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Original Research Article

Development of a *Bacillus subtilis* cell-free transcription-translation system for prototyping regulatory elements

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ABSTRACT

Cell-free transcription-translation systems were originally applied towards *in vitro* protein production. More recently, synthetic biology is enabling these systems to be used within a systematic design context for prototyping DNA regulatory elements, genetic logic circuits and biosynthetic pathways. The Gram-positive soil bacterium, *Bacillus subtilis*, is an established model organism of industrial importance. To this end, we developed several *B. subtilis*-based cell-free systems. Our improved *B. subtilis* WB800N-based system was capable of producing 0.8 μM GFP, which gave a ~72x fold-improvement when compared with a *B. subtilis* 168 cell-free system. Our improved system was applied towards the prototyping of a *B. subtilis* promoter library in which we engineered several promoters, derived from the wild-type P_{grac} (σA) promoter, that display a range of comparable *in vitro* and *in vivo* transcriptional activities. Additionally, we demonstrate the cell-free characterisation of an inducible expression system, and the activity of a model enzyme - *renilla* luciferase.

1. Introduction

Cell-free systems that are based on cellular extracts were originally developed as experimental systems to understand fundamental aspects of molecular biology, cellular biochemistry and for *in vitro* protein production (Hodgman and Jewett, 2012; Nirenberg, 2004; Sullivan et al., 2016; Zubay, 1973). Synthetic biology approaches are enabling the re-purposing of cell-free systems as coupled *in vitro* transcription–translation characterisation platforms for the prototyping of DNA based parts, devices and systems (Kelwick et al., 2014). Cell-free transcription-translation systems have been employed to rapidly prototype DNA regulatory elements (Chappell et al., 2013), logic systems (Niederholtmeyer et al., 2015; Shin and Noireaux, 2012; Sun et al., 2014; Takahashi et al., 2015) and medical biosensor devices (Pardee et al., 2014) with workflows that can be completed within several hours. In contrast, typical *in vivo* approaches may take several days. Another distinguishing advantage of cell-free systems is that they can be coupled with model-guided design strategies to create 'biomolecular-breadboards' that enable the robust cell-free characterisation of bioparts that can then be implemented as final designs *in vivo* (Siegal-Gaskins et al., 2014). These developments are also enabling cell-free protein synthesis driven metabolic engineering approaches for the biochemical characterisation of novel enzymes and prototyping of biosynthetic pathways (Karim and Jewett, 2016).

Several cell-free systems have been developed, of which, the most well established systems use cellular extracts from *Escherichia coli* (Garamella et al., 2016), Wheat Germ (Ogawa et al., 2016), Yeast (Gan and Jewett, 2014) or HeLa cells (Gagoski et al., 2016). Additionally, more specialist cell-free systems including the PURE express system, which uses purified cellular machinery rather than cellular extracts, have also been established (Shimizu et al., 2005). Whilst these cell-free systems have been continually improved through developments in the methods used for their preparation (Shrestha et al., 2012) and optimisation of energy buffers (Caschera and Noireaux, 2015a, 2014), there have been fewer reports of *Bacillus subtilis* cell-free systems. Yet, the development of robust *B. subtilis* cell-free systems could have applicability to a broad array of microbiology, synthetic biology and industrial biotechnology applications. Applications for *B. subtilis* are diverse and include the production of industrial or pharmaceutical proteins, and more recently for use as whole-cell biosensors (Harwood, 1992; Pohl et al., 2013; Webb et al., 2016; Westers et al., 2004). Cell-free systems could be applied to support

Abbreviations: ATP, adenosine triphosphate; CTP, cytidine triphosphate; GTP, guanosine triphosphate; UTP, uridine triphosphate; GFPmut3b, green fluorescent protein mut3b variant; Mg, magnesium; K, potassium; tRNA, transfer ribonucleic acid; 3-PGA, 3-phosphoglycerate; NAD, nicotinamide; CoA, coenzyme A; cAMP, adenosine 3′,5′-cyclic monophosphate; DTT, dithiothreitol

