APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 2003, p. 7480–7491 Vol. 69, No. 12
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.12.7480–7491.2003

Single-Copy Green Fluorescent Protein Gene Fusions Allow Accurate Measurement of *Salmonella* Gene Expression In Vitro and during Infection of Mammalian Cells

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Received 27 June 2003/Accepted 18 September 2003

We developed a reliable and flexible green fluorescent protein (GFP)-based system for measuring gene expression in individual bacterial cells. Until now, most systems have relied upon plasmid-borne *gfp* gene fusions, risking problems associated with plasmid instability. We show that a recently developed GFP variant, GFP$^+$, is suitable for assessing bacterial gene expression. Various *gfp*$^+$ transcriptional fusions were constructed and integrated as single copies into the chromosome of *Salmonella enterica* serovar Typhimurium. A comparison of the expression levels of *proU-lacZ* and *proU-gfp*$^+$ fusions showed that GFP$^+$ reported *proU* activity in individual *Salmonella* cells as accurately as β-galactosidase reported activity for entire populations. The single-copy *gfp*$^+$ fusions were ideal for monitoring up- and downregulation of *Salmonella* virulence genes. We discovered that in vitro induction of the *SPI1* gene *prgH* occurs only in a portion of the population and that the proportion varies with the growth phase. We determined the level of expression of the SPI2 gene *ssaG* in bacteria released from murine macrophages. Our results demonstrate for the first time that single-copy GFP$^+$ fusions reliably report gene expression in simple and complex environments. This approach promises to allow accurate measurement of gene expression in individual bacteria during animal infection.

The increasing incidence of infectious diseases is driving renewed efforts to understand the interactions between bacterial pathogens and their hosts at a new level. This has prompted research to study the transcriptional response of bacteria during infection. The past decade has seen the development of a number of techniques for identifying in vivo-induced bacterial genes and for monitoring gene induction in complex environments. Genetic tools for visualization of gene expression in situ have also been devised. Fluorescent reporters have been developed for studying bacterial infection from both the host point of view and the bacterial point of view (44, 57, 60). The green fluorescent protein (GFP) from *Aequoria victoria* is an excellent tool for monitoring gene expression, because it is naturally fluorescent without any exogenous cofactor or substrate (9). Variants of GFP that have different spectral characteristics, folding properties, levels of chromophore brightness, or half-lives have broadened the spectrum of possible applications for GFP (4). Measurement of fluorescence by flow cytometry has revolutionized the use of fluorescent reporter genes in bacteria (27) and now allows observation of gene expression at the level of individual bacterial cells. Plasmid-borne *gfp* fusions have already been used to monitor gene expression in *Salmonella* strains (6, 61), as well as in other gram-negative or gram-positive bacteria, including *Escherichia coli* (42), *Serratia liquefaciens* (2), *Erwinia herbicola* (37), *Staphylococcus aureus* (45), *Listeria monocytogenes* (64), and *Streptococcus gordonii* (24). Such fusions have proven to be useful for identifying bacterial genes induced during infection of eukaryotic cells or animal hosts and have allowed some assessment of bacterial gene expression levels in such complex environments. However, the variation in plasmid copy number that can occur in individual bacterial cells makes plasmid-borne gene fusions unsuitable for measuring differences in gene expression between single bacteria (34, 35). Furthermore, plasmid-borne GFP fusions are useful only for reporting the induction of genes; any reduction in fluorescence may simply reflect plasmid loss, because it is not possible to guarantee that plasmids are maintained in every bacterial cell in plant or animal hosts. Indeed, high expression levels lead to accumulation of toxic levels of GFP in bacterial cells, which results in dramatic plasmid loss (63; I. Hautefort, J. M. Sidebotham, and J. C. D. Hinton, unpublished data). Hopkins et al. showed that a pBR322-derived plasmid-borne *gfp* fusion was unstable in wild-type *Salmonella enterica* serovar Typhimurium strain 14028 during infection of BALB/c mice, while it remained stable in a PhoP^c mutant (29). More recently, it was reported that a plasmid-borne *tac-gfp* fusion was not stable in a wild-type *Salmonella* strain during murine infection (6). This study showed that the level of plasmid loss was related to promoter strength, meaning that the levels of expression of different promoters cannot be compared by using plasmid-borne gene fusions.

Our interest in determining the diversity of gene expression within bacterial populations in the host prompted us to develop an improved approach for construction of *gfp* transcriptional fusions that could be used reliably during infection. We reasoned that integration of single-copy gene fusions onto the bacterial chromosome was the best way to ensure genetic stability. Single-copy *gfp* gene fusions have been used to monitor the activity of the *marRAB* promoter in large populations of *Salmonella* serovar Typhimurium (46). This approach involves inactivation of the gene of interest, making it unsuitable for studying virulence genes during infection. Recently, the activity

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TABLE 1. Strains and plasmids used

