Rapid Communication

**Streptomyces venezuelae TX-TL – a next generation cell-free synthetic biology tool**

**Simon J. Moore**1,2, **Hung-En Lai**1,2, **Hannah Needham**3, **Karen M. Polizzi**1,3 and **Paul S. Freemont**1,2

1 Centre for Synthetic Biology and Innovation, South Kensington Campus, London, UK
2 Department of Medicine, South Kensington Campus, London, UK
3 Department of Life Science, South Kensington Campus, London, UK

*Streptomyces venezuelae* is a promising chassis in synthetic biology for fine chemical and secondary metabolite pathway engineering. The potential of *S. venezuelae* could be further realized by expanding its capability with the introduction of its own in vitro transcription-translation (TX-TL) system. TX-TL is a fast and expanding technology for bottom-up design of complex gene expression tools, biosensors and protein manufacturing. Herein, we introduce a *S. venezuelae* TX-TL platform by reporting a streamlined protocol for cell-extract preparation, demonstrating high-yield synthesis of a codon-optimized sfGFP reporter and the prototyping of a synthetic tetracycline-inducible promoter in *S. venezuelae* TX-TL based on the tetO-TetR repressor system. The aim of this system is to provide a host for the homologous production of exotic enzymes from Actinobacteria secondary metabolism in vitro. As an example, the authors demonstrate the soluble synthesis of a selection of enzymes (12–70 kDa) from the *Streptomyces rimosus* oxytetracycline pathway.

**Keywords:** Cell-free · Gene expression · *Streptomyces* · Synthetic biology · Tetracycline

1 Introduction

*Streptomyces* belongs to the high G+C (%) Actinomycetes soil bacteria and represents the leading source of natural antibiotics such as streptomycin and tetracycline [1]. Recently, *Streptomyces venezuelae*, the chloramphenicol producer, has been adopted by synthetic biology for its use in metabolic engineering [2], since it is relatively well characterized, has strong promoter tools and genome engineering plasmids for integration [3–5]. Whilst it is not as characterized as *Streptomyces coelicolor* A3(2), in contrast, *S. venezuelae* provides significant advantages such as a fast growth (≈TD = 40 min) and no aggregation during liquid culture [3]. One potential route for the further development of *S. venezuelae* and the characterization of its genetic parts is the introduction of an in vitro transcription-translation system (TX-TL). TX-TL has recently been developed as a highly adaptable tool for bottom-up synthetic biology and is based on a whole-cell extract [6–9] to synthesize recombinant proteins from the chemical building blocks of life.

One potential new application for TX-TL is the direct assembly of natural products from biosynthetic genes, as recently pioneered in *E. coli* TX-TL for the co-synthesis of two large (>100 kDa) non-ribosomal peptide synthetases [10]. Indeed, for expression of genes from *Streptomyces* species, *E. coli* may not be the ideal host chassis in all cases – e.g. poor codon usage, solubility issues, post-translational modification [11] or an absence of exotic precursors, such as coenzyme F420 [12]. Moreover, utilizing a host homologous to the chosen pathway has previously proved successful for acquiring soluble and active pathway enzymes [13], whereas *E. coli* accumulated only inclusion bodies. Another caveat to the use of *E. coli* is the potential inhibition of TX-TL machinery if the target products possess antimicrobial activities, whereas many *Streptomyces* species are known to provide their own resistance strategies to a variety of antimicrobials [14].

**Correspondence:** Prof. Paul Freemont, Department of Medicine, Imperial College London, Exhibition Road, SW7 2AZ, London, UK
**E-mail:** p.freemont@imperial.ac.uk

**Abbreviations:** aTC, Anhydrotetracycline; sfGFP, superfolder green fluorescence protein; TX-TL, in vitro transcription-translation
There is past evidence to suggest that a range of Streptomyces species are suitable for TX-TL [15, 16], however, it is unclear from recent literature what is the true productivity of a Streptomyces TX-TL system. TX-TL can provide a tool to rapidly prototype the cellular machinery of synthetic biology hosts [17]. Herein, we provide evidence for the development of a high-activity S. venezuelae TX-TL system utilizing the kasOp* promoter as a standard for cell-extract optimization [18]. In summary, we demonstrate high-yield synthesis of up to 1.3 µM superfolder GFP (sfGFP), prototype a tetO-TetR gene expression tool [19] and synthesize a selection of enzymes from the S. rimosus (ATCC 10970) oxytetracycline pathway [20].

2 Materials and methods

Materials and methods section is provided in Supporting information.

