed through the glycolytic pathway to produce more ATP. As a result, the ATP regeneration is more efficient and cell-free protein synthesis lasts up to 10 h. Using a commercial *E. coli* strain, we show for the first time that more than 2 mg/ml of protein can be synthesized in run-off cell-free transcription–translation reactions by optimizing the energy regeneration and waste products recycling. This work suggests that endogenous enzymes present in the cytoplasmic extract can be used to implement new metabolic pathways for increasing protein yields. This system is the new basis of a cell-free gene expression platform used to construct and to characterize complex biochemical processes *in vitro* such as gene circuits.

1. Introduction

In vitro protein synthesis is continually expanding its range of applications [1]. Originally developed for fundamental studies, cell-free expression systems are nowadays used for evolutionary biology [2,3], enzymes bioengineering [4,5], NMR-based structural and high-throughput proteomics [6–8], nanobiotechnology [9–13], industrial and medical applications [14,15]. *In vitro* screening of molecular interactions and production of novel potential therapeutics can also be easily performed with these systems [16–19]. In the past decade, DNA-dependent cell-free expression has also become a valuable technique for synthetic biology [20]. Cell-free expression systems are used to rapidly construct, prototype and validate synthetic gene circuits and biological reaction networks [21–26], to emulate complex biological processes [27], and as systems for machine learning optimization [28]. Cell-free protein synthesis is being developed as open platforms for designing and understanding biological systems using reductive bottom-up approaches [29–31].

Recently, cell-free transcription–translation (TX–TL) was used to construct larger self-assembled biological systems. An all *Escherichia coli* platform was used to produce T7 phages [32], including DNA replication and packaging, demonstrating the potential of cell-free TX–TL to construct complex systems from scratch with genome-sized DNA programs. Ribosomes were synthesized using hybrid T7-based *in vitro* TX–TL [33]. Such achievements, carried out with kits capable of producing 1 mg/ml of protein, encourage the development of more powerful *in vitro* TX–TL, to engineer even larger biochemical systems such as evolvable artificial cells [34–36].

One way to increase cell-free protein production of batch mode TX–TL reactions is to improve energy regeneration and byproducts recycling. The recycling of inorganic phosphate (iP) produced during cell-free TX–TL is essential to increase the yield of synthesized protein as its accumulation feedbacks negatively on the reactions [37,38]. Recently, recycling of iP was carried out with maltodextrin, an oligosaccharide hydrolyzed in glucose and glucose-1-phosphate by the enzyme maltodextrin phosphorylase [39]. Using maltodextrin, up to 1.7 mg/ml of protein was synthesized with a T7 hybrid system [40].

In this work, we describe the development of an all *E. coli* cell-free TX–TL system capable of 2.3 mg/ml of protein production in

Abbreviations: TX–TL, transcription–translation; iP, inorganic phosphate; 3-PGA, 3-phosphoglycerate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; NAD, beta-nicotinamide adenine dinucleotide; cAMP, adenosine 3′,5′-cyclic monophosphate; DTT, dithiothreitol; reGFP, recombinant enhanced green fluorescent protein.

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batch mode reactions. The crude extract is prepared in 12 h from a commercial strain. We demonstrate that maltose can be substituted to maltodextrin to recycle iP. We show that maltose also improves ATP regeneration by coupling to 3-phosphoglycerate (3-PGA) metabolism. Cell-free expression lasts up to 10 h in batch mode. Measurements of pH change, kinetics of iP and ATP concentrations are presented. The amount of synthesized green fluorescent protein is measured by fluorescence and SDS-PAGE.

2. Materials and methods

2.1. Crude extract preparation

The crude extract was prepared as described previously [41] using the BL21 Rosetta2 strain and with the following slight modifications. S30A buffer composition: 14 mM Mg-glutamate, 60 mM K-glutamate, 50 mM Tris buffered with acetic acid to pH 8.2. S30B buffer composition: 14 mM Mg-glutamate, 150 mM K-glutamate buffered to pH 8.2 with Tris. The lysate was prepared with a cell press at a pressure of 16,000 LB.