Strain or plasmid	Genotype	Reference
E. coli DH5α	*supE44* Δ*lacU169* (ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	22
S. enterica serovar Typhimurium strains		
LT2	Wild-type LT2A	1
SL1344	Wild type	28[a]
CH946	LT2, *proU1702*::Mud1-8, Amp^{rb}	7
JH3008	SL1344, promoterless *gfp*$^+$, Cm^{rc}	This study
JH3009	SL1344, ϕ(*ssaG′-gfp*$^+$)*1*, Cm^{rc}	This study
JH3010	SL1344, ϕ(*prgH′-gfp*$^+$)*1*, Cm^{rc}	This study
JH3016	SL1344, ϕ(*rpsM′-gfp*$^+$)*1*, Cm^{rc}	This study
JH3017	LT2, ϕ(*proU′-gfp*$^+$)*1*, Cm^{rb}	This study
JH3049	LT2, promoterless *gfp*$^+$, Cm^{rc}	This study
Plasmids		
pWH1012gfp$^+$	pBR322 derivative	51
pKD46	pBAD18 derivative	13
pKD4	pANTSγ derivative (Km^r)	25
pFPV25	pBR322 derivative, ColE1 replicon	61
pFPV25.1	pFPV25, ϕ*rpsM′-gfpmut3*	61
pZEP01	pBR322 derivative (*mob*$^+$/*bla*$^+$), ColE1 replicon	This study
pZEP02	ϕ*rpsM′-gfp*$^+$, pZEP01 derivative	This study
pZEP06	pZEP01 derivative (Cm^r)	This study
pZEP07	pZEP06 derivative, t0T1 (Cm^r)	This study
pZEP08	pZEP07 derivative, (Cm^r Km^r)	This study
pZEP09	ϕ*ssaG′-gfp*$^+$, pZEP08 derivative (Cm^r Km^s)	This study
pZEP10	ϕ*prgH′-gfp*$^+$, pZEP08 derivative (Cm^r Km^s)	This study
pZEP16	ϕ*rpsM′-gfp*$^+$, pZEP08 derivative (Cm^r Km^s)	This study

[a] *Salmonella* strain SL1344 was derived from strain 4/74, which was isolated from a calf bowel.
[b] *proU*::Mud1-8 and the *proU-gfp*$^+$ fusion are both inserted at position 2,956,849 in the LT2 genome (GenBank accession no. AE006468).
[c] The *gfp* gene fusions are inserted at the *putPA* locus at positions 1,210,040 to 1,211,657 in the LT2 genome. ϕ indicates a transcriptional gene fusion.

of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *spac* hybrid promoter (65) was successfully monitored by using a single copy of *gfpmut2* as a reporter in *Bacillus subtilis* (41). We performed preliminary experiments involving expression of single-copy *gfpmut1* fusions in *Salmonella*; and we discovered that the GFPmut1 variant, which is even brighter than GFPmut2 (11), was only fluorescent enough to report particularly high levels of promoter activity in *Salmonella* during infection (Hautefort et al., unpublished). We overcame this problem by using the GFP$^+$ variant (51), which carries the GFP_{uv} mutations F99S, M153T, and V163A (12) along with the EGFP mutations F64L and S65T (11), resulting in better folding of the protein coupled with enhanced brightness. In this study we describe the use of GFP$^+$-based single-copy gene fusions to measure *Salmonella* serovar Typhimurium gene induction in vitro and during infection of mammalian cells.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Bacterial strains used in this study are listed in Table 1. The *Escherichia coli* DH5α strain was used for gene cloning. All *Salmonella* serovar Typhimurium strains used in this study were derived from wild-type strain LT2 or SL1344.

E. coli and *Salmonella* strains were grown in Luria-Bertani (LB) medium (48) at 37°C unless stated otherwise. Cultures were shaken at 250 rpm. Chloramphenicol, ampicillin, and kanamycin were used at final concentrations of 12, 100, and 50 μg ml^{-1}, respectively. For in vitro induction of *proU* expression, *Salmonella* serovar Typhimurium strains CH946, JH3017, and JH3049 were grown at 30°C in LB medium which lacked NaCl (LO medium). This medium was supplemented with 10 mM glucose (7) and sometimes with 0.06, 0.16, or 0.3 M NaCl. In vitro induction of the *ssaG-gfp*$^+$ fusion involved growth of *Salmonella* serovar Typhimurium strains JH3008 and JH3009 in minimum medium at pH 5.8 (MM5.8) (30). MM5.8 is a low-salt, acidic medium based on medium N (40) and contains 38 mM glycerol, 0.1% Casamino Acids, and 100 mM BisTris/Tris-HCl (catalog no. B-6032; Sigma, St. Louis, Mo.) (pH 5.8). Induction of *prgH-gfp*$^+$ in *Salmonella* serovar Typhimurium strain JH3010 was achieved by static growth in either LO medium or LO medium containing 0.3 M NaCl in 30-ml bottles filled to the top with medium to generate microaerophilic conditions.

Recombinant DNA techniques. Plasmid and chromosomal DNA purification was performed by using protocols recommended by the suppliers (Sigma; Qiagen, Hilden, Germany). DNA was digested with restriction endonucleases or ligated with T4 DNA ligase under standard conditions recommended by the manufacturers (New England Biolabs, Beverly, Mass.; Roche, Basel, Switzerland; Promega, Madison, Wis.). Preparation of electrocompetent *E. coli* and *Salmonella* serovar Typhimurium cells and DNA transformation were performed as previously described (15).

Oligonucleotides and PCR. All of the oligonucleotides used in this study are listed in Table 2 and were purchased from Sigma Genosys and MWG AG Biotech (Ebersberg, Germany). PCR amplification was performed in 96-well microtiter plates (MWG AG Biotech) by using a Primus HT thermocycler according to the recommendations of the manufacturer (MWG AG Biotech). For preparative PCR amplification, the *Pfu*Turbo (Stratagene, La Jolla, Calif.) and BioXAct (Bioline, Canton, Mass.) proofreading polymerases were used. PCR products were gel purified by using a Qiagen gel purification kit. PCR screening reactions were performed by using HotStarTaq polymerase (Qiagen).

