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Rapidly maturing red fluorescent protein variants with strongly enhanced brightness in bacteria

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Abstract A rapidly maturing variant of the red fluorescent protein DsRed was optimized for bacterial expression by random mutagenesis. The brightest variant contains six mutations, two of which (S4T and a silent mutation in codon 2) explain most of the fluorescence enhancement. The novel variants are expressed at 9–60-fold higher levels in *Escherichia coli* compared to DsRed.T3, but are not superior fluorophores on a per molecule basis. In contrast to previously available DsRed variants, DsRed.T3_S4T is sufficiently bright to monitor *Salmonella* gene expression in infected animals using flow cytometry. However, no fluorescence enhancement was observed in *Leishmania* or HeLa cells, indicating that these novel variants are specifically useful for bacteria.

Key words: DsRed; Random Mutagenesis; Salmonella; Leishmania; Infection

1. Introduction

The red fluorescent protein DsRed is an attractive reporter for gene expression in living cells [1]. Due to its orange-red fluorescence, DsRed can be easily distinguished from the green fluorescent protein (GFP) and thus permits dual-color applications. Initial technical problems with DsRed such as intracellular aggregation and slow maturation of the chromophore have recently been solved by random and directed mutagenesis [2]. However, bacteria expressing such DsRed variants are still much less fluorescent compared to bacteria expressing analogous GFP constructs and this prevents DsRed applications demanding high signal to noise ratios such as pathogen detection in infected animals.

In this study, we identified nucleotide exchanges in a rapidly maturing DsRed variant that greatly improve its expression level in *Escherichia coli* and *Salmonella enterica* without changing the absorbance and emission spectra. The resulting variant DsRed.T3_S4T can be successfully used together with GFP in dual-color gene expression analysis of *Salmonella* in infected animals.

2. Materials and methods

2.1. Bacterial strains, plasmids, identification and characterization of DsRed variants

E. coli ElectroTen-Blue (Stratagene) and *S. enterica* serovar Typhimurium SL1344 [3] were transformed by electroporation and transformants were cultured at 37°C and 200 rpm in LB medium supplemented with 100 μg ml^{-1} ampicillin and 30 μg ml^{-1} kanamycin (ElectroTen-Blue transformants), or 100 μg ml^{-1} ampicillin and 90 μg ml^{-1} streptomycin (SL1344 transformants), respectively.

The genes encoding the rapidly maturating DsRed.T3 and DsRed.T4 variants [2] were kindly provided by B. Glick, University of Chicago. The dsred.t3 gene was randomly mutagenized by polymerase chain reaction (PCR) amplification under error-prone conditions (25 cycles, 8.8 mM $MgCl_2$, 0.5 mM $MnCl_2$ [4]) using primers t3_up 5′-G*CTCTAGA*TTTAAGAAGGAGATATACATATGGCCTCCTCCGA containing a *Xba*I site (italic), a ribosomal binding site of gene10 of phage T7 (underlined), and the first few codons of DsRed, and t3_down 5′-CCGCCAAAACA*GAAGCTT*CTACAGGAACAGGTGGTG containing a *Hin*dIII site (italic) and the last few DsRed codons (underlined). The amplified fragments were digested with *Xba*I and *Hin*dIII and ligated into pBAD18 downstream of an arabinose-inducible P_{araC} promoter. In this construct, translation is initiated at the DsRed start codon. Transformation of the ligation mixture into *E. coli* yielded 1.5×10^7 independent transformants that were cultivated on plates in the presence of glucose which represses P_{araC}-driven DsRed expression to preserve the full diversity of the library.

