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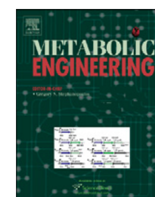

A translation-coupling DNA cassette for monitoring protein translation in *Escherichia coli*

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ABSTRACT

A major challenge to using heterologous expression in metabolic engineering experiments is the inability to quickly dissect experiments that have failed at the stage of translating mRNA. While many methods of detecting proteins exist, methods that detect untagged proteins at low levels are limited. Here, we describe a method to quickly determine whether *Escherichia coli* is capable of expressing the product of any target gene by coupling translation of a target gene to a detectable response gene. A translational coupling cassette was designed to encode a mRNA sequence that forms a secondary structure in the absence of translation and contains the translational start sequence of a detectable response gene. The translational coupling method was successfully tested with fluorescent proteins and antibiotic resistance markers. Only when the target gene was fully translated was the response observed. Further characterization demonstrated that translational coupling functions at both low and high levels of expression and that the response signal is proportional to the amount of target gene product. The translational coupling system was used to determine that a large multi-domain enzyme was not actively translated in *E. coli*, to isolate the translation problems to the C-terminal domains, and to optimize conditions for expressing a codon-optimized sequence variant.

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1. Introduction

For metabolic engineers, heterologous gene expression has become a preferred tool for imparting novel biochemistries, bypassing native pathways, or enhancing metabolic activity in model hosts (Keasling, 2008, 2010; Pfeifer and Khosla, 2001; Tyo et al., 2007). Of the model microorganisms used in metabolic engineering studies, *Escherichia coli* remains a popular choice because of the availability of a large knowledge base, a wide variety of engineering tools (e.g. vectors, promoters, construction methodologies), and well characterized genetic systems (Boyle and Silver, 2012; Keasling, 2012; Michener et al., 2012). Despite these advantages, heterologous gene expression in *E. coli* is not always straightforward. The development of algorithms for codon optimization (Angov et al., 2008) and inexpensive gene synthesis (Carlson, 2009) methods have dramatically increased success rates but have not solved all problems. The major challenge to using heterologous expression in metabolic engineering experiments lies in the inability to quickly dissect experiments that have failed. If an expected phenotype is not observed, a researcher must determine which step in the path from gene to activity is the cause of failure.

While facile assays for the presence of any target mRNA can be developed (e.g. qPCR, hybridization), assays for monitoring protein activity are highly dependent on the properties of the target gene product. Conventional assays for detecting the presence of target protein, such as SDS-PAGE, Western blot, mass spectrometry, are not always feasible (for both technical and economic reasons) or compatible with producing active protein in vivo. For example, a target protein must be found in high concentration in or purified from a crude cell lysate in order for a specific band to be detected by SDS-PAGE analysis. Western blots offer a lower limit of detection in complex mixtures but require the existence of an antibody that specifically recognizes the target protein. Fusion tags (e.g. 6X-histidine, maltose binding protein, FLAG, fluorescent proteins) can facilitate both purification and Western blot detection but can also interfere with protein folding and activity. For these reasons, the ability to monitor protein expression directly in hosts without significant sequence modification is limited. Therefore, a tool than can be used in *E. coli* to test for

Abbreviations: RBS, ribosome binding site; RFP, red fluorescent protein, DsRed; YFP, yellow fluorescent protein; Ols, olefin synthase; SDS-PAGE, sodium dodecyl sulfate poly-acrylamide gel electrophoresis; qPCR, quantitative polymerase chain reaction; LIC, ligation independent cloning; KM, kanamycin resistance; CM, chloramphenicol resistance; PKS, polyketide synthase; LD, loading domain, acyl-ACP synthetase; ACP, acyl-carrier protein; KS, ketosynthase domain; AT, acyl-transferase domain; KR, ketoreductase domain; ST, sulfotransferase domain; TE, thioesterase domain

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translation in vivo without affecting the activity of the target protein is of great interest. Such a method could be used to simplify and speed the process of optimizing heterologous expression of novel genes in both protein production and metabolic engineering applications.

To address this challenge, we have developed a synthetic biology method of indirectly detecting protein translation that is based on a microbial method of gene regulation. Often, each cistron of a polycistronic mRNA has its own Shine–Delgarno sequence, which in principle initiates translation independent of other cistrons. However, it has been observed that translation of some genes in a polycistronic mRNA is dependent on the translation of a contiguous upstream gene. This interdependence of translation efficiency of neighboring genes, called translational coupling, is used in phage and bacteria to regulate expression of some proteins (Ivey-Hoyle and Steege, 1989). In *E. coli*, translational coupling has been observed for genes encoding ribosomal proteins (Lindahl et al., 1989; Lindahl and Zengel, 1986) and genes *trpBA* (Aksoy et al., 1984), *trpDE* (Oppenheim and Yanofsky, 1980) and *galKT* (Schumperli et al., 1982). Translational coupling is mediated by mRNA secondary structures that outcompete formation of the mRNA:rRNA base pairs that lead to translation initiation. When the gene in the 5′ position is not translated, the secondary structure forms and prevents translation of the gene in the 3′ position. Conversely, when the gene in the 5′ position is translated, the helicase activity of the ribosome (Takyar et al., 2005) melts the secondary structure, thereby promoting binding of the ribosome and translation initiation of the gene in the 3′ position.