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developments across these applications, particularly, where the functionality of the engineered system relates to aspects of the biochemistry, metabolism and/or regulatory processes of *B. subtilis* as well as potentially other Gram-positive bacteria. For instance, the cell-free prototyping of *B. subtilis* regulatory elements (*e.g.* promoter libraries) may provide synergistic benefits when coupled with *in vivo* studies, such that multiple rounds of cell-free characterisation workflows may result in more rapid iterations of the design cycle towards the final *in vivo* design (Chappell et al., 2013; Karim and Jewett, 2016; Tuza et al., 2013).

However, the initially reported *B. subtilis* cell-free systems were typically encumbered by the requirement to use exogenous mRNA, protease inhibitors, DNAse treatments or less efficient energy systems (Legault-Demare and Chambliss, 1974; Leventhal and Chambliss, 1979; Nes and Eklund, 1983; Okamoto et al., 1985; Zaghloul and Doi, 1987) which is perhaps why, despite their potential, these systems have been largely neglected. In the present study, we report on the development and improvement of a *B. subtilis* cell-free system, using a standardised workflow that has no such limitations. We demonstrate the utility of *B. subtilis* cell-free transcription-translation systems as a useful tool for genetic regulatory element prototyping through the characterisation of an engineered promoter library that enables a range of comparable *in vitro* and *in vivo* transcriptional activities. Additionally, as a step towards additional applications for *B. subtilis* cell-free systems, we characterise an inducible expression system (a precursor to genetic circuit prototyping) and, characterise the activity of the Renilla (sea pansy) luciferase (a model enzyme).

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

Bacterial strains used in this study are listed in Table S1. *E. coli* strains were grown in Luria-Bertani (LB) medium at 37 °C whilst *B. subtilis* strains were grown in 2× YTP medium (31 g/L 2× YT, 40 mM potassium phosphate dibasic, 22 mM potassium phosphate monobasic) at 30 °C. When applicable, the medium was supplemented with the following antibiotics: *E. coli* cultures - ampicillin (Amp) 100 μg/ml; chloramphenicol (Cam) 50 μg/ml; kanamycin (Kan) 35 μg/ml; *B. subtilis* WB800N cultures – chloramphenicol 5 μg/ml; kanamycin 10 μg/ml. Kanamycin is used to select for the neomycin (Neo) resistance gene in *B. subtilis* WB800N.

2.2. Strain and plasmid construction

Oligonucleotide primers for plasmid construction and sequencing are listed in Table S2.

2.2.1. GFPmut3b expression vector

The GFPmut3b expression vector pHT01-*gfpmut3b* was constructed as follows. The insert *gfpmut3b* was amplified from plasmid pAJW26 (BBa_K316008) using primer pair AJW289/AJW290, the resultant PCR product was purified, digested with enzymes *Bam*HI and *Xba*I and ligated with the vector pHT01, which had been digested with the same enzymes, resulting in the construction of plasmid pHT01-*gfpmut3b* (pAJW107). To remove LacI control, *lacI* was deleted from plasmids pHT01 and pHT01-*gfpmut3b* as follows: inverted PCR reactions using primer pair AJW320/AJW321 and plasmids pAJW9 and pAJW107 as templates were undertaken, the DNA products were purified, phosphorylated, self-ligated and transformed into *E. coli* NEB10-beta, resulting in the plasmids pHT01-Δ*lacI* (pAJW118) and pHT01-Δ*lacI*-*gfpmut3b* (pWK-WT). To generate a pHT01-Δ*lacI*-*gfpmut3b* construct lacking the −35 and −10 boxes and the region between the two boxes, plasmid pWK-WT was used as the template in an inverted PCR reaction with primers WK5/WK6. The resultant DNA product was purified, phosphorylated, self-ligated and transformed into *E. coli* NEB10-beta, resulting in the plasmid pHT01-Δ*lacI*-Δbox-*gfpmut3b* (pWK-Δbox).