Aliquots of the library were induced with 0.2% L-arabinose in M9 minimal medium for 6 h. Some 1300 highly fluorescent bacteria were then sorted using a flow cytometric cell sorter (FacsDiva, Beckton and Dickinson) and recovered on agar plates. Twenty-six clones were individually tested for inducible orange fluorescence and one clone (MS165) with the highest fluorescence intensity was selected for further characterization. Absorption and emission spectra of bacterial sonicates were measured using a spectrophotometer (SpectraMAX 250, Molecular Devices) and a fluorimeter (PC1000, Sentronic) with excitation at 488 nm, respectively. Fluorescence of individual cells was measured by flow cytometry (FacSort, Beckton and Dickinson). DsRed maturation kinetics were determined as described using shortly induced cultures treated with chloramphenicol to block further protein synthesis [2]. The size of DsRed-expressing bacteria was determined using a light microscope (DMR, Leica) equipped with a CCD-camera (DMX 1200, Nikon) and image analysis with Scion Image software (PC-version of NIH image, free download at http://www.scioncorp.com). The results from 100 to 200 cells per strain were used to normalize flow cytometric fluorescence measurements for DsRed-expressing bacteria with differential size distributions. Protein expression was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting with a monoclonal antibody to DsRed (Clonetech). To obtain quantitative data, staining intensities for various numbers of bacteria were compared using Scion image software (see Fig. 1B).

The coding sequence of DsRed.MS165 was determined by double-strand sequencing using primers seq_1 5′-TATCGCAACTCTCTACTGTT and seq_2 5′-CGCCAGGCAAATTCT. To determine the

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individual effects of two amino acid exchanges present in DsRed.MS165, the corresponding mutations were separately introduced into DsRed.T3 by site-directed mutagenesis using primers mut_1 5′-GCTCTAGATTTAAGAAGGAGATATACATATGGCCTCCACCGAGGACGTCATCAAGGAGT and t3_down (see above), or mut_2 5′-GCTCTAGATTTAAGAAGGAGATATACATATGGCCTCCTCCGAGGACGACATCAAGGAGT and t3_down, respectively. In addition, the S4T mutation alone or together with one silent mutation was introduced into the variant DsRed.T4 using primers mut_1 and t3_down (see above), or mut_1silent 5′-GCTCTAGATTTAAGAAGGAGATATACATATGGCATCCACCGAGGACGTCATCAAGGAGT and t3_down. For expression in *Salmonella*, the *dsred.T3_S4T* gene was exchanged for *gfp_ova* in pMW57 carrying the P_{pagC} promoter [5].

2.2. *Construction of* Leishmania mexicana *parasites with chromosomally integrated dsred.T3_S4T*

L. mexicana (strain MNYC/BZ/62/M379) expressing Dsred.T3_S4T was generated as described [6]. Briefly, *dsred.T3_S4T* was cloned into pSSU-int and the resulting construct was integrated into a rDNA locus by homologous recombination.

2.3. *Mice,* Salmonella *and* Leishmania *infection, confocal microscopy, and flow cytometry*

Female 8–12 week old BALB/c mice were obtained from the Bundesamt für gesundheitlichen Verbraucherschutz und Veterinärmedizin, Berlin, and kept under specific-pathogen-free conditions.

Mice were intravenously infected with 10 000 colony-forming units (cfu) of 1:1 mixtures of *Salmonella* expressing GFP or DsRed.T3_S4T, respectively. One day post infection, mice were killed under anesthesia and spleens were prepared. A small piece was embedded in OCT medium and snap-frozen in liquid nitrogen. Cryosections (20 μm) were analyzed using a confocal microscope (DM IRBC, Leica). Stack projections (15 μm) of optical sections (0.2 μm) are shown. The residual spleen was homogenized, treated with 0.1% Triton Tx-100 to liberate intracellular *Salmonella* cells, and fixed with 2% formalin. The suspensions were analyzed with a FACSort flow cytometer (Beckton and Dickinson) using a two-color method that allows to distinguish GFP, DsRed, and autofluorescence based on green/orange emission ratios [5].

For *Leishmania* infection, recombinant *L. mexicana::DsRed.T3_S4T* were grown in selective medium [6] to stationary phase and injected into the hind footpad of BALB/c mice. Three weeks later, mice were killed and tissue samples of lesions and draining lymph nodes were fixed in 2% paraformaldehyde. Cryosections (10 μM) were stained with monoclonal antibodies to LAMP-1 and CD11b, and analyzed by confocal microscopy.