Plasmids. All of the plasmids used in this study are listed in Table 1. Plasmids pZEP01 and pZEP02 were constructed by PCR as follows. PCR amplification of part of the promoterless *gfpmut3* gene or of the *rpsM-gfpmut3* fusion was performed with primers pFPV_F1 and pFPV_R1 (Table 2) and plasmid templates pFPV25 and pFPV25.1 (61). The two resulting PCR products contained the common 5′ end DNA coding sequence of both the *gfpmut3* and *gfp*$^+$ genes, either with no promoter (from pFPV25; designated product 1) or fused to the *rpsM* promoter (from pFPV25.1; designated product 2). The *gfp*$^+$-specific 3′ region, designated product 3, was amplified from the pWH1012gfp$^+$ plasmid (51) with primers GFP_F1 and GFP_R1 (which contained an additional *Eco*RV site [Table 2]). Products 1 and 2 obtained from pFPV25 and pFPV25.1 and product 3 obtained from pWH1012gfp$^+$ had a 118-bp homologous sequence located in their 3′ and 5′ ends, respectively. A crossover PCR approach was subsequently used (33). Left and right products 1 and 3 or products 2 and 3 were annealed at the overlapping region and amplified by PCR as single fragments with the outer primers pFPV_F1 and GFP_R1, generating ~1,600-bp product 4 containing the new *gfp*$^+$ reporter fused to the *rpsM* promoter and the corresponding 980-bp product 5 that lacked a promoter. PCR products 4 and 5 were

TABLE 2. Oligonucleotides used

Oligonucleotide	Sequence[a]
pFPV_F1	5′-GAATTCGAGCTCGGTACCCGG-3′
pFPV_R1	5′-CGTATGTAGCATCACCTTCACC-3′
GFP_F1	5′-GGTGAAGGTGATGCTACATACG-3′
GFP_R1	5′-ATGCGATATCGCCACCTGACGTCTAAGAAACC-3′
Cam_F3	5′-GCATGATATCCGTCATTTCTGCCATTCATCC-3′
Cam_R3	5′-GCATGATATCGGGCTAGCCGGCCCGACGC-3′
t0T1_F4	5′-ATGGGGTACCGGATCCGTCGACCTGCAGCC-3′
t0T1_R4	5′-GCTCTAGAATAAGAATGCGGCCGCTCCCCGGGGGACCGAAACGCGCGAGGCAGC-3′
Kan_F1	5′-CATGCGACGCTAGCAGCCCGGGCTGCGGCCGCACCAAGCGAACCGGAATTGCCAGC-3′
Kan_R1	5′-GTCGCCATTCTAGAACGCTCAGAAGAACTCGTCAAGAAGG-3′
PssaG_F2	5′-ACGTCCCGGGCGATTGCTAAAGCCGTCTCC-3′
PssaG_R2	5′-CGATTCTAGACCATGTGGGAGAGCATATCC-3′
PprgH_F1	5′-ACGTCCCGGGGATGACTATTACTTACAAAGG-3′
PprgH_R1	5′-CGATTCTAGACGAACTATGTATGGCCCTGG-3′
PrpsM_F3	5′-CATGCGACCCGGGGAAAGGCTACGGCCGTTAAT-3′
PrpsM_R2	5′-GTCGCCATTCTAGACCAGCCAGGATGGCTTTAGAA-3′
proUgfp+_F2	5′-TGAAATTATTACAGGACGAAGACCGTGAATATGGTTACGTCATTGAGCGTTAAGAAGGAGATATACATATGAG-3′
proUgfp+_R2	5′-AATGCCGCTTTTAATGAGTCGATGGACACGACGCCCACGAATTTATTGCCTTATCACTTATTCAGGCGTA-3′
T1_F1	5′-GCAGGTCACATTTAACGCGGTTGCACAAGTTGCAACATGGCCTGGGGTAATGACTCTCTAGC-3′
Cam_R5	5′-GACCCGGATAGTAATTTTGCCCGGCCAGATGATAAATCGCGACGTCATTTCTGCCATTCATCC-3′

[a] Sequences underlined indicate locations of restriction sites used for cloning.

digested with *Kpn*I and *Eco*RV and cloned into the pFPV25 vector digested with *Kpn*I and *Eco*RV to generate plasmids pZEP01 and pZEP02, respectively, in which the *gfpmut3* gene was replaced by *gfp*$^+$.

Plasmid pZEP07 was constructed as follows. A chloramphenicol resistance cassette was amplified by PCR from pACYC184 (10) by using primers Cam_F3 and Cam_R3 (Table 2). The resulting 1,004-bp product was digested with *Eco*RV and subsequently cloned into *Eco*RV-digested pZEP01 in the same orientation as the *gfp*$^+$ gene, generating the pZEP06 plasmid. A 1,067-bp fragment, containing the strong t0 and T1 transcriptional terminators (49, 50), was amplified by PCR from the pQE9 plasmid (Qiagen) with primers t0T1_F4 and t0T1_R4 (Table 2), which added *Kpn*I and *Xba*I restriction sites to the 5′ and 3′ ends of the product, respectively. After *Kpn*I-*Xba*I digestion, the 1,067-bp fragment was cloned into *Kpn*I-*Xba*I-digested pZEP06 to generate pZEP07.