2.2. Cell-free reaction in batch mode

The reaction mixture was prepared as described before [20]. The reaction buffer is composed of: 50 mM Hepes pH 8, 1.5 mM ATP and GTP, 0.9 mM CTP and UTP, 0.2 mg/ml tRNA, 0.26 mM coenzyme A, 0.33 mM NAD, 0.75 mM cAMP, 0.068 mM folinic acid, 1 mM spermidine, 30 mM 3-PGA, 1 mM DTT, 2% PEG8000. A typical cell-free reaction is composed of 33% (volume) of *E. coli* crude extract. The other 66% of the reaction volume are composed of the plasmids and the reaction buffer containing the nutrients. The amino acid concentration was adjusted to either 1.5 mM or 3 mM of each of the 20 amino acids. Mg-glutamate and K-glutamate concentrations were adjusted according to the plasmids used (60 mM K-glutamate and 5 mM Mg-glutamate for pBEST-OR2-OR1-Pr-UTR1-deGFP-T500, 20 mM K-glutamate and 5 mM Mg-glutamate for the T7 cascade). The concentration of synthesized deGFP was determined from a calibration curve (Ex 485 nm and Em 528 nm) done with pure recombinant eGFP (reGFP) purchased from Cell Biolabs Inc. End-point fluorescence was measured in a 384 format well plate using a BioTek plate reader. deGFP synthesis kinetics were measured in a 96-well plate sealed with a plastic cap to avoid evaporation. Cell-free reactions (5–15 μl) were incubated at 29 °C.

2.3. Plasmids

The plasmids used in this study have been described before [20]. The plasmid pBEST-OR2-OR1-Pr-UTR1-deGFP-T500 was used for cell-free protein synthesis based on the endogenous core RNA polymerase and sigma factor 70. The plasmids pBEST-p15A-OR2-OR1-Pr-UTR1-T7rnap-T500 and pIVEX2.3d-deGFP were used for the expression of deGFP through the T7 transcriptional activation cascade. pH kinetics measurements were performed using pBEST-OR2-OR1-Pr-UTR1-Luc-T500. Plasmids were quantified with the fluorescent assay QuantiFluor (Promega).

2.4. SNARF-5F pH change kinetic measurements

pH of cell-free reactions was measured with the SNARF-5F dye, 5-(and-6)-carboxylic acid (Invitrogen). The dye was dissolved in DMSO at 10 mM and stored at −20 °C. Before usage, the dye was diluted 100 fold in water and used at 100 μM in the reaction mixture. The dye was excited at 514 nm and the ratio of the emissions at 580/640 nm was used to monitor the pH. The pH change was monitored in real-time (cell-free reaction incubated at 29 °C in a 384-well plate). To avoid interference between the fluorescence signals of the dye and the expressed protein, a plasmid encoding for the non-fluorescent protein Luciferase was used for expression.

2.5. iP and ADP/ATP assays

A spectrophotometry assay for inorganic phosphate was performed according to a procedure described in the literature [42]. Briefly, the protocol is based on absorbance measurements at 850 nm of the phosphomolybdate complex reduced by ascorbic acid at pH 5 with zinc in solution. Measurements were taken with Genesys10UV spectrophotometer (Thermofisher). The ADP/ATP assay was done using the ApoSENSOR ADP/ATP ratio assay kit (BioVision). The assay was performed in a 96 format well plate using a plate reader (BioTek). For both assays, aliquots were collected during cell-free reaction and diluted accordingly before each measurement.

2.6. SDS-Page

A 12% polyacrylamide gel was prepared according to standard procedures. To quantitatively estimate the total amount of deGFP (25.4 kDa) synthesized, known amounts of rEGFP (29 kDa, Cell Biolabs Inc) were added into each sample to be analyzed. A blank cell-free reaction was used for subtracting the background for quantification of deGFP and reGFP. The image analysis was done with the software ImageJ.

2.7. Visual display of data

Histograms are used for endpoint measurements (after 10–12 h of incubation), lines are used for kinetics.

