

RESEARCH NOTE

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Codon-optimized *DsRed* fluorescent protein for use in *Mycobacterium tuberculosis*

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Abstract

Objective: We have previously codon-optimized a number of red fluorescent proteins for use in *Mycobacterium tuberculosis* (mCherry, tdTomato, Turbo-635). We aimed to expand this repertoire to include *DsRed*, another widely used and flexible red fluorescent protein.

Results: We generated expression constructs with a full length *DsRed* under the control of one of three strong, constitutive promoters (P_{hsp60} , P_{rpsA} or P_{G13}) for use in mycobacteria. We confirmed that full length *DsRed* (225 amino acids) was expressed and fluoresced brightly. In contrast to mCherry, truncated versions of *DsRed* lacking several amino acids at the N-terminus were not functional. Thus, we have expanded the repertoire of optimized fluorescent proteins for mycobacteria.

Keywords: Fluorescent protein, Mycobacteria, Reporter system

Introduction

Fluorescent proteins (FPs) have become the work horses of molecular biology and microbiology, with numerous applications. A plethora of variants of *Aequorea victoria* green fluorescent protein (GFP) [1] and *Discosoma* sp red fluorescent protein (*DsRed*) [2] are available covering almost the whole light spectrum from green to infrared [3]. Mutant derivatives have been engineered with altered excitation and emission wavelengths, increased or decreased stability, resistance to photo bleaching, sensitivity to environmental stimuli and substrates, as well as time for fluorophore maturation, intrinsic brightness and multimeric formats [3, 4]. We previously described the use of a range of red reporters, of which the brightest was mCherry [5]. We wanted to expand our repertoire of proteins. Since *DsRed* has been widely used as a bright and stable reporter, we optimized constructs for its expression in *M. tuberculosis*.

Main text

Materials and methods

Bacterial culture

Escherichia coli DH5 α was cultured in LB medium or on LA agar. *M. tuberculosis* H37Rv was grown in Middlebrook 7H9 medium plus 10% v/v OADC (oleic acid, albumen, dextrose, catalase) supplement (Becton–Dickinson) and 0.05% w/v Tween 80 or on Middlebrook 7H10 agar (Becton–Dickinson) plus 10% v/v OADC. Hygromycin was used at 100 μ g/ml where required.

Construction of expression vectors

The *DsRed* expression vectors were constructed as follows: a partial *DsRed* sequence was codon optimized for *M. tuberculosis*, synthesised and cloned into pUC57 (Genscript USA Inc.) to generate pRed1. The *DsRed* ORF was excised from pUC57 as a BamHI/HindIII fragment and cloned into pSMT3 [6] to generate pBlazel1. The *DsRed* ORF was extended three times by PCR to generate pRedA1, pRedB1 and pRedC1 using primers *DsRed*-F1 5'-GGA TCC ATG CGC TTC AAG GTG CGC ATG GAG GGC TCG GTG AAC-3', *DsRed*-F2 5'-GGA TCC GAC GTG ATC AAG GAG TTC ATG CGC TTC AAG GTG CGC-3' and *DsRed*-F3 5'-GGA TCC ATG GCC TCG TCG GAG GAC GTG ATC AAG

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GAG TTC together with the reverse primer DsRed-R 5'-AAG CTT TTA CAG GAA CAG GTG GTG CCG-3'. The restriction sites are underlined, potential start codons are in bold. The ORFs were excised and cloned into pSMT3 [6] as *Bam*HI/*Hind*III fragments to generate pBlazeA1, pBlazeB1 and pBlazeC1 with *DsRed* under the control of the *hsp60* promoter (Table 1). Plasmids pBlazeC8 and pBlazeC10 were generated by replacing P_{hsp60} with P_{rpsA} and P_{G13} respectively. All three promoters should drive constitutive high level expression [5, 7, 8].

Quantitation of fluorescence in whole cells

Mycobacterium tuberculosis was electroporated as described [9] and transformants selected with hygromycin. *M. tuberculosis* was grown to stationary phase, harvested, washed twice in 10 mM Tris pH 8.0 and resuspended in 10 mM Tris pH 8.0 to an OD₅₈₀ of 0.25, 0.10, 0.05 and 0.01 in 12 × 100 mm glass culture tubes. Fluorescence was measured on a Shimadzu RF-1501 spectrofluorimeter (Shimadzu) with a detection range of 0–1015 relative fluorescent units at Ex/Em 558/583 nm [5].

Western analysis of fluorescent proteins

Cell extracts were prepared from liquid cultures. Cells were harvested by centrifugation, washed twice in 10 mM Tris (pH 8.0), resuspended in 1 ml of 10 mM Tris (pH 8.0), and added to lysing matrix B tubes (QBiogene). Cells were disrupted using the Fastprep (QBiogene) set at speed 6.0 for 30 s. Samples were centrifuged at 4000 rpm for two min, and the supernatant was recovered and filter sterilized (0.2 micron filter). Protein was quantified using a BCA kit (Pierce), and 10 µg of total protein was subjected to Western blot using a rabbit anti-body (Clonetech). The primary antibody was detected using horseradish peroxidase

goat-anti-rabbit (Sigma), and activity was detected using an ECL kit (GE Healthcare).