Plasmid pZEP08 was derived directly from pZEP07. A kanamycin resistance cassette was amplified from pKD4 (13) by using primers Kan_F1 and Kan_R1 (Table 2), which introduced an *Nhe*I site, a *Sma*I site, and a *Not*I site into the 5′ end and an *Xba*I site into the 3′ end of the resulting 993-bp fragment. The product was digested with *Nhe*I and *Xba*I and cloned into *Xba*I-digested pZEP07 to generate pZEP08.

Plasmids pZEP09, pZEP10, and pZEP16 containing the *ssaG-gfp*$^+$, *prgH-gfp*$^+$, and *rpsM-gfp*$^+$ fusions were constructed as follows. The *ssaG*, *prgH*, and *rpsM* promoters were amplified from purified *Salmonella* LT2 chromosomal DNA with the following primer pairs: PssaG_F2 plus PssaG_R2, PprgH_F1 plus PprgH_R1, and PrpsM_F3 plus PrpsM_R2 (Table 2). All forward primers contained a 5′ *Sma*I site, and all reverse primers carried a 5′ *Xba*I site. Each PCR product was digested with *Sma*I-*Xba*I before gel purification and cloning into *Sma*I-*Xba*I-digested pZEP08, which resulted in deletion of the kanamycin cassette (Fig. 1A). The chloramphenicol-resistant (Cm^r) and kanamycin-sensitive (Km^s) *E. coli* transformants that harbored the new plasmid-borne fusions were identified.

Chromosomal integration of single-copy fusions in *Salmonella* strains. All *gfp*$^+$ constructs were integrated into the chromosome of *Salmonella* strain LT2 or SL1344 by using the Lambda Red system as previously described (13, 43).

For construction of the single-copy *proU-gfp*$^+$ fusion, a fragment containing the promoterless *gfp*$^+$ gene and the chloramphenicol resistance cassette was amplified from the pZEP07 plasmid with primers proUgfp$^+$_F2 and proUgfp$^+$_R2 (Table 2). Each of these primers has a 5′ 50-nucleotide region that exhibits perfect homology with an internal part of the *proV* gene coding sequence (positions 2,956,849 to 2,956,948 on the *Salmonella* LT2 chromosome; GenBank accession no. AE006468).

Plasmids pZEP09, pZEP10, pZEP16, and pZEP07 were used as templates for PCR amplification of a fragment containing the T1 terminator, the *ssaG*, *prgH*, and *rpsM-gfp*$^+$ fusions or the promoterless *gfp*$^+$ gene, and the chloramphenicol resistance cassette by using primers T1_F1 and Cam_R5 (Table 2). Both of these primers had a 5′ 40- to 42-nucleotide region exhibiting perfect homology with the *putPA* locus of the *Salmonella* serovar Typhimurium SL1344 chromosome (Fig. 1) (H2 at positions 1,210,040 to 1,210,079 and H1 at positions 1,211,618 to 1,211,657 on the *Salmonella* LT2 chromosome). The PCR fragments were between 2 and 2.7 kb long.

For all single-copy fusions, between 500 ng and 1 μg of each linear PCR product was used for integrating fusions on the chromosome of *Salmonella* strain LT2 (*proU* fusion) or SL1344 (*ssaG*, *prgH*, and *rpsM* fusions) by the Lambda Red method (13). Between 10 and 15 transformants were obtained for each gene fusion. The loss of the pKD46 helper plasmid was monitored on LB medium plates at 37°C by using MAST ID Intralactam circles (MAST Diagnostics, Bootle Merseyside, United Kingdom) to screen for the absence of beta-lactamase in bacterial colonies. Putative constructs were verified by colony PCR by using specific primer pairs that annealed externally and internally with respect to the *gfp*$^+$ fusions. The chromosomal regions containing the *gfp*$^+$ fusions were sequenced on both DNA strands with specific primers by using an ABI 3700 sequencer and a Big Dye version 3 sequencing kit (ABI Prism).

β-Galactosidase assay. The method used to measure β-galactosidase activity was adapted from the Miller method (36). Serial twofold dilutions of purified β-galactosidase (Sigma) were used at concentrations ranging from 100 to 1.56 mU/ml to establish a standard curve. Cells were permeabilized with chloroform-sodium dodecyl sulfate, and chlorophenol red β-D-galactopyranoside (Roche) was used as the substrate. Reactions were performed in 96-well microtiter plates, and the results were read with a Spectramax spectrophotometer (Molecular Devices, Sunnyvale, Calif.). The kinetics of substrate hydrolysis was determined for 20 min, and the V_{max} was used to convert the data into milliunits per milliliter by using the SoftMaxPro 3.1.2 software (Molecular Devices) and the linear function formula $y = (Ax + B)/OD_{600}$, where y is the β-galactosidase activity to be determined, x is the reading value, A is the slope of the reading curve, B is the y intercept of the line, and OD_{600} is the optical density at 600 nm of the culture resuspended in reaction buffer.

Fixation, immunostaining, and flow cytometric analysis. For measurement of GFP in *Salmonella*, samples were immediately fixed for 1 min at room temperature in 4% (wt/vol) formalin (Sigma) freshly prepared in phosphate-buffered saline (PBS) (pH 7.4) (48). Fixed bacteria were subsequently washed, resuspended in PBS, and kept in the dark at 4°C until analysis. The PBS used in this study was filtered through a 0.22-μm-pore-size filter (Millipore, Billerica, Mass.) to reduce the background noise during flow cytometric analysis.

