Optimization of GFP levels for analyzing *Salmonella* gene expression during an infection

Meike Wendland, Dirk Bumann*

Max-Planck-Institute for Infection Biology, Department of Molecular Biology, Schumannstr. 21/22, D-10117 Berlin, Germany

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Abstract Green fluorescent protein (GFP) is an attractive reporter for *Salmonella* gene expression analysis but might interfere with virulence when expressed at high levels. To identify suitable GFP levels, we constructed a series of *Salmonella* strains expressing different amounts of GFP and measured their fluorescence and colonization levels in infected mice. The results show that GFP concentrations in the range of 7000–200 000 molecules per *Salmonella* cell are detectable in ex vivo samples using flow cytometry, and cause no major *Salmonella* virulence defect. Appropriate GFP levels can be obtained with weak promoters and stable GFP, or strong promoters and destabilized GFP. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Green fluorescent protein; Flow cytometry; *Salmonella enterica*

1. Introduction

The green fluorescent protein (GFP) is widely used as a quantitative reporter for gene expression in diverse organisms. One interesting application is the analysis of pathogen adaptation to host environments during an infection [1]. However, GFP may pose a metabolic burden on the recombinant pathogen and can even be toxic when expressed at high levels [2]. An impaired pathogen may in turn alter its gene expression pattern so that the analysis technique would affect the variable of interest. For minimal interference, GFP levels should thus be as low as possible. On the other hand, sufficient GFP is needed for fluorescence detection with an acceptable signal-to-background ratio. We have recently shown that the spectral separation of GFP emission and tissue autofluorescence by two-color flow cytometry significantly improves the detection of GFP-expressing *Salmonella* cells in infected mouse tissues [3,4], but the range of in vivo GFP levels that are both tolerable and detectable is still unknown for *Salmonella* and most other pathogens in spite of the numerous infection studies using GFP constructs.

The GFP concentration depends on its expression rate, its degradation rate, and the growth rate of the expressing cell [5]. In this study, we varied both the GFP expression and degradation rates in a pathogenic *Salmonella enterica* strain using different promoters and well-characterized GFP lifetime variants, and analyzed the effects on *Salmonella* growth and fluorescence detection in infected mice.

2. Materials and methods

2.1. Bacterial strains and plasmids

Salmonella enterica serovar *typhimurium* SL1344 is a streptomycin-resistant wild-type isolate [6]. For cloning *Escherichia coli* ElectroTen-Blue (Stratagene) was used. Both strains 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 90 μg ml^{-1} streptomycin (SL1344 transformants), or 100 μg ml^{-1} ampicillin and 30 μg ml^{-1} kanamycin (ElectroTen-Blue transformants), respectively.

GFP variants with different lifetimes in Gram-negative bacteria (GFP.mut3, lifetime >24 h; GFP[ASV], lifetime about 110 min; and GFP[LVA], lifetime about 40 min) [7] were obtained from plasmids pJBA27, pJBA113, and pJBA111 generously provided by Dr. S. Molin, Technical University Lyngby, Denmark. These GFP variants were expressed from two promoters with differential activity levels in vivo (P_{pagC}, P_{spvA}; [8]) by exchanging *Xba*I/*Hin*dIII fragments of plasmids pJBA27, pJBA113, and pJBA111 carrying the *gfp* variants, for the GFP_OVA fusion genes in pP_{pagC}GFP_OVA or pP_{spvA}GFP_OVA [8] yielding plasmids pP_{pagC}GFP.mut3, pP_{pagC}GFP[ASV], and pP_{pagC}GFP[LVA], and plasmids pP_{spvA}GFP.mut3, pP_{spvA}GFP[ASV], and pP_{spvA}GFP[LVA], respectively. The chromosomal P_{pagC} promoter drives expression of the putative outer membrane protein PagC that is required for *Salmonella* virulence in the mouse model [9]. P_{spvA} on the *Salmonella* virulence plasmid drives expression of the *spvABCD* operon. SpvB can ADP-ribosylate actin thereby inhibiting its polymerization in host cells [10]. Both SpvB and SpvC are required for systemic virulence in the mouse model [11]. For in vitro studies, P_{pagC} was induced in M9 minimal medium containing 10 μM $MgCl_2$, while P_{spvA} was induced during overnight growth on agar plates.