3 Results

3.1 Optimizing a high-activity S. venezuelae cell-extract

A general protocol for Streptomyces TX-TL was previously developed by Hopwood, Bibb and colleagues [21]. Significantly, a number of costly and therefore undesirable components are present in this original protocol, such as Staphylococcus nuclease and pyruvate kinase. To try a different low-cost strategy, we prepared a S. venezuelae cell-extract using the original Streptomyces method and tested its activity with a 3-phosphoglyceric acid (3-PGA) energy regeneration buffer derived from E. coli TX-TL [22]. Cell-extracts were tested for activity using sfGFP reporter coupled to a high-activity kasOp* promoter.

The process of cell-extract preparation can be divided into five stages in the order of (1) cell-growth, (2) washing, (3) sonication, (4) run-off and (5) dialysis. We merged the Streptomyces method for stages (1–2) with the E. coli...
TX-TL methodology for stages (3–5). This new protocol provided a significant baseline level of sfGFP fluorescence (154 nM), whereas by following the separate protocols on their own, only trace levels of sfGFP fluorescence (≈5 nM) were observed (data not shown). Next, by focusing on the preparation of the cell-extract and the reaction conditions, key variables (Fig. 1A–F) were optimized such as cell-lysis by sonication, run-off, dialysis and the concentration of TX-TL reaction components including polyethylene glycol (PEG), Mg-glutamate and K-glutamate. Each of these single components was varied and assessed during two rounds of parameter optimization to establish a streamlined protocol for *S. venezuelae* TX-TL, providing a maximum yield of 1.31 µM sfGFP (36 µg mL⁻¹), which demonstrated an 8.5-fold increase over the original base levels. In brief, a significant gain in activity was observed by varying the levels of the molecular crowding agent PEG and Mg-/K-glutamate salt (Fig. 1B and 1C), whilst the sonication duration did not appear to alter the activity. Dialysis in S30-SC buffer was also found to reduce cell-extract activity by 18%.

### 3.2 TX-TL protein synthesis requires 20–40 nM of DNA for translation saturation

The *S. venezuelae* cell-extracts were active for up to 4 h of TX-TL batch synthesis. Interestingly, the signal intensity of sfGFP in both real-time TX-TL fluorescence and Western blot end-point samples demonstrated a proportional increase in sfGFP production with plasmid DNA concentration, which saturate between 20–40 nM of DNA in three independent batches (Fig. 2A and 2B). For comparison, in *E. coli* Rosetta TX-TL extracts, saturation requires 10–15 nM [17]. In addition, for *S. venezuelae* TX-TL, the fluorescence signal for sfGFP was observed to decay after approximately 4 h of incubation. This also occurred with incubations pre-spiked with purified sfGFP (Supporting information, Fig. S2). The signal decay was suspected to be due to host proteases, however, a Western blot prepared with anti-GFP primary antibody confirmed that only single full-length sfGFP (27 kD) species was present in the extracts (Fig. 2C). A possible explanation for this fluorescence decay is non-specific unfolding or aggregation of sfGFP within the cell-extract.

**Figure 2.** Saturation of *S. venezuelae* TX-TL occurs at 20–40 nM DNA.  
(A) Time-course reaction with increasing DNA concentration. (B) Saturation curve of end-point samples with Extracts A–C. Error bars (standard deviation) are representative of three technical repeats. (C) Western blot of end-point TX-TL samples with mouse anti-GFP primary antibody. Lane abbreviations: M, PageRuler Plus (ThermoFisher) and positive control (PC), purified His₆-sfGFP (29 kDa). (D) Western blot of *S. venezuelae* and *E. coli* TX-TL of sfGFP and oxytetracycline enzymes. Red star (*) indicates negative synthesis of OxyD in *E. coli* TX-TL. Positive bands are individually cropped, with the original blots and SDS-PAGE gels shown in Supporting information, Fig. S3.
### 3.3 TX-TL synthesis of the OxyB, -C, -D, -J, -K and -T enzymes

To test the ability of *S. venezuelae* TX-TL to synthesize proteins from secondary metabolism, a selection of genes (oxyB, -C, -D, -J, -K and -T) from the *S. rimosus* oxytetracycline pathway [20] were assembled by Golden Gate with a T7 promoter, strong RBS and C-terminal His$_6$-tag.