When appropriate, *Salmonella* cells were labeled with specific antibodies. A 1:200 final dilution of a rabbit anti-*Salmonella* lipopolysaccharide polyclonal primary antibody (catalog no. 2948-47-6; Biosciences Pharmingen, San Diego, Calif.) and a 1:40 final dilution of a goat R-phycoerythrin-conjugated anti-rabbit secondary antibody (catalog no. 4010-09; Southern Biotechnology Associates, Inc., Birmingham, Ala.) were used. Primary antibody staining and secondary antibody staining were performed in PBS containing 10% normal horse serum (Sigma) for 30 min at room temperature, followed by three washes in PBS.

For flow cytometric analysis, samples were diluted in 1 to 2 ml of PBS to obtain a maximum of approximately 10^6 particles per ml and were analyzed with a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, N.J.) equipped with a 15-mW air-cooled argon ion laser as the excitation light source (488 nm).

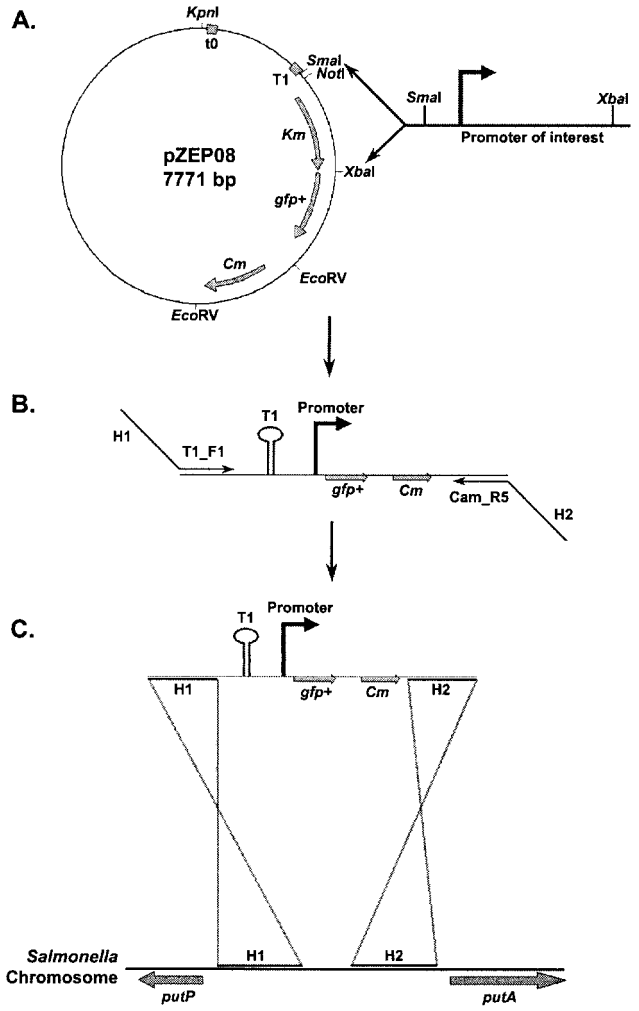

FIG. 1. Strategy used for construction of single-copy *gfp*$^+$ fusions. Promoters of interest were amplified by PCR from the *Salmonella* chromosome and inserted into the pZEP08 plasmid in place of the kanamycin resistance cassette (A). The resulting plasmid was then used as a template for PCR amplification of the fragment that contained the T1 terminator, the new *gfp*$^+$ transcriptional fusion, and the chloramphenicol resistance cassette by using primers that had 40- to 50-nucleotide tails (H1 and H2) exhibiting perfect homology with the chromosomal site of insertion (i.e., *putPA* locus) (B). The linear PCR product containing the new fusion was moved to the chromosome of the recipient *Salmonella* strain by recombination by using the Lambda Red system (13) (C).

For analysis of bacterial cells released from macrophages, samples were gated for *Salmonella*-like particles by using the orange fluorescence of the anti-*Salmonella* labeling to identify bacterial cells and to exclude mammalian cell debris and background noise. Fluorescence compensation settings were determined in parallel under identical conditions by using the constitutively GFP$^+$-expressing *Salmonella* strain JH3016 or the nonexpressing strain JH3008, with and without anti-*Salmonella* antibody labeling. All parameters were collected by using amplification gains set on LOG mode. Approximately 15,000 events identified as *Salmonella* cells were collected per sample. GFP fluorescence intensity values are presented below as medians for the populations after analysis with CellQuest 3.3 software (Becton Dickinson).

Macrophage infection by *Salmonella* strains. Murine J774-A.1 macrophage-like cells (European Collection of Cell Cultures [ECACC] no. 91051511) were grown in RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, Calif.) supplemented with 20% fetal bovine serum, 2 mM L-glutamine (Sigma), and 20 mM HEPES buffer (Sigma). For infection with *Salmonella*, 10^8 J774-A.1 cells were seeded in six-well plates (Becton Dickinson) at 37°C in the presence of 5% CO_2 as described previously (17). *Salmonella* cells were grown overnight on LB agar plates, washed, and resuspended in sterile PBS. Complement opsonization of bacteria and macrophage infection were performed as described previously (18) by using a multiplicity of infection of 100 bacteria per macrophage. Contact with the macrophage monolayer was maximized by 5 min of centrifugation at 453 $\times$ *g* at room temperature. Infected J774-A.1 cells were immediately incubated for 1 h at 37°C in the presence of 5% CO_2. Time zero of an experiment was defined as the beginning of this incubation. *Salmonella* cells that remained outside the macrophages were subsequently removed, immediately fixed, and used as a control in flow cytometry. The remaining extracellular bacteria were killed by addition of HEPES-buffered RPMI 1640 containing 10% fetal bovine serum and 30 μg of gentamicin per ml and incubation for an additional 1 h at 37°C in the presence of 5% CO_2. The medium was then replaced by HEPES-buffered RPMI 1640 containing 5 μg of gentamicin per ml, and the preparation was incubated at 37°C in the presence of 5% CO_2 until the end of the assay. At the end of the experiment, infected monolayers were washed with PBS and lysed under hypotonic conditions (17). Bacteria released from the intracellular environment were immediately fixed and kept at 4°C in PBS before anti-*Salmonella* labeling and flow cytometric analysis.