3. Results and discussion

3.1. *Identification of a DsRed variant with enhanced brightness in* E. coli

To generate DsRed variants that are well suited for bacterial expression, we used the rapidly maturing DsRed.T3 variant as a starting point [2]. This variant is efficiently excited by the 488 nm line of standard argon lasers in commercial flow cytometers and can thus be used for high-throughput single-cell analysis. To obtain improved variants, we randomly mutagenized the *dsred.t3* gene by PCR amplification under error-prone conditions. In contrast to previous similar optimization attempts, we used conditions favoring high mutation rates [4]. A high mutation rate can yield a high proportion of non-functional proteins since frequent stop codons result in truncated variants. On the other hand, a high mutation rate allows to screen many exchanges in parallel and can yield superior protein variants compared to several consecutive molecular evolution cycles using conventional low mutation rates [7].

Sorting of a library of 1.5×10^7 independent clones yielded some 1300 clones with improved brightness. Among 26 individually tested clones, clone MS165 had the highest orange-

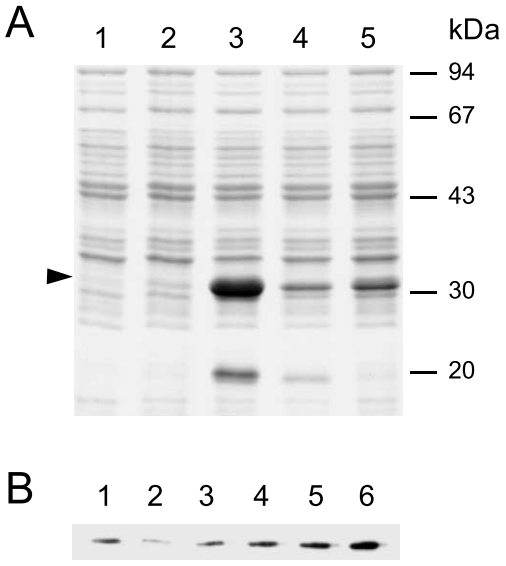

Fig. 1. A: SDS–PAGE analysis of *E. coli* expressing various DsRed.T3 variants (lane 1, pBAD18 control; lane 2, DsRed.T3; lane 3, DsRed.MS165; lane 4, DsRed.T3_S4T; lane 5, DsRed.T3_V7D). The arrowhead indicates the apparent molecular mass of DsRed. B: Quantification of relative expression levels by Western blotting using a monoclonal antibody to DsRed. 10^8 *E. coli* expressing DsRed.T3 were loaded on lane 1, whereas lanes 2–6 contain 8×10^6, 1×10^7, 1.3×10^7, 1.7×10^7, or 2.5×10^7 *E. coli* expressing DsRed.T3_S4T. Densitrometric analysis revealed that 1.08×10^7 *E. coli* expressing DsRed.T3_S4T would give an equivalent staining intensity compared to lane 1 indicating that DsRed.T3_S4T is expressed at 9.3-fold higher levels compared to DsRed.T3. The other variants were analyzed in the same way (see Table 1).

Table 1
Properties of DsRed.T3 variants

DsRed variant	Absorption maximum (nm)	Emission maximum (nm)[a]	Fluorescence intensity in *E. coli*[b]	Expression level[c]	Extinction coefficient (488 nm)[d]	Quantum yield[e]	Maturation time (h)[f]
DsRed.T3	560	589	1.0	1.0	1.0	1.0	2.5 ± 0.5
DsRed.165	560	593	30.1	60	0.49	0.84	2.9 ± 0.5
DsRed.T3_S4T	560	589	10.7	9.3	0.77	0.96	n.d.
DsRed.T3_V7D	560	589	1.7	15.8	0.16	0.53	n.d.

[a]Excitation at 488 nm.
[b]Mean fluorescence of induced bacteria as determined by flow cytometry, the fluorescence intensity of DsRed.T3 was defined as 1.0.
[c]Expression level relative to DsRed.T3 as determined by Western blotting (see Fig. 1B).
[d]Absorbance of lysates at 488 nm normalized for DsRed protein content, the extinction coefficient of DsRed.T3 was defined as 1.0.
[e]Quantum yield (emission at 590 nm, excitation at 488 nm), the quantum yield of DsRed.T3 was defined as 1.0.
[f]Time after induction at which bacteria reach 50% of their maximal fluorescence.