2.2. Mice, infection, and flow cytometry

Female 8–12 weeks old BALB/c mice were obtained from the Bundesamt für gesundheitlichen Verbraucherschutz und Veterinärmedizin, Berlin, and kept under specific-pathogen-free conditions. For oral infection, *Salmonella* cultures were grown to the late logarithmic phase (OD_{600} 1.5; ca. 3×10^9 CFU ml^{-1}) and 100 μl were orogastrically administered with a round-tip stainless-steel needle. At 4 days post infection, mice were sacrificed under anesthesia and the Peyer's patches were prepared. After homogenization between the sanded ends of two microscope slides, the suspensions were treated with 0.1% Triton X-100 to liberate intracellular *Salmonella* cells. Serial dilutions were plated on streptomycin-containing LB medium with or without ampicillin to determine colonization levels and plasmid stability.

For flow cytometric analysis of *Salmonella* in vivo GFP expression, Triton-treated homogenates were fixed with 2% formalin and analyzed with a FACSort flow cytometer (Becton and Dickinson). Linearity and constant amplification of the flow cytometer were validated with intensity calibration beads (Molecular Probes). For suppression of background autofluorescence from tissue fragments, both the green and orange emission channels (FL-1, 515–545 nm; FL-2, 563–607 nm) were recorded. GFP has an almost 10-fold lower orange/green emission ratio compared to tissue autofluorescence which permits the spec-

*Corresponding author. Fax: (49)-30-28460 401.
E-mail address: bumann@mpiib-berlin.mpg.de (D. Bumann).

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tral separation of these two populations with two-color flow cytometry [3,4]. To convert fluorescence measurements to the number of GFP molecules per *Salmonella* cell, in vitro cultures of GFP-expressing *Salmonella* cells were analyzed in parallel by flow cytometry and SDS–PAGE. Coomassie brilliant blue-stained gels were scanned with a HP ScanJet 6300C and analyzed using Scion Image software (PC-version of NIH image, free download at http://www.scioncorp.com). After correction for background staining in SL1344 samples, *Salmonella* GFP expression was quantified by comparison with standards containing 20, 30, 40, and 50 ng of purified GFP (Clonetech) and converted into number of GFP molecules per *Salmonella* cell which was then used to calibrate the fluorescence measurements.

3. Results and discussion

To determine what GFP levels are compatible with full *Salmonella* virulence in a mouse infection model, we constructed a series of *Salmonella* strains that contain different GFP concentrations in vivo. In general, the GFP concentration depends on the expression rate (including transcription, translation, and maturation), the degradation rate, and the growth rate which reflects the dilution due to cell division [5]. To modulate the GFP expression rate, we used the strong P_{pagC} promoter or the weak P_{spvA} promoter [8]. To modulate the GFP degradation rate, we compared stable GFP.mut3 [12] to two well-characterized destabilized GFP variants (GFP[ASV] and GFP[LVA]) that contain C-terminal recognition sequences for the tail-specific protease [7].

3.1. In vitro characterization

During in vitro growth under inducing conditions, all constructs containing P_{pagC} upstream of GFP are highly fluorescent with intensities in the order GFP.mut3 ≈ GFP[ASV] > GFP[LVA] (Fig. 1A) in agreement with previous data for these GFP variants in *E. coli* [5,7]. All three recombinant *Salmonella* strains have similar in vitro division times (pP_{pagC}GFP.mut3, 37 ± 5 min; pP_{pagC}GFP[ASV], 35 ± 5 min; pP_{pagC}GFP[LVA], 38 ± 5 min) compared to wild-type SL1344 (33 ± 5 min) suggesting that at the tested concentrations, GFP expression is well tolerated in vitro. In contrast to the P_{pagC} constructs, only small subpopulations of *Salmonella* cells in which P_{spvA} drives GFP expression exhibit detectable fluorescence suggesting that our in vitro induction conditions for this promoter (stationary growth phase on agar plates) are suboptimal. Again, the fluorescence intensity of expressing cells is in the order of GFP.mut3 > GFP[ASV] > GFP[LVA] (data not shown).