RESULTS AND DISCUSSION

Fixation does not impair fluorescence of GFP$^+$. Visualization of fluorescence from single-copy transcriptional fusions requires a particularly bright version of GFP. Therefore, we compared the levels of fluorescence of the most promising GFP variants. These included the GFPmut3 variant (11) and the GFP$^+$ variant, which was reported to be 130-fold brighter than the wild-type protein (51). The *rpsM* promoter was chosen because it was reported to be expressed at similar levels in various environments, including growth media and mammalian cells (62). We compared the *rpsM-gfpmut3* plasmid-borne fusion pFPV25.1 with the pZEP02 plasmid (see Materials and Methods), which carried the *gfp*$^+$ gene under control of the same transcriptional and translational signals as *gfpmut3* in pFPV25.1. Both pZEP02 and pFPV25.1 were transformed into *E. coli* DH5α, and the levels of green fluorescence were determined by flow cytometry with and without formalin fixation.

Figure 2A shows that the plasmid-borne *rpsM-gfp*$^+$ fusion is approximately three times brighter than the corresponding *rpsM-gfpmut3* fusion. Use of fluorescent proteins to monitor bacterial gene expression by flow cytometry requires the use of chemical fixation to stop gene expression. It has previously been reported that formalin has less effect on GFP fluorescence than other fixatives have (4). Here, we compared the effects of formalin fixation on GFPmut3 fluorescence and GFP$^+$ fluorescence, and we observed that the treatment reduced GFPmut3 fluorescence by about 30% compared to the fluorescence in unfixed *E. coli* (Fig. 2B). We discovered that GFP$^+$ is the first GFP variant which is not adversely affected by fixation (Fig. 2C), probably due to the greater stability of the GFP$^+$ protein. This confirms that the presence of the F64L, S65T, F99S, M153T, and V163A mutations results in increased fluorescence, making GFP$^+$ a promising reporter for poorly expressed promoters in individual bacterial cells. We therefore constructed single-copy *gfp*$^+$ fusions to monitor *Salmonella* gene expression.

GFP$^+$ is a reliable reporter of gene expression. Since 1979, *lacZ* fusions have been used to obtain robust gene expression data for hundreds of bacterial genes (8, 26, 53). To assess the reliability of single-copy *gfp*$^+$ fusions for monitoring gene expression, we compared a *proU-gfp*$^+$ fusion with a well-charac-