2.2.2. Promoter library construction

To construct the promoter library of clones with changes to the −35 and −10 boxes, inverted PCR was undertaken using pWK-WT as the template and primer pair WK1/WK2. The resultant PCR product was purified, phoshporylated, self-ligated, transformed into *E. coli* NEB10-beta and the colonies cultured on plates incubated at either 30 °C or 37 °C. This resulted in the production of the pWK(n) plasmid promoter variants. To create targeted changes to the −10 box, inverted PCR was undertaken using pWK-WT and pWK5 as the templates and primer pair WK7/WK8. The products were purified, phosphorylated, self-ligated and transformed into *E. coli* NEB10-beta, resulting in the plasmids pWK403 and pWK501 respectively. Promoter library clones tested in this study are listed in Table S6.

2.2.3. GFPmut3b purification vector

Primer pair RK003 and RK004 were designed to PCR amplify *gfpmut3b* along with the addition of restriction sites *Bam*HI and *Hind*III from plasmid pRK1. The subsequent PCR product was designed so that it could be digested with *Bam*HI and *Hind*III and ligated into pre-digested vector pPROEX HTb to form pRK2 – a vector in which N-terminally His-tagged GFPmut3b protein production could be induced.

2.2.4. Renilla luciferase vector

Primer pair RK005/RK006 were designed to PCR amplify the *renilla* luciferase gene along with the addition of restriction sites *Bam*HI and *Xba*I from plasmid pRK5. The subsequent PCR product was designed so that it could be digested with *Bam*HI and *Xba*I and ligated into pre-digested vector pWK-WT to form pRK6 – a vector in which *Renilla* Luciferase enzyme could be constitutively expressed.

The DNA of all inserts/constructs were verified by the sequencing service provided by Eurofins Genomics GmbH (Ebersberg, Germany). Primers AJW10 and AJW11 were used to sequence pSB1C3 based constructs and primers AJW77, AJW78, AJW322 and AJW376 were used to sequence either pHT01 or pHT01-Δ*lacI* based constructs. Primer WK3 was used to sequence the *gfpmut3b* constructs whilst primers RK001 and RK002 were used to sequence pPROEX HTb His-*gfpmut3b*.

2.3. Cell-free extract preparation

To prepare cell-free extracts, *B. subtilis* 168 cells were revived from glycerol stocks onto LB plates whilst *B. subtilis* WB800N cells were revived from glycerol stocks onto LB plates supplemented with kanamycin (Kan;10 μg/ml). Once streaked, plates were incubated for 48 h at 30 °C. Individual colonies were inoculated into 5 ml 2× YTP medium and incubated for 10 h with shaking (180 rpm) at 30 °C. The resultant cultures were diluted (1:500) into flasks containing 50 ml 2× YTP medium and incubated for 10 h with shaking (180 rpm) at 30 °C. Resultant cultures were either harvested for cell lysis or, for larger scales of production, they were diluted (1:500) into flasks containing 500 ml 2× YTP medium and incubated for 10 h with shaking (180 rpm) at 30 °C. To harvest cells, 500 ml cultures were centrifuged at 3,220*g* for 15 min. Cell pellets were re-suspended into 20 ml S30-A buffer (14 mM Magnesium (Mg) glutamate, 60 mM Potassium (K) glutamate, 50 mM Tris, 2 mM DTT, pH 7.7) and transferred into a pre-weighed 50 ml Falcon tube. Each 50 ml Falcon tube was centrifuged (2,000*g*, 10 min, 4 °C), pellets washed with 20 ml S30-A buffer and subsequently re-centrifuged (2,000*g*, 10 min, 4 °C) to form the final cell pellets in preparation for cell lysis. To determine the weight of the cell pellet, the weight of the 50 ml falcon tube was subtracted from the combined weight of the 50 ml tube and cell pellet. Pellets were stored at −80 °C for no more than 48 h, prior to cell lysis.