Results

We were interested in the use of FPs in *M. tuberculosis* and had previously used these as reporters of bacterial viability for in vitro and in vivo studies [5, 8]. We were successful in obtaining high level expression by using codon-optimized versions of red fluorescent proteins driven by strong mycobacterial promoters [5].

Optimization of *DsRed* expression

We wanted to expand the range of reporters available for use to increase flexibility and allow dual reporter expression and monitoring. We selected *DsRed* for optimization, based on its Ex/Em wavelengths, and the fact that it is a well-characterized FP in wide use [3, 4, 10–14].

Expression of *DsRed* uses a different translational start site than *mCherry*

Our initial attempts to obtain expression of a codon-optimized *DsRed* were unsuccessful. We constructed a synthetic gene for *DsRed* using a similar approach as we used with another red fluorescent protein mCherry (Fig. 1). We designed a codon-optimized version based on the *DsRed*-T3 protein previously used. We cloned the synthetic version into a mycobacterial expression vector and tested for fluorescence in *M. tuberculosis*. Surprisingly, we did not detect any fluorescence from this construct (Fig. 1c).

mCherry is a variant of *DsRed* and we expected the two proteins would be similarly functional. Our previous work demonstrated that mCherry is expressed from a distal translational start site than the one annotated in the databases [15]. Sequence alignment shows the few mutations which differ between the two (Fig. 2a); these include loss of the translational start site we identified for mCherry, although there are still multiple translational start sites (Fig. 1a). The version we used for the

Table 1 Plasmids used in this study

Plasmid	Description	Promoter	Fluorescent protein	Source
pSMT3	Shuttle vector, P_{hsp60} HygR			[6]
pRED1	Codon-optimized <i>DsRed</i> in pUC57	None	<i>DsRed</i> 208	This study
pBlaze1	<i>DsRed</i> expression vector. HygR	<i>Hsp60</i>	<i>DsRed</i> 208	This study
pBlazeA1	<i>DsRed</i> expression vector. HygR	<i>Hsp60</i>	<i>DsRed</i> 214	This study
pBlazeB1	<i>DsRed</i> expression vector. HygR	<i>Hsp60</i>	<i>DsRed</i> 220	This study
pBlazeC1	<i>DsRed</i> expression vector. HygR	<i>Hsp60</i>	<i>DsRed</i> 225	This study
pBlazeC8	<i>DsRed</i> expression vector. HygR	<i>RpsA</i>	<i>DsRed</i> 225	This study
pBlazeC10	<i>DsRed</i> expression vector. HygR	<i>G13</i>	<i>DsRed</i> 225	This study

a
MASSED**V**IKE**F****M**RFK**V****M**EGSVNGHEFEIEEGEGEGRPYEGTQTAKLKVTKGGPLP
 FAWDILSPQFQYGSKVYVKHPADIPDYKKLSFPEGFKWERVMNFEDGGVVTVTQD
 SSLQDGCFIYKVKFIGVNFPSDGPVMQKKTMGWEPSTERLYPRDGVLKGEIHKAL
 KLDGGHYLVEFKSIYMAKKPVQLPGYYYVDSKLDITSHNEDYTIVEQYERTEGR
 HHLFL

b
ATGGCCTCGTCCGAGGAC**G**T**G**ATCA**A**GGAGTTC**ATG**CGCTTCAAGGTGCG**ATGG**
 AGGGCTCGGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCC
 GTACGAGGGCACCCAGACCGCCAAGCTGAAGGTCACCAAGGGCGGCCCGCTGCCG
 TTCGCCTGGGACATCCTGTCGCCGCAGTTCAGTACGGCAGCAAGGTGTACGTCA
 AGCACCCGGCCGACATCCCGGACTACAAGAAGCTGAGCTTCCCGGAGGGCTTCAA
 GTGGGAGCGGGTGATGAACTTCGAGGACGGCGGCGTGGTCACCGTCACCCAGGAC
 TCGAGCCTGCAGGACGGCTGCTTCATCTACAAGGTGAAGTTCATCGGCGTCAACT
 TCCCGAGCGACGGCCCGGTGATGCAGAAGAAGACCATGGGCTGGGAGCCGTGCAC
 CGAGCGCCTGTACCCGCGGGACGGCGTGCTGAAGGGCGAGATCCACAAGGCCCTG
 AAGCTGAAGGACGGCGGCCACTACCTGGTCGAGTTCAAGTCGATCTACATGGCGA
 AGAAGCCGGTGCAGCTGCCGGGCTACTACTACGTGACTCGAAGCTGGACATCAC
 CAGCCACAACGAGGACTACACCATCGTCGAGCAGTACGAGCGCACCCGAGGGCCGG
 CACCACCTGTTCCCTGTAA