To determine if the intrinsic brightness of the various GFP variants is comparable, SL1344(pP_{pagC}GFP.mut3), SL1344(pP_{pagC}GFP[ASV]), and SL1344(pP_{pagC}GFP[LVA]) cultures were analyzed by SDS–PAGE (Fig. 1B). Samples that were normalized for equal fluorescence intensity as determined by flow cytometry contained similar amounts of GFP suggesting that the different GFP variants have a comparable fluorescence quantum yield. Defined amounts of purified GFP were used as standards to estimate that 10^6 SL1344(pP_{pagC}GFP.mut3) cells contain 20 ± 5 ng GFP (average of two independent measurements). Based on the molecular mass of GFP of 26 900 g mol^{-1} and the Avogadro number of 6.023×10^{23} molecules mol^{-1}, this corresponds to 0.74 ± 0.2 fmol or $4.4\pm1\times10^{11}$ molecules in 10^6 bacteria which is equivalent to 440 000 ± 100 000 copies per cell. We combined this result with flow cytometric data of the same cultures to calibrate the GFP fluorescence units. Some GFP variants form non-fluorescent inclusion bodies when expressed to high levels which would result in an underestimation of the

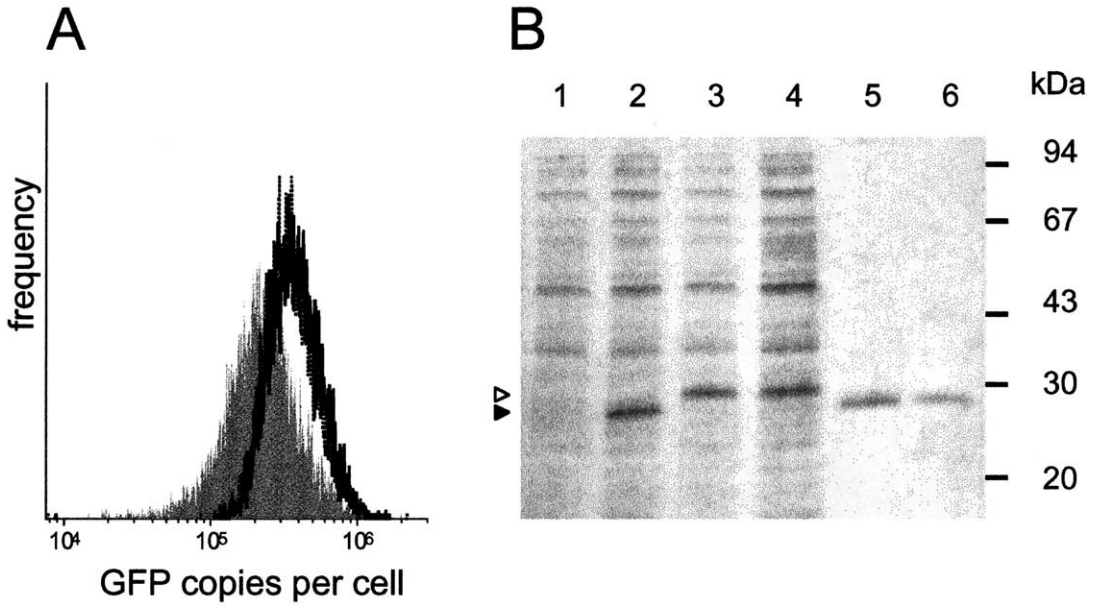

Fig. 1. In vitro characterization of GFP-expressing *Salmonella* strains. A: GFP expression in induced in vitro cultures of SL1344(pP_{pagC}GFP.mut3) (thick line), SL1344(pP_{pagC}GFP[ASV]) (dotted line), and SL1344(pP_{pagC}GFP[LVA]) (shaded). The fluorescence intensity is given in GFP equivalents (see text for explanation). Plasmid-free SL1344 cells have no detectable fluorescence in the range shown. B: SDS–PAGE of the same cultures as shown in (A). To normalize for equal fluorescence intensities, different cell numbers were applied (lane 2: SL1344(pP_{pagC}GFP.mut3), 2.4×10^6 CFU; lane 3: SL1344(pP_{pagC}GFP[ASV]), 2.7×10^6 CFU; lane 4: SL1344(pP_{pag}GFP[LVA]), 4.1×10^6 CFU). The solid arrowhead indicates the apparent molecular weight of GFP.mut3, the open arrowhead indicates the apparent molecular weights of GFP[ASV] and GFP[LVA] that are larger due to the fused C-terminal recognition sequences of the tail-specific protease [7]. For comparison, 2.4×10^6 CFU plasmid-free SL1344 (lane 1), and purified GFP (lane 5: 50 ng, lane 6: 25 ng) were also applied.