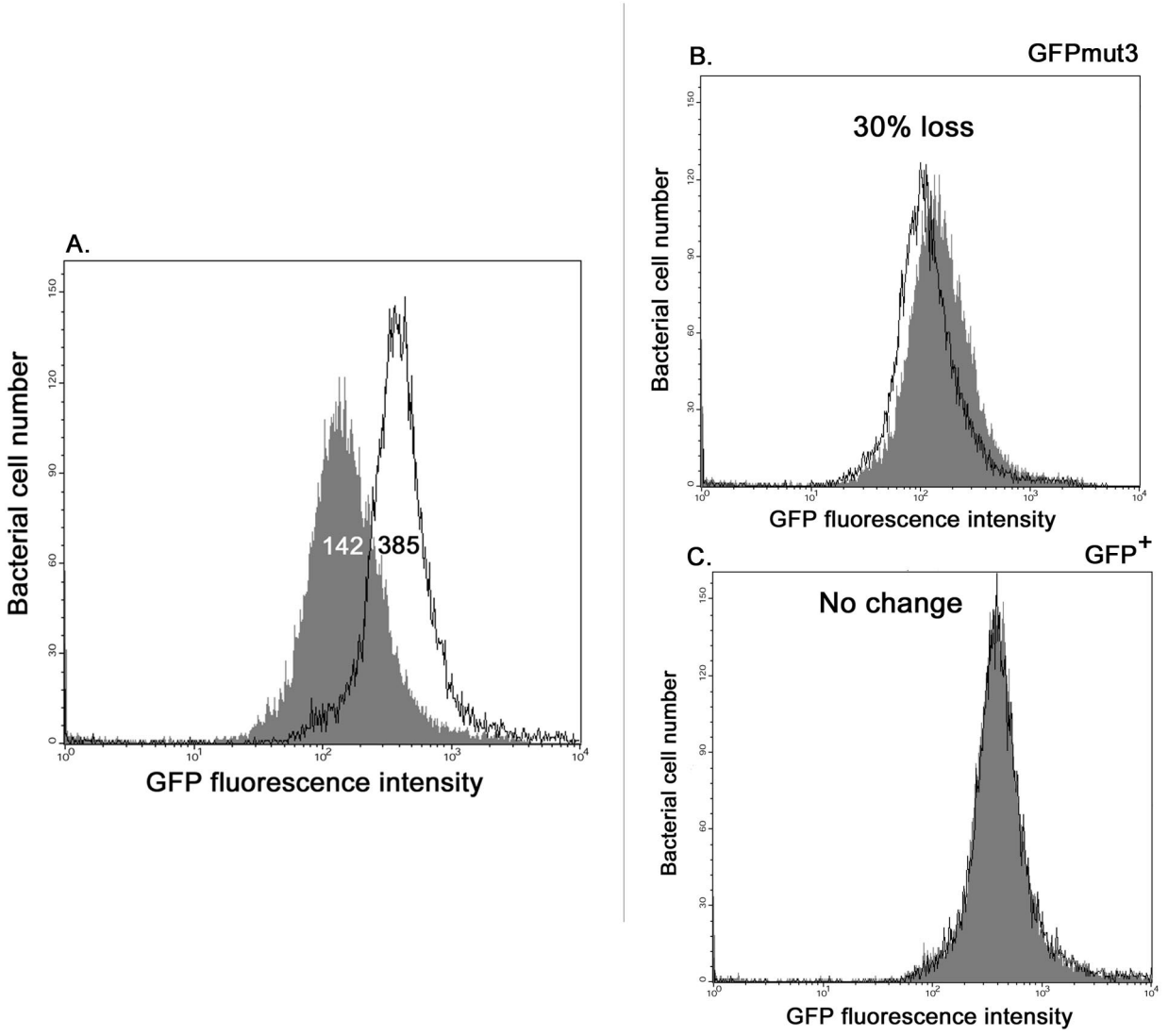

FIG. 2. Comparison of *rpsM-gfpmut3* and *rpsM-gfp*$^+$ expression in LB medium and after formalin fixation. *E. coli* strains harboring either pFPV25.1 (*rpsM-gfpmut3*) or pZEP02 (*rpsM-gfp*$^+$) were grown overnight in LB broth containing ampicillin. Live or fixed (4% formalin) bacteria were immediately analyzed by flow cytometry. (A) Unfixed bacteria harboring either pFPV25.1 (shaded graph) or pZEP02 (solid line). (B and C) Fixed (solid line) and unfixed (shaded graph) bacteria harboring either pFPV25.1 expressing GFPmut3 (B) or pZEP02 expressing GFP$^+$ (C). The values are the median values for fluorescence intensity for all individual bacteria in a population.

terized, salt-inducible *proU-lacZ* fusion in *Salmonella* (7). The *proU* operon encodes a betaine transport system that is involved in the adaptation of the bacteria to increases in environmental osmolarity (20). Cairney et al. reported the pattern of osmoregulation of *proU* expression in *Salmonella* serovar Typhimurium strain CH946, which carries a Mud1-8(*lacZ*) insertion in the *proV* gene (7, 56). We directly compared salt induction of the *proU-lacZ* fusion in CH946 with salt induction of a *proU-gfp*$^+$ fusion that was constructed at exactly the same location in strain JH3017 (see Materials and Methods).

The LT2 strain JH3049 carrying a promoterless *gfp*$^+$ gene was used as a negative control. Expression of *proU-lacZ* was assessed by measuring the β-galactosidase activity of the entire population, and *proU-gfp*$^+$ expression was monitored by flow cytometry of fixed bacteria (Fig. 3). Figure 3A shows that in the presence of 0.06 M NaCl, neither the *proU-lacZ* fusion nor the *proU-gfp*$^+$ fusion was induced. When 0.16 M NaCl was added, a low level of induction was observed for both *proU-lacZ* and *proU-gfp*$^+$ 20 min after the salt was added; this level of induction stabilized after 40 min and remained very low until the end of the assay (Fig. 3B). Figure 3C shows that both *proU-lacZ* and *proU-gfp*$^+$ were highly induced by 0.3 M NaCl and exhibited similar expression patterns through time. Induction of both *proU-lacZ* and *proU-gfp*$^+$ was detected just 10 min after salt was added. The slight difference in expression observed for the two fusions at 20 min probably reflected the greater variation in the β-galactosidase measurements for that time point. For both reporter systems maximum induction of the *proU* promoter was obtained 60 min after addition of 0.3 M NaCl. Subsequently, *proU* expression slowed, and both reporters ex-