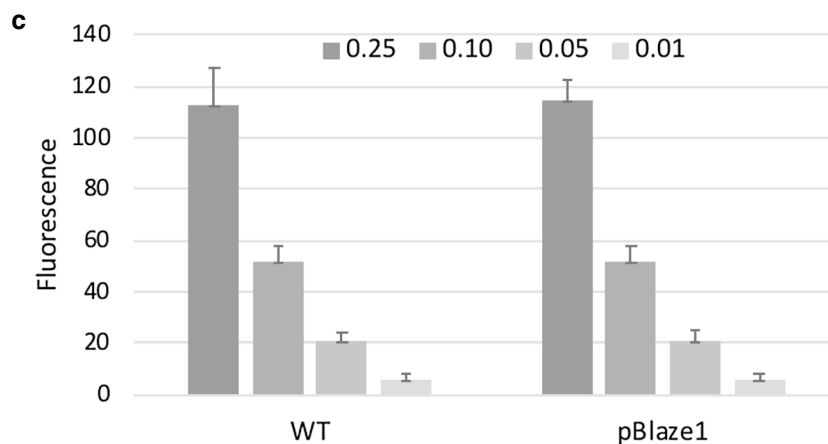


Fig. 1 Expression of non-functional *DsRed*. **a** *DsRed* Protein sequence. Three potential translational start sites (methionine) are indicated in bold. The valine which corresponds to the methionine start site of mCherry in *M. tuberculosis* is also indicated in bold. **b** DNA sequence of *DsRed*. The 5' end of the synthetic gene designed to codon-optimize *DsRed* for *M. tuberculosis* is boxed. Potential starts sites are indicated in bold. The Shine Delgarno sequence is underlined. **c** *M. tuberculosis* was resuspended in 10 mM Tris pH 8.0 to an OD₅₈₀ of 0.25, 0.10, 0.05 and 0.01 in 12 × 100 mm glass culture tubes. Fluorescence was measured at Ex/Em 558/583 nm. WT—wild-type (no plasmid). pBlaze1—recombinant strain carrying *DsRed* 208aa. Data are the average ± SD of three cultures

coding region into the expression vector under the control of the constitutive *hsp60* promoter (Fig. 2b).

In order to test this, we used PCR amplification to extend the region sequentially. We extended the gene to incorporate both additional start sites and generate proteins of 214, 220 and 225 amino acids. These variants were cloned into the same mycobacterial expression system and tested. Plasmids were transformed into *M. tuberculosis* and fluorescence was monitored. In contrast to mCherry, expression of a functional fluorescent DsRed was not seen with any truncated versions of the protein. In fact fluorescence could only be detected when the full length amino acid sequence (as annotated) was cloned into the expression vector; high level fluorescence was seen with transformants carrying the plasmid pBlazeC1 (Fig. 2b).

We constructed two alternative vectors with DsRed under the control of either P_{rpsA} or P_{G13} (pBlazeC8 and pBlazeC10 respectively); both of these constructs gave high level expression in *M. tuberculosis*. Western blotting using an anti-DsRed antibody in *E. coli* demonstrated that a protein of the expected size was only seen in bacteria carrying the full length construct (pBlazeC series), but not in the strains carrying the truncated version (Fig. 2c; lanes 5–9). Two additional bands are seen in the Western, these are unknown proteins, but are also present in the control *E. coli* (no plasmid, Fig. 2c, lane 1).

Discussion

We have determined that the functional translational start sites for two closely related FPs are different in *M. tuberculosis*. Although mCherry was functional even when a truncated version was being expressed, DsRed was non-functional in a truncated form and only fluoresced when expressed as a full length protein (225 amino acids). Western blotting suggested that the lack of fluorescence was most likely due to a lack of protein expression, since proteins could not be detected in the plasmids carrying truncated forms. This difference may relate to protein stability, with the extended N-terminal portion of DsRed increasing stability or protein maturation; alternatively this could be attributed to the physical state of the active proteins, since mCherry functions as a monomer, whereas DsRed is a tetramer which might also affect protein degradation.

Fluorescent proteins have proved useful in multiple applications in mycobacteria; our previous constructs using mCherry have been widely disseminated to the community and used in a range of methods. For example, we have used these for high throughput drug testing [16], and imaging infection using animal models [8]. Other approaches have used mCherry to develop reporter strains for environmental sensing [17].

In conclusion, we have codon-optimized DsRed for use in *M. tuberculosis* and demonstrated its high level fluorescence in that species from three different promoters of slightly varying strength (*hsp60*, *rpsA*, and *G13*). These vectors extend our current repertoire of functional fluorescent proteins for mycobacteria. They will be useful for generating fluorescent strains of *M. tuberculosis* for use in multiple studies, such as monitoring drug efficacy in vitro and in vivo [5, 8, 16, 18] and will allow for detection of multiple reporters simultaneously.

Limitations

- We have monitored the expression of DsRed under aerobic conditions only.
- We have not monitored long term stability of expression in the absence of antibiotic selection to maintain the plasmid.
- We have not monitored stability of expression in vivo.

Abbreviations

FP: fluorescent protein; OADC: oleic acid, albumin, D-glucose, catalase.

Authors' contributions

Experimental design: PC, JM, TP. Experimental work: PC, JM. Data analysis: PC, TP. Writing manuscript: PC, TP. Reviewing manuscript: PC, JM, TP. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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