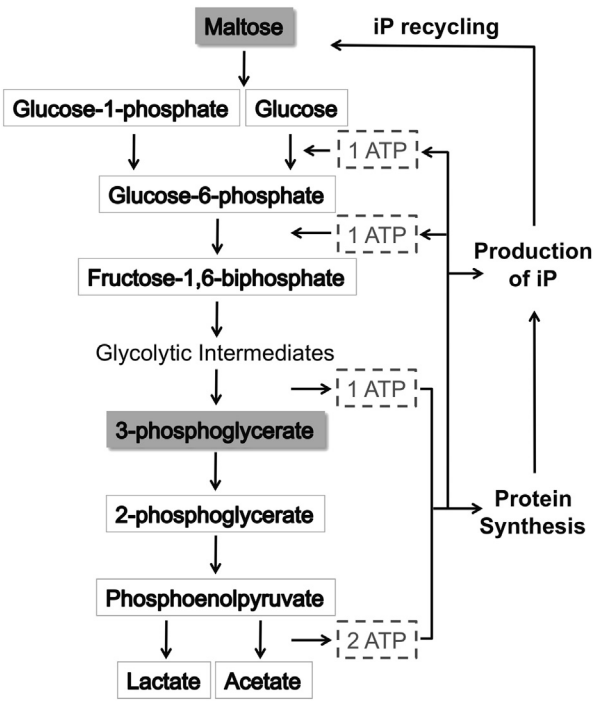

Fig. 1. Schematic illustration of the maltose-based metabolism for recycling inorganic phosphate (iP) and regenerating ATP. Chemical components added to cell-free reactions are shaded in gray.

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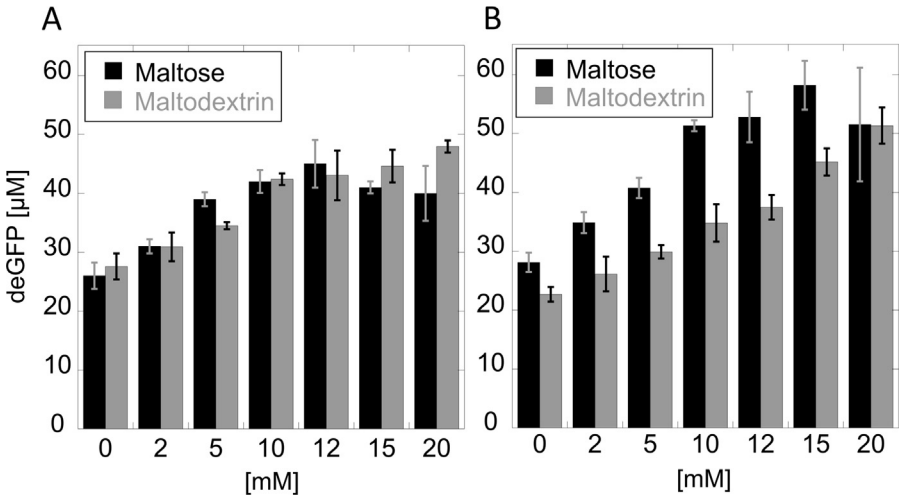

Fig. 2. Cell-free expression of deGFP in batch mode with 5 nM plasmid pBEST-OR2-OR1-Pr-UTR1-deGFP-T500 measured after 10 h of incubation. Maltose and maltodextrin concentration ranges with 1.5 mM amino acids (A) and 3 mM amino acids (B). Error bars represent standard deviation of three different samples.

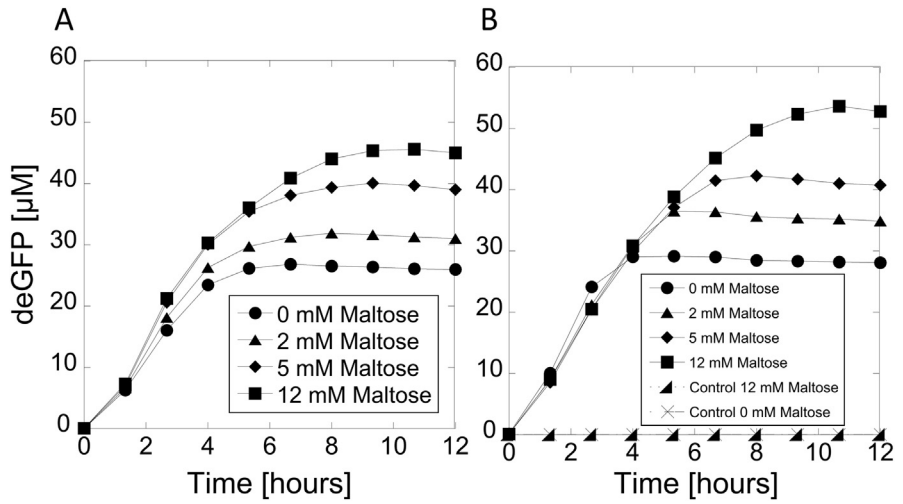

Fig. 3. Kinetics of cell-free reaction with 5 nM plasmid pBEST-OR2-OR1-Pr-UTR1-deGFP-T500 and different concentrations of maltose with 1.5 mM amino acids (A) and 3 mM amino acids (B). In addition, two negative controls expressing the non-fluorescence protein Luciferase were performed.