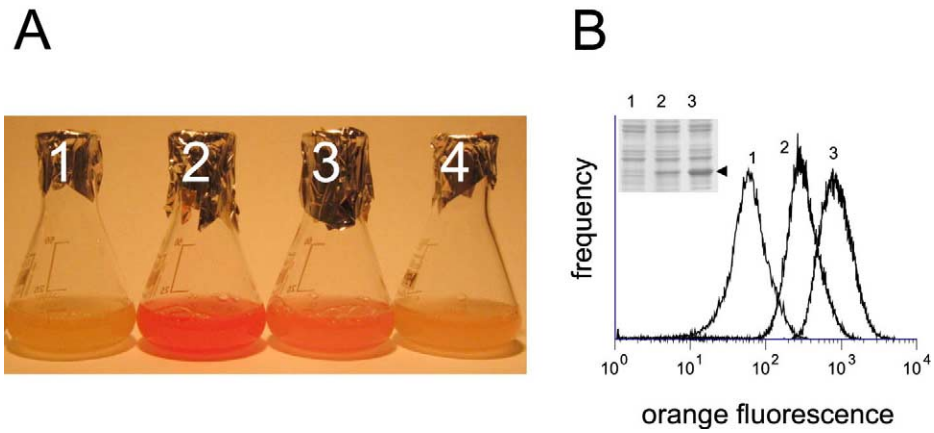

Fig. 2. A: *E. coli* cultures expressing various DsRed.T3 variants (1, DsRed.T3; 2, DsRed.MS165; 3, DsRed.T3_S4T; 4, DsRed.T3_V7D). B: Fluorescence intensity of *E. coli* expressing various DsRed.T4 variants (1, DsRed.T4; 2, DsRed.T4_S4T; 3, DsRed.T4_S4T_sil2) as determined by flow cytometry. The inset shows SDS–PAGE analysis of the same cultures, the arrowhead indicates the apparent molecular mass of DsRed.

red emission intensity (approximately 30-fold enhancement compared to DsRed.T3). The *dsred.ms165* gene was subcloned into another expression vector (pQE30) and again transformed into *E. coli*. Upon induction with IPTG, these cells exhibited again a much stronger fluorescence compared to an analogous DsRed.T3 construct (not shown) suggesting that mutations within the *dsred.ms165* gene instead of unrelated episomal or chromosomal mutations in strain MS165 are responsible for the high fluorescence intensity.

DsRed.MS165 retains the spectral characteristics and the rapid maturation of DsRed.T3 but has a lower extinction coefficient and quantum yield (Table 1) indicating that it is not a superior fluorophore on a per molecule basis. However, SDS–PAGE and Western blotting revealed that it is expressed at dramatically higher levels compared to the original DsRed.T3 variant (Fig. 1). Strains expressing DsRed.T3 or DsRed.MS165 have similar division times under inducing conditions (T3, 2.1 ± 0.2 h; MS165, 2.0 ± 0.2 h) suggesting that this high expression is still well tolerated by expressing *E. coli*. A similar increase of both protein expression and fluorescence intensity has recently been observed for slowly maturing DsRed variants [8].

Micrograph image analysis revealed that after 24 h of induction, cells expressing DsRed.MS165 have a similar diameter but a 2.1-fold greater length compared to the other strains (data not shown) corresponding to an about two-fold difference in cell volume. Such an increase in cell size is commonly observed for *E. coli* expressing very high levels of foreign proteins [9].

3.2. Identification of relevant mutations

To identify mutations that are responsible for enhanced expression, the sequence of DsRed.MS165 was determined and compared to DsRed.T3. DsRed.MS165 contains six mutations (Table 2), two of which are non-synonymous (S4T, V7D). Synonymous and non-synonymous mutations can increase expression if codons with superior translational efficiency are generated. However, comparison of the respective

