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

Paul Carroll¹, Julian Muwanguzi-Karugaba¹ and Tanya Parish¹,²*

**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 (Phsp60, PrpsA or PG13) 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* DH5α 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 μg/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 pBlaze1. 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 AAG GTG ATC AAG...
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 BamHI/HindIII 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 | None         | DsRed208               | [6]        |
| pRED1     | Codon-optimized DsRed in pUC57 | Hsp60        | DsRed208               | This study |
| pBlaze1   | DsRed expression vector, HygR | Hsp60        | DsRed214               | This study |
| pBlazeA1  | DsRed expression vector, HygR | Hsp60        | DsRed220               | This study |
| pBlazeB1  | DsRed expression vector, HygR | Hsp60        | DsRed225               | This study |
| pBlazeC1  | DsRed expression vector, HygR | G13          | DsRed225               | This study |
| pBlazeC8  | DsRed expression vector, HygR | RpsA         | DsRed225               | This study |
| pBlazeC10 | DsRed expression vector, HygR | None         | DsRed225               | This study |
a

\text{MASSEDVIKEFMRFKVRMEGSVNHEFEIEEGEGRYPEGTQTALKVTKGGPLP}
\text{FNDILSPQQFQYSKSKYHPADIPDYKLKLSFPEGFKWRMNFEEDGKVVTQD}
\text{SSLQDCFIFVVKFIGVFPSDGPVMQKKTGMWEPSTERLYPRDGVLKGEIHKAL}
\text{KLKDGGHYLVEFKSIYMAKPKPVQLPGYVVDSKLDITSNEDYTVIEQYERTEGR}
\text{HHLFL}

b

\text{ATGGCCTCGTCGGAGGACGTGATCAAGGAGTTCTAGGCGCTTCAAGGTGCATGG}
\text{AGGGCTCGGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCC}
\text{GTACGAGGGCCACCCAGCCGCAAGCTGAGTGTCACCAAGGCGCCGCTGCGG}
\text{TTCCGCTGGGACATCTGTGCGCGCAGTTCCAGTCGACGGCAAGGGTGTAAGCTCA}
\text{AAGCACCAGCGCCATCCGCGGCTAACAAGAAGCTGACTTCCGGAGGGCTTCAA}
\text{AGTGGAGCCGGTGTAGTAACCTCTCAGAGCCGCGCGCTGATCAGGCACCCTGACG}
\text{TCCGAGGCTCGACGAGCTGCTCAGTCTATGTTCAAGGTAAGTTGCTACGCGCTCAACT}
\text{TCCCGAGCGCCGGCCGCGTGGTAGCTGAAGAAGACCTGGGGTGGGAGCGCGTACGACG}
\text{CGAGCGCTGTACCCGCGGACGGCGCGTGGCAGTGACGGCGAGATCCACAAGGCGCTG}
\text{AAGCTGAGGGCAGCGGGCGTGAGTCCAGGAGGAGGCTACCCGACCTGCGATCTACATGG}
\text{AGAAGGCGGCGTGACGTCCCGCTGGCGACTGCTACGCTACGCTAGGCTACATCAGTACAC}
\text{CAGGCCAACAAGGAGACTACACCATCGTCCAGCAGTACCGAGCCACCGAGCGCAGGCGC}
\text{CACCACCTGTTCTGTAA}

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 OD\text{\textsubscript{600}} of 0.25, 0.10, 0.05 and 0.01 in 12 x 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.
synthetic gene used a downstream translation start site and would produce a truncated version of DsRed as compared to mCherry. Therefore it was possible that we did not express the full protein (Fig. 1b). In order to determine the functional start site for DsRed we used a different approach in which we cloned several versions of the
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 PrpsA or PG13 (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.

Author details

1 Queen Mary University of London, Barts & The London School of Medicine and Dentistry, London, UK. 2 Infectious Disease Research Institute, Seattle, WA 98102, USA.

Acknowledgements

We thank Amanda Brown and Lise Schreuder for technical assistance and helpful discussion.

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.

Funding

This work was supported by the Bill & Melinda Gates Foundation Grant OPP42786.