In addition, a T7-driven sfGFP (-/-+ His$_6$-tag) was used as a positive control, with T7 RNA polymerase added to the *S. venezuelae* extracts to drive mRNA synthesis. Interestingly, for all of the oxytetracycline enzymes OxyB, -C, D, -J, -K, -T and sfGFP, these were detected by Western blotting as full-length His$_6$-tagged proteins (Fig. 2D), ranging in size from 12 kDa (OxyC) to 70 kDa (OxyD). This provided an indication of the potential of *S. venezuelae* TX-TL to

![Figure 3. Synthetic tetO-TetR gene expression in *S. venezuelae* TX-TL.](image-url)

(A) Plasmid design of the kasOp*-tetO synthetic promoter. (B) Purified TetR and aTC was spiked into *S. venezuelae* TX-TL to modulate gene expression. (C) End-point readings of sfGFP with varied TetR and aTC concentrations in combination with the kasOp*-tetO-sfGFP plasmid. aTC inhibition with (D) kasOp*-sfGFP and (E) kasOp*-tetO-sfGFP. (F) Repression with TetR and (G) release with 1 µM aTC (non-inhibitory level). Error bars (standard deviation) are representative of three technical repeats.
synthesize proteins from high G+C (%) genes from secondary metabolism. In comparison, with E. coli TX-TL, although higher yields (>2–10 µM) of sfGFP and the OxyB, -C, -J, -K and -T proteins were obtained, the OxyD protein could not be detected. For further information please refer to Supporting information, Fig. S3.

3.4 Inducible gene expression with the TetR system

Utilizing the Tn10-derived TetR gene expression tool [19], we introduced a tetO operator site immediately downstream of the kasOp* promoter. By assembling this synthetic promoter with sfGFP, an anhydrotetracycline (aTC) inducible gene expression tool was rapidly prototyped in S. venezuelae TX-TL by utilizing purified cognate TetR repressor, aTC and the TX-TL reaction components (Fig. 3). Firstly, 20 nM kasOp*-tetO synthetic promoter produces 3.5-fold less sfGFP in comparison to the equivalent kasOp*-sfGFP control plasmid (20 nM) lacking the tetO operator. Additionally, gene expression from the kasOp*-sfGFP plasmid is unaltered with either TetR (5 µM) or 1 µM aTC, whilst a 25% decrease in signal is observed with 10 µM aTC. However, in the presence of the tetO operator coupled to the kasOp* promoter and sfGFP, a clear switch-off in gene expression is observed by titrating an increasing concentration of TetR (1–5 µM), which was recovered by the presence of aTC (1–10 µM).

4 Concluding remarks

Herein, we have developed a S. venezuelae TX-TL system as a new tool for synthetic biology. This system demonstrates high-yield synthesis of sfGFP (up to 1.3 µM) and a range of enzymes from the oxytetracycline pathway, using a simple and cost-efficient protocol for extract preparation. The development of a S. venezuelae TX-TL system potentially provides a fast route to obtaining enzymes from Streptomyces secondary metabolism using a homologous host for protein folding. Whilst each protein target is unique, we will investigate this tool for the synthesis of specialized enzymes that require post-translational modification [10, 11] or exotic precursors for protein folding, such as coenzyme F420 [12]. We will now focus on enhancing this initial S. venezuelae TX-TL platform, prototype gene circuits and investigate its use for the characterization of cryptic gene clusters located within the Actinomycetes bacteria [23, 24].

The authors declare no financial or commercial conflict of interest.