3. Results and discussion

3.1. Cell-free system and maltose metabolism

Preparation of the all *E. coli* cell-free system has been described previously [20,41,43]. Only slight modifications have been made in this work: pH of buffer S30B was adjusted to 8.2; potassium concentration of buffer S30B was increased to 150 mM; a cell press was used to scale up the volume of crude extract, rather than a bead beater. Cell-free expression occurs without the addition of external TX–TL components, and it was previously shown that 0.5–075 mg/ml of proteins can be synthesized with this system after 5–6 h of incubation. The reporter protein deGFP used in this work was also described previously [43].

Our goal was to increase the yield of protein synthesis in batch mode reactions by improving ATP regeneration and using the same commercial *E. coli* strain BL21 Rosetta2. Inspired by the utilization of maltodextrin to recycle byproducts of reactions [39], we decided to test maltose, a disaccharide, as a new metabolite with the hypothesis that it could be used both for iP recycling and ATP regeneration (Fig. 1). First, we simply added maltose or maltodextrin in cell-free reactions containing 5 nM of plasmid pBEST-OR2-OR1-Pr-UTR1-deGFP-T500. The reporter protein deGFP is expressed through a strong *E. coli* promoter specific to sigma 70 and an untranslated region containing a strong ribosome binding site. With a concentration of 1.5 mM of each amino acids, we observed a net increase of deGFP synthesis with both maltose and maltodextrin (Fig. 2A). A concentration of up to 45 μM (1.14 mg/ml) of active reporter protein was measured versus 25 μM only with no maltose. A larger amount of protein was produced by increasing the concentration of amino acids to an optimum of 3 mM each (Fig. 2B). We observed that about 60 μM (1.5 mg/ml) of active deGFP was produced with either 12–15 mM maltose or 25–30 mM maltodextrin added to the reactions (Fig. S1). Maltose acts better at lower concentrations than maltodextrin. We also observed that maltose had an inhibitory effect on expression at concentrations higher than 15–20 mM. Most likely this was due to an excessive production of inorganic acids, which decrease the pH in the reaction mixture too quickly [40]. These experiments demonstrate that different concentration of the two sugar molecules can be used to equally increase cell-free synthesis of deGFP.

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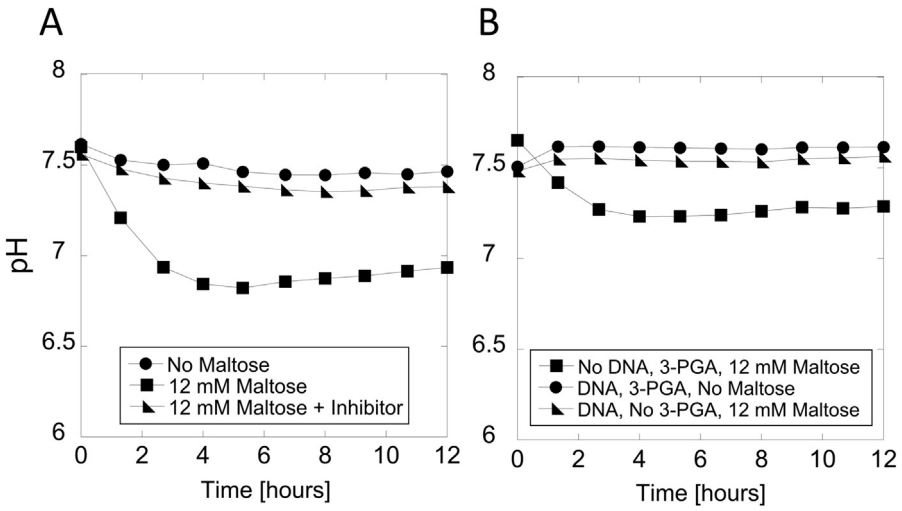

Fig. 4. pH variation during cell-free reaction with 5 nM plasmid pBEST-OR2-OR1-Pr-UTR1-Luc-T500 monitored using the fluorescence dye SNARF-5F. (A) Kinetics with and without maltose and in the presence of 12 mM glycolysis pathway inhibitor, 2-deoxy-D-glucose. (B) The effect on the pH of the main system components involved in the metabolic pathway: protein synthesis (DNA), reaction buffer with 3-phosphoglycerate (3-PGA) and maltose.