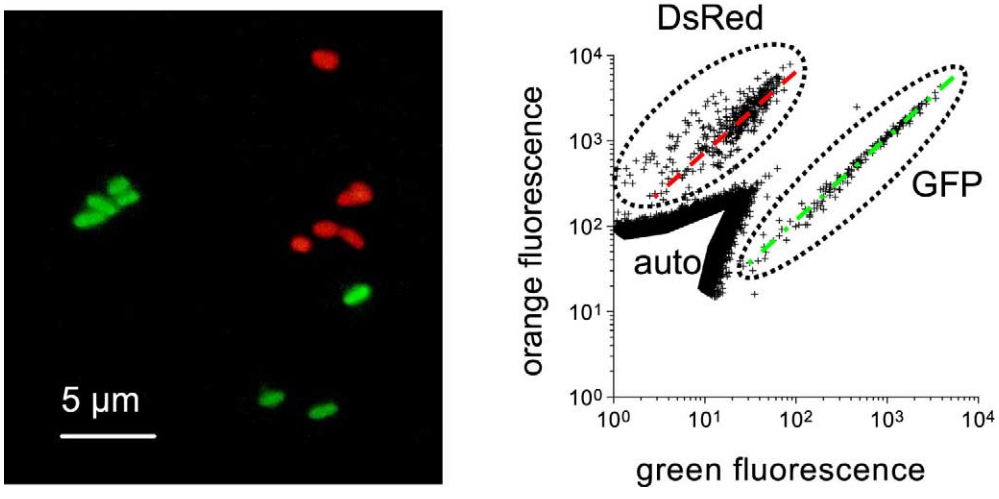

Fig. 3. Simultaneous detection of GFP- and DsRed.T3_S4T-expressing *S. enterica* in infected mouse spleen. Left: Confocal microscopy of a cryosection. Right: Two-color flow cytometry of a detergent-treated spleen homogenate. The dashed lines represent typical spectral characteristics of *Salmonella* in vitro cultures expressing GFP (green) or DsRed.T3_S4T (red). The majority of autofluorescent tissue fragments (auto) was rejected by appropriate gating during data acquisition.

codon frequencies for *E. coli* [10] showed that none of the mutations yields superior codons (data not shown).

Both non-synonymous exchanges are located in the N-terminal region which is far from the DsRed chromophore both in primary and tertiary structure in agreement with their negligible effects on spectral properties (Table 1). The N-terminus of DsRed has already been extensively mutagenized to improve its biochemical properties. In particular, the high positive net charge of the N-terminus of the original DsRed protein has been shown to be responsible for its pronounced aggregation [11,2].

Interestingly, the V7D mutation in DsRed.MS165 further decreases the positive net charge of the N-terminus of DsRed.T3 from +2 to +1 by introducing an additional aspartate residue. In contrast, the S4T mutation involves chemically similar amino acids serine and threonine. Steric effects of the slightly larger threonine are unlikely to affect folding since amino acid residue 4 appears to reside in a structurally flexible part of the DsRed molecule [12].

To determine the individual effect of the two amino acid exchanges, the corresponding mutations were separately introduced into DsRed.T3 by site-directed mutagenesis. Interestingly, only DsRed.T3_S4T but not DsRed.T3_V7D yielded an increased fluorescence (Table 1; Fig. 2A). Similar to DsRed.MS165, DsRed.T3_S4T is an inferior fluorophore compared to the original DsRed.T3 with somewhat lower extinction coefficient and quantum yield, but this is compensated for by an increased expression level (Fig. 1). Surprisingly, DsRed.T3_V7D is also expressed at high levels (Fig. 1), but its very poor extinction coefficient and quantum yield prevent increased bacterial fluorescence (Table 1). Both mutations thus improve protein expression, but only DsRed.T3_S4T retains sufficient fluorophore formation.

DsRed.T3 is efficiently excited by 488 nm light which makes it attractive for flow cytometry. In comparison, DsRed.T4 containing an additional T217A mutation matures even faster and has significantly less absorption at 488 nm and emission around 520 nm [2]. The lower spillover into GFP fluorescence detection channels makes this variant especially attractive for microscopy. When introduced into DsRed.T4, the S4T mutation also enhances expression level and bacterial fluorescence (Fig. 2B). Interestingly, introduction of a silent mutation in codon 2 that is present in MS165 (Table 1) in addition to S4T results in a variant DsRed.T4_S4T_sil2 that is expressed at even higher levels and yields more fluorescence (Fig. 2B). As for the DsRed.T3 variants, both DsRed.T4 variants do not yield superior fluorophores on a per molecule basis (data not shown).