Publisher’s Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.
Received: 8 August 2018  Accepted: 27 September 2018  Published online: 01 October 2018

References

1. Tsien RY. The green fluorescent protein. Annu Rev Biochem. 1998;67:509–44.
2. Matz MV, Fradkov AF, Labas YA, Savitsky AP, Zaraisky AG, Markelov ML, Lukyanov SA. Fluorescent proteins from nonbioluminescent Anthozoa species. Nat Biotechnol. 1999;17:969–73.
3. Rodriguez EA, Campbell RE, Lin JY, Lin MZ, Miyawaki A, Palmer AE, Shu X, Zhang J, Tsien RY. The growing and glowing toolbox of fluorescent and photoactive proteins. Trends Biochem Sci. 2017;42:111–29.
4. Stepanenko OV, Stepanenko OV, Shcherbakova DM, Kuznetsova IM, Turoverov KK, Verkhusha VV. Modern fluorescent proteins: from chromophore formation to novel intracellular applications. BioTechniques. 2011;51:313–4.
5. Carroll P, Schreuder LJ, Muwanguzi-Karugaba J, Wiles S, Robertson BD, Ripoll J, Ward TH, Bancroft GJ, Schaible UE, Parish T. Sensitive detection of gene expression in mycobacteria under replicating and non-replicating conditions using optimized far-red reporters. PLoS ONE. 2010;5:e9823.
6. O’Gaora P. Expression of genes in mycobacteria. In: Parish T, Stoker NG, editors. Mycobacteria protocols, vol. 101. New York: Springer; 1998. p. 261–73.
7. Andreu N, Zelmer A, Fletcher T, Elkingston PT, Ward TH, Ripoll J, Parish T, Bancroft GJ, Schable U, Robertson BD, et al. Optimisation of bioluminescent reporters for use with mycobacteria. PLoS ONE. 2010;5:e10777.
8. Zelmer A, Carroll P, Andreu N, Hagens K, Mahlo J, Redinger N, Robertson BD, Wiles S, Ward TH, Parish T, et al. A new in vivo model to test antituberculosis drugs: using fluorescent imaging. J Antimicrob Chemother. 2012;67:1948–60.
9. Goude R, Roberts DM, Parish T. Electroporation of mycobacteria. Methods Mol Biol. 2015;1285:117–30.
10. Telford WG, Hawley T, Subach F, Verkhusha V, Hawley RG. Flow cytometry of fluorescent proteins. Methods. 2012;57:318–30.
11. Shrestha S, Deo SK. Anthozoa red fluorescent protein in biosensing. Anal Bioanal Chem. 2006;386:515–24.
12. Horn C, Schmid BG, Pogoda FS, Wimmer EA. Fluorescent transformation markers for insect transgenesis. Insect Biochem Mol Biol. 2002;32:1221–35.
13. Hadjantonakis AK, Dickinson ME, Fraser SE, Papaioannou VE. Technicolour transgenesis: imaging tools for functional genomics in the mouse. Nat Genet. 2003;34:613–25.
14. Berg RH, Beachy RN. Fluorescent protein applications in plants. Methods Cell Biol. 2008;85:153–77.
15. Carroll P, Muwanguzi-Karugaba J, Milief E, Files M, Parish T. Identification of the translational start site of codon-optimized mCherry in Mycobacterium tuberculosis. BMC Res Notes. 2014;7:566.
16. Ollinger J, Bailey MA, Moraski GC, Casey A, Florio S, Alling T, Miller MJ, Parish T. A dual read-out assay to evaluate the potency of compounds active against Mycobacterium tuberculosis. PLoS ONE. 2013;8:e60531.
17. Tan S, Sukumar N, Abramovitch RB, Parish T, Russell DG. Mycobacterium tuberculosis responds to chloride and pH as synergistic cues to the immune status of its host cell. PLoS Pathog. 2013;9:e1003282.
18. Bonnett SA, Ollinger J, Chandrasekera S, Florio S, O’Malley T, Files M, Jee JA, Ahn J, Casey A, Ovechkin Y, et al. A target-based whole cell screen to identify potential inhibitors of Mycobacterium tuberculosis signal peptide. ACS Infect Dis. 2016;2:893–902.
19. Thompson JD, Higgins DG, Gibson TJ.CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994;22:4673–80.