3.2. Maltose metabolism is based on endogenous enzymes

Maltodextrin was previously reported to improve ATP regeneration by recycling iP accumulated during cell-free expression [39]. This process was shown to require the addition of two purified enzymes (maltodextrin phosphorylase and phosphoglucomutase) to the reactions. The two enzymes catalyze the hydrolysis of maltodextrin with simultaneous addition of iP to form glucose-1-phosphate, which is then converted into glucose-6-phosphate [39]. Addition of the two enzymes in our cell-free reactions did not improve the protein synthesis, with either maltose or maltodextrin (Fig. S2A and B). We concluded that maltose and maltodextrin metabolisms with our cell-free system are carried out by endogenous enzymes present in the crude extract.

Kinetics of deGFP production were performed to visualize the effect of maltose on cell-free protein synthesis. While no effect was observed on the initial rate of protein synthesis, a clear extension of expression occurred with an increasing concentration of maltose (Fig. 3). Up to 9 and 10 h of protein synthesis were observed with 1.5 mM and 3 mM amino acids respectively. With no maltose, deGFP synthesis stopped after 5 h as previously observed [43]. The rate of protein synthesis is constant on longer period of times with maltose. It should be mentioned that a maturation time of ≈7 min was previously estimated for deGFP reporter gene [44]. Negative controls using non-fluorescent proteins for expression showed that maltose does not artificially increase fluorescence signals.

3.3. pH kinetics during cell-free reactions

The catabolism of maltose supposedly activates the glycolysis biochemical pathway (Fig. 1). A direct consequence is an increased production of lactate and acetate, which is known to decrease the pH [45,46]. The pH of cell-free reactions was monitored over time using the pH sensitive fluorescence dye SNARF-5F [47]. We first made a calibration curve of the dye fluorescence versus pH (Fig. S3). The firefly Luciferase reporter protein was expressed to avoid interferences in the fluorescence signals between the reporter gene (deGFP) and the pH sensitive dye. With no maltose added to the reaction, the pH is stable for more than 12 h (Fig. 4A). With 12 mM maltose in the reaction, the pH dropped from 7.5 to 6.8, thus suggesting activation of the glycolysis biochemical pathway [45,46]. To confirm that glycolytic intermediates are involved in the maltose catabolism, an inhibitor of the glycolysis pathway was used in the reaction [47]. The inhibitor, 2-deoxy-D-glucose, is a competitive analog of glucose that inhibits the production of glucose-6-phosphate. Upon addition of the analog molecule to the reaction, no pH change was detected (Fig. 4A, Fig. S4A). This observation confirmed that glycolysis intermediates are exploited to produce ATP used for protein synthesis.

The effects of protein synthesis (addition of plasmid DNA), maltose and 3-phosphoglycerate were tested on the pH change (Fig. 4B, Fig. S4B). No pH change was observed when only 3-phosphoglycerate or maltose was added to the reaction. Conversely, a small drop in pH from 7.5 to 7.25 was observed with co-addition of 3-phosphoglycerate and maltose, although no DNA was added to activate protein synthesis. Overall, these observations

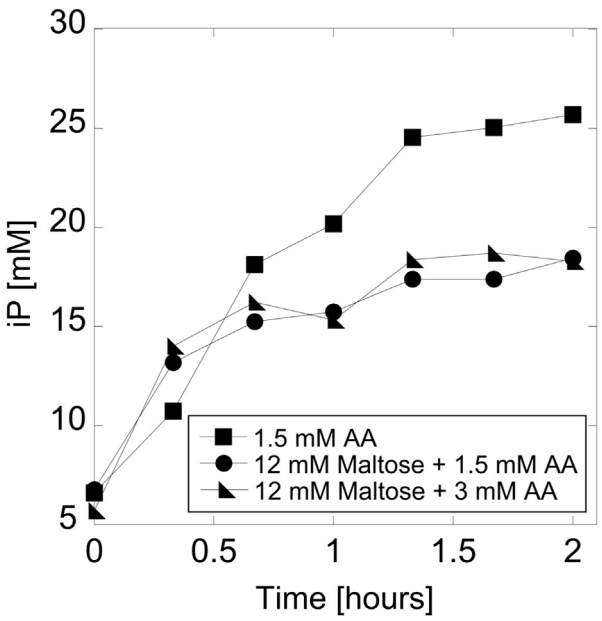

Fig. 5. Kinetics of the accumulation of inorganic phosphate during cell-free reaction with 5 nM plasmid pBEST-OR2-OR1-Pr-UTR1-deGFP-T500.