The strong effects of a silent mutation and a single serine to threonine exchange in the N-terminus suggest that instead of differential properties of the corresponding proteins, changes in mRNA folding might contribute to the enhanced expression and this is supported by mRNA folding predictions for our DsRed variants (data not shown). Sequence alterations affecting mRNA folding close to the translational initiation site have previously been shown to have dramatic effects on expression in *E. coli* [13,14]. Interestingly, in the original manuscript describing the DsRed.T3 variant, one enhancing mutation (P-4L) was found in a N-terminal leader sequence linking a hexahistidine tag to the DsRed sequence [2]. We did not include this leader sequence into our variants but it is possible that the (P-4L) mutation might similarly affect expression of the original variant.

Table 2
Mutations in DsRed.MS165

Codon	DsRed.T3	DsRed.MS165
2	gcc (A)	gca (A)
4	tcc (S)	acc (T)
7	gtc (V)	gac (D)
15	aag (K)	aaa (K)
188	cag (Q)	caa (Q)
215	gag (E)	gaa (E)

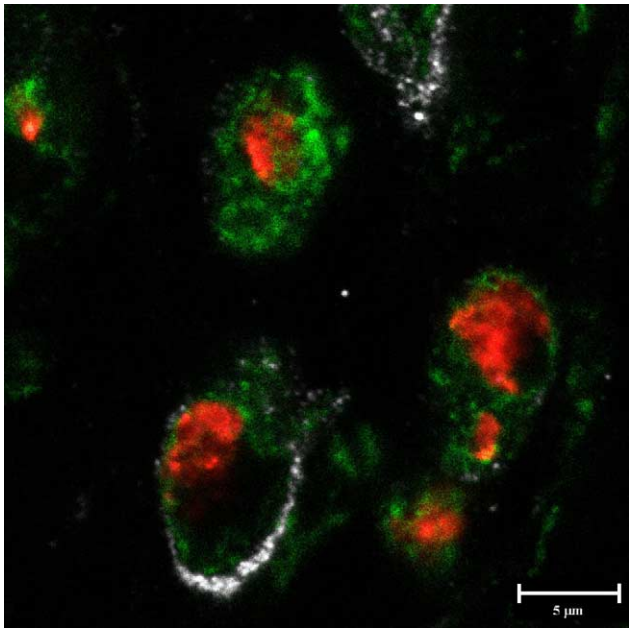

Fig. 4. Confocal microscopy of DsRed.T3_S4T expressing *L. mexicana* in murine footpad lesions. The parasites reside in LAMP-1 (stained green) containing vacuoles of $CD11b^-$ or $CD11b^+$ (stained white) host cells.

3.3. *Application for* Salmonella *gene expression analysis during infection*

Our previous attempts to use the original DsRed or DsRed.T3 as quantitative reporters for *Salmonella* gene expression during infection were all unsuccessful (data not shown). Even when expressed from strong promoters, the DsRed fluorescence was always too weak for flow cytometric detection against the background of autofluorescent tissue fragments. To determine if the new bright DsRed variants solve this problem, *S. enterica* serovar Typhimurium was transformed with a plasmid on which the strong P_{pagC} promoter [15] drives DsRed.T3_S4T expression. Mice were infected with a mixture of *Salmonella* strains expressing either GFP or DsRed.T3_S4T. One day later, mice were killed and spleens were prepared. Fluorescence microscopy of unstained spleen cryosections showed the presence of brightly red and green fluorescing particles with typical *Salmonella* morphology (Fig. 3, left panel). We have recently shown by immunohistochemistry that the green particles indeed represent GFP-expressing *Salmonella* [16]. Both red and green particles are absent in control mice infected with *Salmonella* not expressing fluorescent proteins (not shown).

Two-color flow cytometry of detergent-treated spleen homogenates revealed two particle populations with spectral characteristics typical for GFP- and DsRed-expressing bacteria, respectively (Fig. 3, right panel). We have recently shown that the GFP-like particles indeed represent GFP-expressing *Salmonella* [5]. Both green and red particles are absent in infected control mice, and the number of particles in a sample with defined volume as measured by flow cytometry correlates well with the number of cfu as determined by plating (data not shown). These results demonstrate that DsRed.T3_S4T can be used as an easily distinguishable fluorescence marker together with GFP to localize and quantify gene expression of *Salmonella* in infected animals.