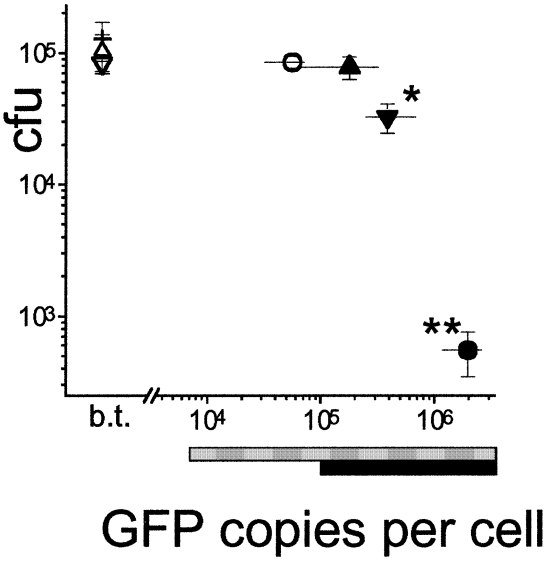

Fig. 2. GFP content and colonization levels of various *Salmonella* strains in murine Peyer's patches at 4 days post infection: P_{pagC} constructs (solid), P_{spvA} constructs (open), GFP.mut3 (circle), GFP[ASV] (down triangle), GFP[LVA] (up triangle). The cross represents plasmid-free SL1344. For each construct, the median fluorescence intensity and 25 and 75 percentiles were plotted against the mean CFU and the standard error of the mean of six mice from two independent experiments. Statistical differences to SL1344 colonization levels were tested using the *t*-test ($*P < 0.05$; $**P < 0.01$). The detection range for GFP-expression in infected mouse tissues using one-color (black bar) or two-color flow cytometry (gray bar) are also shown; b.t. below detection threshold.

actual GFP concentrations by fluorescence measurements. In our *Salmonella* in vitro cultures, no insoluble GFP.mut3 could be detected SDS–PAGE of fractionated lysates even at expression levels exceeding 1 000 000 copies per cell (our unpublished data) in agreement with previous data for this GFP variant [12]. For most constructs, in vivo and in vitro fluorescence levels are similar (see below) suggesting that in vivo also little if any GFP inclusion bodies are formed.

3.2. In vivo characterization

To characterize the in vivo properties of the various constructs, BALB/c mice were orally infected with either strain and 4 days later, Peyer's patches were prepared, homogenized, and analyzed using two-color flow cytometry. All three constructs containing the P_{pagC} promoter have detectable in vivo levels of GFP in the order GFP.mut3 $\gg$ GFP[ASV] $>$ GFP-[LVA] (Fig. 2). The in vivo levels of GFP[ASV] and GFP[LVA] are similar to induced in vitro levels (see Fig. 1A), while the GFP.mut3 construct contains in vivo some five-fold more GFP compared to in vitro conditions. The only P_{spvA} construct with detectable in vivo GFP levels contains GFP.mut3. This construct requires two-color flow cytometry to distinguish GFP-expressing bacteria from autofluorescent tissue fragments (see Section 2) while all P_{pagC} constructs are bright enough for conventional one-color measurements (Fig. 2).