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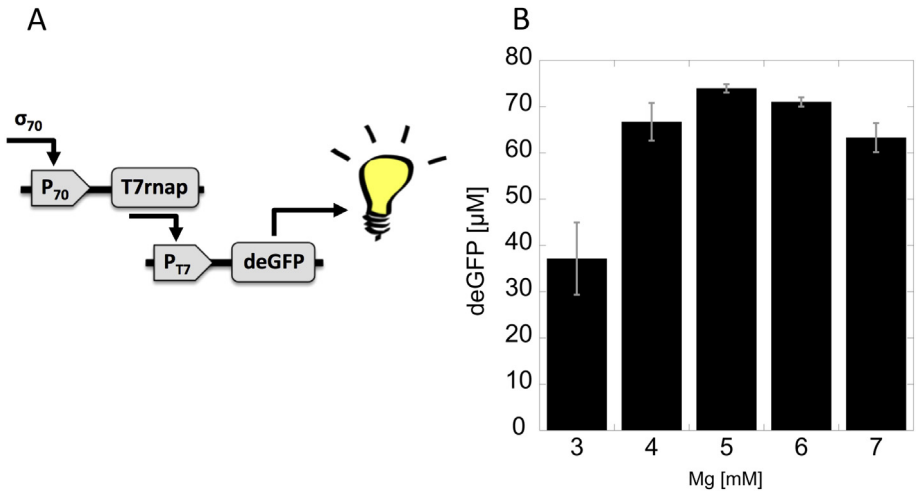

Fig. 6. (A) Schematic illustration of a two-stage transcriptional activation cascade to express deGFP cloned under the T7 promoter (0.1 nM pBEST-p15A-OR2-OR1-Pr-UTR1-T7rnap-T500, 1 nM pIVEX2.3d-deGFP). (B) Histogram of deGFP expression in batch mode at different concentrations of Mg-Glutamate (20 mM K-glutamate).

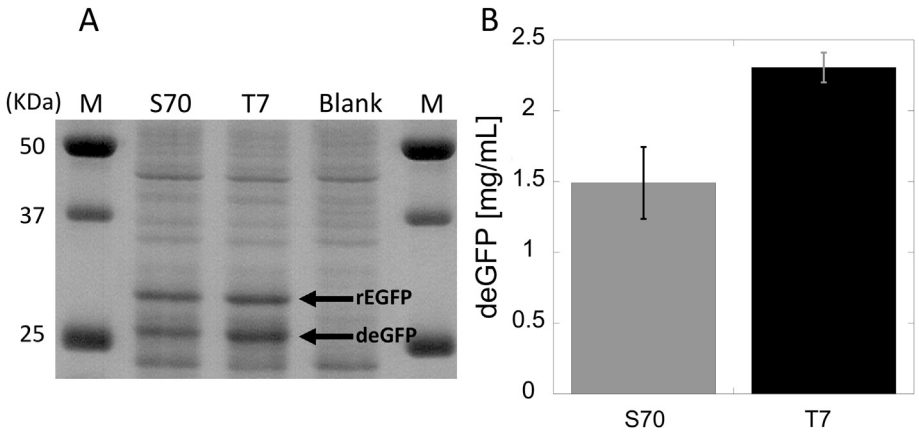

Fig. 7. (A) SDS-PAGE of deGFP expressed in the cell-free reactions with either pBEST-OR2-OR1-Pr-UTR1-deGFP-T500 or through the T7 cascade. M: marker, S70 (5 nM plasmid pBEST-OR2-OR1-Pr-UTR1-deGFP-T500), T7 (cascade as shown in Fig. 6.), blank (blank cell-free reaction). reGFP: commercial recombinant eGFP, deGFP (reporter used in this work). (B) Histogram of deGFP quantification using an image analysis software. Errors bars represent the standard deviation of two different samples.

demonstrate that 3-PGA and maltose activate the metabolic reactions involved in glycolysis. Production of lactate and acetate is increased due to protein synthesis, which causes an accumulation of iP necessary for maltose metabolism.