3.4. Eukaryotic expression

To evaluate DsRed.T3_S4T as a reporter for eukaryotic pathogens, we expressed both the original DsRed.T3 and DsRed.T3_S4T in the protozoan parasite *L. mexicana*. Flow cytometry revealed that *L. mexicana::DsRed.T3 and L. mexicana::DsRed.T3_S4T* display a similar fluorescence intensity when excited at 488 nm (mean orange fluorescence of individual clones: 485 ± 101 vs. 469 ± 124 arbitrary units) suggesting that the S4T mutation has no effect on DsRed expression in *Leishmania*. We obtained similar data for DsRed expression from the P_{CMV} promoter in HeLa cells (data not shown) indicating that the beneficial effect of the S4T mutation is restricted to bacteria. This is compatible with the hypothesis that changes in mRNA folding predominantly affecting prokaryotic translation might influence DsRed expression in *E. coli* and *Salmonella* (see above). Strong differences between prokaryotic and eukaryotic expression have been previously observed for variants DsRed1 and DsRed2 [2].

DsRed.T3-S4T-expressing *Leishmania* are fully virulent in the murine infection model as demonstrated by footpad lesion formation within 3 weeks of infection (not shown). During this infection period, the recombinant *Leishmania* retain their bright red fluorescence. Three-color microscopic analysis revealed that they reside in LAMP-1 containing vacuoles of $CD11b^+$ and $CD11b^-$ host cells in the lesion and the draining lymph node (Fig. 4) in agreement with previous data [17]. These data show that DsRed.T3-S4T can be used as a reporter for *Leishmania* in infected mice, but the original DsRed.T3 variant is probably equally suitable.

3.5. Conclusion

In conclusion, we describe two nucleotide exchanges in rapidly maturing DsRed variants that improve their expression in bacteria but not eukaryotic cells. These variants might be useful for various infection models and other applications.

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References

[1] Matz, M.V., Fradkov, A.F., Labas, Y.A., Savitsky, A.P., Zaraisky, A.G., Markelov, M.L. and Lukyanov, S.A. (1999) Nat. Biotechnol. 17, 969–973.
[2] Bevis, B.J. and Glick, B.S. (2002) Nat. Biotechnol. 20, 83–87.
[3] Hoiseth, S.K. and Stocker, B.A. (1981) Nature 291, 238–239.
[4] Fromant, M., Blanquet, S. and Plateau, P. (1995) Anal. Biochem. 224, 347–353.
[5] Bumann, D. (2002) Mol. Microbiol. 43, 1269–1283.
[6] Misslitz, A., Mottram, J.C., Overath, P. and Aebischer, T. (2000) Mol. Biochem. Parasitol. 107, 251–261.
[7] Daugherty, P.S., Chen, G., Iverson, B.L. and Georgiou, G. (2000) Proc. Natl. Acad. Sci. USA 97, 2029–2034.
[8] Knop, M., Barr, F., Riedel, C.G., Heckel, T. and Reichel, C. (2002) Biotechniques 33, 592–602.
[9] Nakamura, Y., Gojobori, T. and Ikemura, T. (2000) Nucleic Acids Res. 28, 292.
[10] Yanushevich, Y.G., Staroverov, D.B., Savitsky, A.P., Fradkov, A.F., Gurskaya, N.G., Bulina, M.E., Lukyanov, K.A. and Lukyanov, S.A. (2002) FEBS Lett. 511, 11–14.
[11] Yarbrough, D., Wachter, R.M., Kallio, K., Matz, M.V. and Remington, S.J. (2001) Proc. Natl. Acad. Sci. USA 98, 462–467.
[12] Soriano, E., Borth, N., Katinger, H. and Mattanovich, D. (2002) Biotechnol. Bioeng. 80, 93–99.
[13] Devlin, P.E., Drummond, R.J., Toy, P., Mark, D.F., Watt, K.W. and Devlin, J.J. (1988) Gene 65, 13–22.
[14] Baneyx, F. (1999) Curr. Opin. Biotechnol. 10, 411–421.
[15] Bumann, D. (2001) Infect. Immun. 69, 7493–7500.
[16] Bumann, D. (2001) Infect. Immun. 69, 4618–4626.
[17] Antoine, J.C., Prina, E., Lang, T. and Courret, N. (1998) Trends Microbiol. 6, 392–401.