Here, we describe a method to quickly determine whether *E. coli* is capable of expressing the product of any target gene by coupling translation of a target gene to a detectable response gene. A translational coupling cassette was designed to encode a mRNA sequence that forms a secondary structure in the absence of translation and contains the translational start sequence of a detectable response gene. The translational coupling method was successfully tested with fluorescent proteins and antibiotic resistance markers. Only when the target gene was fully translated was the response observed. Further characterization demonstrated that translational coupling functions at both low and high levels of expression and that the response signal is proportional to the amount of target gene product. The translational coupling system was used to determine that a large, multi-domain enzyme was not actively translated in *E. coli*, to isolate the translation problems to the C-terminal domains, and to optimize conditions for expressing a codon-optimized sequence variant. We anticipate that the translational coupling system can be used to detect complete translation, select for conditions and/or sequences that maximize expression, and tightly regulate multiple genes at desired ratios.

2. Methods

2.1. Plasmid construction and oligonucleotides

Plasmid construction and maintenance was performed using *E. coli* DH10B (Invitrogen, Carlsbad, CA). Plasmids were prepared by alkaline lysis (Qiagen, Valencia, CA). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, Iowa). Cloning enzymes, including Phusion® DNA polymerase, restriction enzymes, and T4 DNA ligase were purchased from New England Biolabs (NEB, Ipswich, MA) and used according to the manufacturer's instructions. Plasmids used in this paper are listed in Table 1. New plasmid sequences are deposited in the NCBI nucleotide collection. All cloning procedures were confirmed by DNA sequencing.

Table 1
Plasmids constructed and used in this manuscript.

Plasmid	Genotype/features	Source
pTRC99A	Backbone for translational coupling cassette	Amann et al. (1988)
pJ206	Used to amplify *aphII* and *cat*	DNA 2.0
$pRFP^{EC}$	Used to amplify RFP gene	Pfleger et al. (2005)
pAQ1Ex-PcpcBA::YFP	Used to amplify YFP gene	Xu et al. (2011)
pBP18	Translational coupling cassette using *cat* as response gene	This report
pLIC-KM	Translational coupling cassette using *aphII* as response gene	This report
pLIC-CM	Translational coupling cassette using *cat* as response gene	This report
pRFP-KM	pLIC-KM with RFP as the target gene	This report
$pRFP^{nf}$-KM	pLIC-KM with RFP with premature stop codon as response gene	This report
pRFP-CM	pLIC-CM with RFP as the target gene	This report
$pRFP^{nf}$-CM	pLIC-CM with RFP with premature stop codon as response gene	This report
pLIC-RFP	Translational coupling cassette using RFP as response gene	This report
pYFP-RFP	pLIC-RFP with YFP as the target gene	This report
pYFP-RFP-library	pYFP-RFP with different ribosome binding sites upstream YFP	This report
	RBS sequence (5′–3′) of characterized clones (start codon in bold)	
pYFP-RFP1	TGTTAGCGCCGAGGGAATTAAGTAGCTAATTAGA**ATG**	
pYFP-RFP 2	TGTGAGCGGCGAGGGAATTAAGGAGTTAATTAGA**ATG**	
pYFP-RFP 3	TGTTAGCGTTAGGTAGGTAATTAGA**ATG**	
pYFP-RFP 4	TGTGAGCGCCGAGGGAATTATGGAGATAATTAGA**ATG**	
pYFP-RFP 5	TGTTAGCGTCGAGGGAATTATGCAGTTAATTAGA**ATG**	
pYFP-RFP 6	TGTTAGCGGCGAGGGAATTAAGGAGATAATTAGA**ATG**	
pYFP-RFP 7	TGTCAGCGGCGAGGGAATTATGGAGTTAATTAGA**ATG**	
pYFP-RFP 8	TGTCAGCGGCGAGGGAATTATGAAGATAATTAGA**ATG**	
pYFP-RFP 9	TGTGAGCGTCGAGGGAATTATGGAGATAATTAGA**ATG**	
pYFP-RFP 10	TGTAAGCGACGAGGGAATTAGGGAGGTAATTAGA**ATG**	
pYFP-RFP 11	TGTCAGCGACGAGGGGAATTAAGGAGGTAATTAGA**ATG**	
pOLS-WT	pLIC-KM with wild type *ols* as the target gene	This report
pLD-ACP1	pLIC-KM with LD and ACP1 as target gene	This report
pKS-AT-KR	pLIC-KM with KS, AT, KR as target gene	This report
pLD-ACP1-KS-AT-KR	pLIC-KM with LD, ACP1, KS, AT, KR as target gene	This report
pACP2-ST-TE	pLIC-KM with ACP2, ST, TE as target gene	This report
pACP2-ST-TE-His	pACP2-ST-TE including a N-terminal 6X-histidine tag	This report
pACP2-ST-TE-Opt	pLIC-KM with codon optimized ACP2, ST, TE as target gene	This report
pOLS-Opt	pLIC-KM with *ols* gene with codon optimized ACP2-ST-TE domains as target gene	This report

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