High in vivo GFP levels could pose a metabolic burden on *Salmonella* cells that may interfere with their ability to adapt to the hostile host environments. This could result in plasmid instability and/or lower colonization levels both of which are undesirable for gene expression studies. To test this hypothesis, tissue homogenates from infected mice were replica plated on media with or without ampicillin. While all constructs stably maintain their plasmids ($>90\%$ in all cases), SL1344(pP_{pagC}GFP.mut3) had colonization levels at 4 days post infection that are several orders of magnitude lower than those of plasmid-free SL1344 (Fig. 2) suggesting that the rather high GFP levels in these *Salmonella* cells indeed impair their in vivo growth. While SL1344(pP_{pagC}GFP[ASV]) is also significantly impaired, SL1344(pP_{pagC}GFP[LVA]) reached colonization levels comparable to those of plasmid-free SL1344. As the three P_{pagC} constructs differ solely in the GFP degradation rate, this result suggests that *Salmonella* virulence is impaired by a high steady state GFP concentration but less affected by strong *gfp* transcription and translation, or plasmid maintenance.

The large differences in both colonization levels and in vivo GFP concentrations between SL1344(pP_{PagC}GFP.mut3) and SL1344(pP_{pagC}GFP[ASV]) are surprising based on their very similar in vitro behavior (Fig. 1). Interestingly, we made similar observations when we compared stable GFP.mut2 [12] with its degradable variant GFP_OVA [8] expressed from the identical promoter. Both variants are similarly abundant in exponential *Salmonella* in vitro cultures but in vivo, GFP.mut2 levels are up to 20-fold higher compared to GFP_OVA levels (our unpublished data). During fast in vitro growth (division times around 35 min, see above), GFP dilution due to cell division may be the dominating mechanism of GFP concentration loss while GFP degradation may be less important [5]. Hence, subtle differences in the degradation rates of stable GFP.mut3 vs. slowly degradable GFP[ASV] (or stable GFP.mut2 vs. degradable GFP_OVA) might not translate into a large GFP concentration difference. In contrast, *Salmonella* growth in vivo is much slower with division times in the range of 170–300 min [13] possibly resulting in more obvious effects of the different lifetimes of GFP.mut3 and GFP[ASV] [5]. The high GFP concentration in SL1344(pP_{PagC}GFP.mut3) apparently results in an even slower in vivo growth, which in turn further increases the GFP concentration, as GFP is less diluted by cell division.

All tested P_{spvA} constructs including SL1344-(pP_{spvA}GFP.mut3) colonize well (Fig. 2) indicating that stable GFP.mut3 can be successfully used when expressed from a weak promoter instead of the strong P_{pagC}. This further supports that the GFP concentration is critical for virulence while unrelated intrinsic properties of the various GFP variants seem to be less relevant.

3.3. Conclusion

GFP is an attractive reporter for gene expression of pathogens in infected hosts. However, it has been unclear if GFP levels that are high enough for detection are compatible with an unimpaired virulence of the pathogen. Here we show that GFP levels below ca. 200 000 molecules per *Salmonella* cell are well tolerated in vivo. Such levels are high enough for conventional one-color detection even in infected tissue samples with high background autofluorescence (Fig. 2). For lower expression levels, background suppression by two-color flow cytometry is preferable as it decreases the detection threshold from about 100 000 to about 7000 molecules per cell. This value is still rather high compared to *Salmonella* in vitro cultures in which GFP levels down to 500 molecules per cell can be detected (our unpublished data).

To obtain in vivo GFP levels in the suitable range between 7000 and 200 000 molecules per cell with weak promoters, one may use stable GFP variants expressed from plasmids with medium copy number such as pP_{spvA}GFP.mut3. High copy number plasmids would yield even more GFP but such plasmids interfere with *Salmonella* virulence [14]. On the other hand, strong promoters are best studied with unstable GFP variants such as GFP[LVA]. As an additional advantage, the short lifetimes of unstable variants (minutes to hours instead of >1 day for GFP.mut3 [5,7]) allow to resolve downregulation that is difficult to detect with stable GFP. Alternative approaches to decrease GFP concentration such as unfavorable ribosomal binding sites, or chromosomal integration of the expression cassette to lower the gene copy number are also suitable for experiments involving strong promoters.

In summary, the combination of GFP lifetime variants with two-color flow cytometry allows to characterize *Salmonella* promoters with a wide range of activities during infection.

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