5 References

[1] Fischbach, M. A., Walsh, C. T., Antibiotics for emerging pathogens. Science 2009, 325, 1089–1094.
[2] Park, J. W., Park, S. R., Nepal, K. K., Han, A. R. et al., Discovery of parallel pathways of kanamycin biosynthesis allows antibiotic manipulation. Nat. Chem. Biol. 2011, 7, 841–852.
[3] Phelan, R. M., Sachs, D., Petkiewicz, S. J., Barajas, J. F. et al., Development of next generation synthetic biology tools for use in Streptomyces venezuelae. ACS Synth. Biol. 2017, 6, 159–168.
[4] Phehan, R. M., Sekurova, O. N., Keasing, J. D., Zotchev, S. B., Engineering terpene biosynthesis in Streptomyces for production of the advanced biofuel precursor bisabolene. ACS Synth. Biol. 2015, 4, 393–399.
[5] He, J., Van Theeck, B., Nguyen, H. B., Melançon, C. E., Development of an unnatural amino acid incorporation system in the actinobacterial natural product producer Streptomyces venezuelae ATCC 15439. ACS Synth. Biol. 2016, 5, 125–132.
[6] Garamella, J., Marshall, R., Rustad, M., Noireaux, V., The all E. coli TX-TL Toolbox 2.0: A platform for cell-free synthetic biology. ACS Synth. Biol. 2016, 5, 344–355.
[7] Jewett, M. C., Swartz, J. R., Mimicking the Escherichia coli cytoplasmic environment activates long-lived and efficient cell-free protein synthesis. Biotechnol. Bioeng. 2004, 86, 19–26.
[8] Siegal-Gaskins, D., Tuza, Z. A., Kim, J., Noireaux, V., Murray, R. M., Gene circuit performance characterization and resource usage in a cell-free “breadboard.” ACS Synth. Biol. 2014, 3, 416–426.
[9] Harris, D. C., Jewett, M. C., Cell-free biology: Exploiting the interface between synthetic biology and synthetic chemistry. Curr. Opin. Biotechnol. 2012, 23, 672–678.
[10] Goering, A. W., Li, J., McClure, R. A., Thomson, R. J. et al., In Vitro reconstruction of nonribosomal peptide biosynthesis directly from DNA using cell-free protein synthesis. ACS Synth. Biol. 2017, 6, 39–44.
[11] Takahashi, H., Kumagai, T., Kitani, K., Mori, M. et al., Cloning and characterization of a Streptomyces single module type non-ribosomal peptide synthetase catalyzing a blue pigment synthesis. J. Biol. Chem. 2007, 282, 9073–9081.
[12] Wang, P., Baezir, G., Gao, X., Sawaya, M. R., Tang, Y., Uncovering the enzymes that catalyze the final steps in oxytetracycline biosynthesis: J. Am. Chem. Soc. 2013, 135, 7138–7141.
[13] Moore, S. J., Lawrence, A. D., Biedenbiek, R., Deery, E. et al., Elucidation of the anaerobic pathway for the corrin component of cobalamin (vitamin B12). Proc. Natl. Acad. Sci. USA 2013, 110, 14906–14911.
[14] Niemis, O., Suicide, A. V., Cundiff, E., How antibiotic-producing organisms avoid suicide. Annu. Rev. Microbiol. 1989, 43, 207–233.
[15] Thompson, J., Rae, S., Cundiff, E., Coupled transcription—translation in extracts of Streptomyces lividus. Mol. Gen. Genet. 1984, 195, 39–43.
[16] Jones, G. H., Macromolecular synthesis in Streptomyces antibioticus: In vitro systems for aminoacylation and translation from young and old cells. J. Bacteriol. 1975, 124, 364–372.
[17] Sun, Z. Z., Yeung, E., Hayes, C. A., Noireaux, V., Murray, R. M., Linear DNA for rapid prototyping of synthetic biological circuits in an Escherichia coli based TX-TL cell-free system. ACS Synth. Biol. 2014, 3, 387–397.
[18] Wang, W., Li, X., Wang, J., Xiang, S. et al., An engineered strong promoter for streptomycetes. Appl. Environ. Microbiol. 2013, 79, 4484–4492.

SM is a postdoctoral researcher funded by the EPSRC grant EP/K038648/1 (Frontier Engineering). HL is funded by a PhD studentship award from Imperial College London. We thank Mr. Ciaran McKeown and Dr. Luke Yates for technical assistance.
[19] Rodríguez-García, A., Combes, P., Pérez-Redondo, R., Smith, M. C. A., Smith, M. C. M., Natural and synthetic tetracycline-inducible promoters for use in the antibiotic-producing bacteria Streptomyces. Nucleic Acids Res. 2005, 33, e87.

[20] Zhang, W., Watanabe, K., Cai, X., Jung, M. E. et al., Identifying the minimal enzymes required for anhydrotetracycline biosynthesis. J. Am. Chem. Soc 2008, 130, 6068–6069.

[21] Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F., Hopwood, D. A., Practical Streptomyces Genetics. John Innes Foundation, Norwich 2000.

[22] Sun, Z. Z., Hayes, C. A., Shin, J., Caschera, F. et al., Protocols for implementing an Escherichia coli based TX-TL cell-free expression system for synthetic biology. J. Vis. Exp. 2013, 79, e50762.

[23] Challis, G. L., Mining microbial genomes for new natural products and biosynthetic pathways. Microbiology 2008, 154, 1555–1569.

[24] Weber, T., Blin, K., Duddela, S., Krug, D. et al., antiSMASH 3.0—a comprehensive resource for the genome mining of biosynthetic gene clusters. Nucleic Acids Res. 2015, 43, W237–W243.