3.4. iP and ADP/ATP ratio kinetics during cell-free reactions

One of the maltose functions is to recycle iP accumulated during protein synthesis (Fig. 1). An assay was performed to measure the accumulation of iP during cell-free reactions [42]. A calibration was made to retrieve the iP concentration through absorbance measurements (Fig. S5). First, the concentration of iP was monitored for 2 h in a cell-free reaction without maltose (Fig. 5). iP accumulated to a concentration of 25 mM after 1.3 h. We observed a net decrease of iP accumulation in the presence of maltose. With either 1.5 mM or 3 mM amino acids, iP accumulated to a concentration of 17 mM. iP is recycled in the presence of maltose, independently of the amino acid concentration used.

To confirm that maltose also improves ATP regeneration, the ratio ADP/ATP was monitored during cell-free reactions for the first 4 h (Fig. S6). A constant increase of the ADP/ATP ratio was observed with no maltose, whereas a slower increase of the ratio was observed for the first 2 h in the presence of maltose. This was more pronounced when 3 mM amino acids were used.

3.5. More than 2 mg/ml of protein in batch mode

Our cell-free system is unique in that it is based on both the endogenous transcription and translation of *E. coli*. Transcription is performed by the core RNA polymerase and the housekeeping sigma factor 70. As shown previously, the primary transcription machinery can be used to construct gene circuits by simply cascading other sigma factors or bacteriophage RNA polymerases for example [20]. The strongest promoter specific to sigma 70 that we have found so far for our system is the promoter OR2-OR1-Pr from the lambda phage genome. Associated with the highly efficient untranslated region UTR1, a maximum of 1.5 mg/ml of active deGFP was produced with the plasmid pBEST-OR2-OR1-Pr-UTR1-deGFP-T500 in the presence of 12–15 mM maltose in the reaction (Fig. 2B). We then tested a T7 transcriptional activation cascade (Fig. 6A). The T7 RNA polymerase is known for its high promoter specificity its high transcription efficiency. We found that a maximum of 1.9 mg/ml of active deGFP can be produced in the presence of 12–15 mM maltose (Fig. 6B).

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The total amount of protein produced was determined by SDS-PAGE (Fig. 7A). Using pure reGFP and image analysis (Fig. S7), we determined that 1.5 mg/ml and 2.3 mg/ml of deGFP were produced through a single *E. coli* promoter and the T7 cascade respectively (Fig. 7B). In the case of the T7 cascade, more than 80% of the total amount of deGFP produced is active.

4. Conclusions

Using maltose as a new metabolite, we developed an all *E. coli* cell-free TX–TL system that delivers 1.5 mg/ml and 2.3 mg/ml of protein through a single bacterial promoter and a transcriptional activation cascade respectively. Similar yields can be obtained with maltodextrin. We observe that maltose works better at lower concentrations (optimum around 12–15 mM) compared to maltodextrin (optimum around 30–35 mM). Exploiting endogenous enzymes present in the cytoplasmic extract, we showed that maltose is synergistically involved in both ATP regeneration and iP recycling, which results in a longer TX–TL reaction and greater protein production. By coupling maltose or maltodextrin with 3-phosphoglycerate, we were able to reach higher protein yield than previously described system using dual energy sources [48]. For the first time, more than 2 mg/ml of protein is synthesized *in vitro* using a commercial *E. coli* strain. Preparation of the crude extract takes 12 h, starting from cell cultures to lysate storage. All the parts and chemical mixtures needed for cell-free TX–TL reactions are prepared in the laboratory (crude extract, 3-PGA reaction buffer, amino acids and plasmids), which substantially decreases the cost per reaction.

Understanding reaction networks that can be exploited using only endogenous enzymes present in the crude extract may lead to future applications for *in vitro* metabolic engineering [49]. New products such as, therapeutics [17] and biofuels [50], could be quickly and efficiently synthesized by rewiring and expanding preexisting biochemical networks. This new system is interesting for synthetic biology applications as it uses the TX–TL machineries from *E. coli*. We anticipate that larger synthetic gene networks can be executed in batch mode reactions or artificial cell systems [51–54].

Author contributions

F.C. and V.N. performed the experiments, analyzed the data and wrote the manuscript. The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2013.11.025.

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