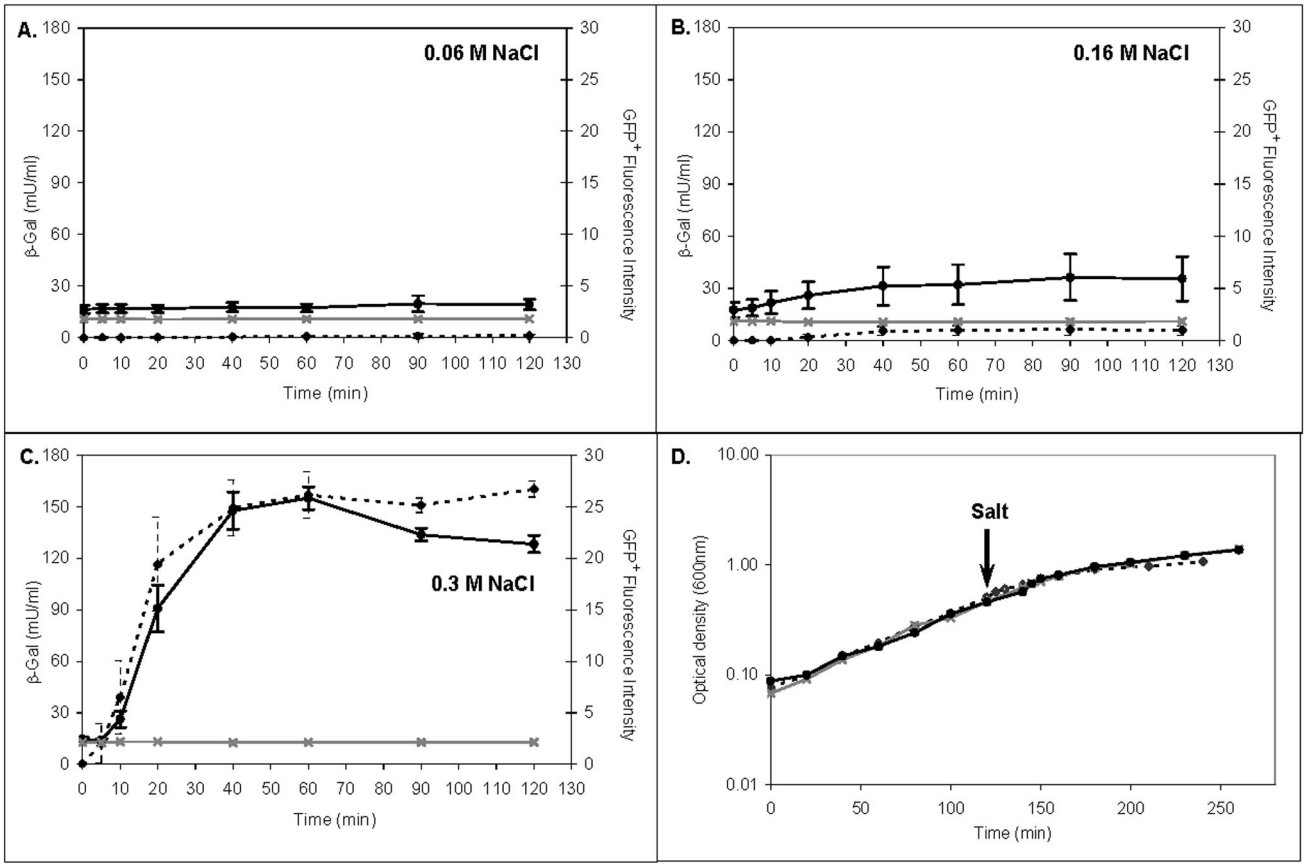

FIG. 3. Osmotic induction of *proU*. Induction of *proU-lacZ* and *proU-gfp*$^+$ expression was tested as follows. *Salmonella* strains CH946 (*proU-lacZ*; β-galactosidase [β-Gal] activity indicated by the dashed line), JH3017 (*proU-gfp*$^+$; GFP fluorescence indicated by the solid black line), and JH3049 (promoterless *gfp*$^+$; GFP fluorescence indicated by the solid grey line) were grown in LO medium containing glucose at 30°C to an optical density at 600 nm of 0.5. NaCl was then added to a final concentration of 0.06 M (A), 0.16 M (B), or 0.3 M (C). Samples were collected at 0, 5, 10, 20, 40, 60, 90, and 120 min after addition of the salt. The optical density of each culture was measured at 600 nm (panel D shows the growth curves obtained before and after addition of 0.3 M NaCl). The arrow indicates when the salt was added to each mid-log-phase culture. The median β-galactosidase activities for three independent experiments are shown (see Materials and Methods). The GFP$^+$ fluorescence intensities are the median values for the intensities of all individual bacteria in a population for seven independent experiments. The error bars indicate the standard deviations.

hibited constant or slightly reduced expression in the stationary phase (Fig. 3D).

In summary, these results confirmed the previously described osmoregulation of *proU* (7) and demonstrated that GFP$^+$ reports *proU* activity as accurately as β-galactosidase does. These data agree with a previous study involving plasmid-borne fusions (51). However, insertion of a reporter gene into a gene of interest, as described above, necessarily generates a mutation that could have a polar effect. To maintain an intact copy of each wild-type promoter and to avoid production of virulence mutants while allowing direct comparison of many promoters in the same chromosomal context, we developed a system for insertion of single-copy transcriptional fusions at a different chromosomal location.

Construction of single-copy *gfp*$^+$ chromosomal fusions in *Salmonella* serovar Typhimurium. Rapid construction of single-copy GFP$^+$ fusions in *Salmonella* involved the pZEP08 plasmid (see Materials and Methods), which carried transcriptional terminator T1, a promoterless *gfp*$^+$ gene, a chloramphenicol resistance cassette, and a kanamycin resistance cassette (Fig. 1A). Fragments containing the promoter regions of genes of interest were amplified by PCR and cloned upstream of the *gfp*$^+$ gene. The whole constructs were amplified by PCR (Fig. 1B) and integrated onto the *Salmonella* chromosome (13). A similar approach was used to generate the negative control construct; this approach involved amplification of the corresponding fragment from parental plasmid pZEP07, which carried the promoterless *gfp*$^+$ gene located directly downstream of the T1 terminator, preventing production of GFP$^+$. The gene fusions were integrated at the *putPA* locus (Fig. 1B and C). We have shown that interruption of *putPA* does not affect the ability of *Salmonella* to colonize the spleen and liver in the BALB/c mouse model (Hautefort, Proença, and Hinton, unpublished data).

The *rpsM*, *ssaG*, and *prgH* gene fusions were integrated as a single copy into the *putPA* locus on the *Salmonella* chromosome. In parallel, the promoterless *gfp*$^+$ gene derived from pZEP07 was inserted at exactly the same position. Colony PCR and subsequent DNA sequencing (see Materials and Methods) were used to verify that all colonies carried the correct fusion.

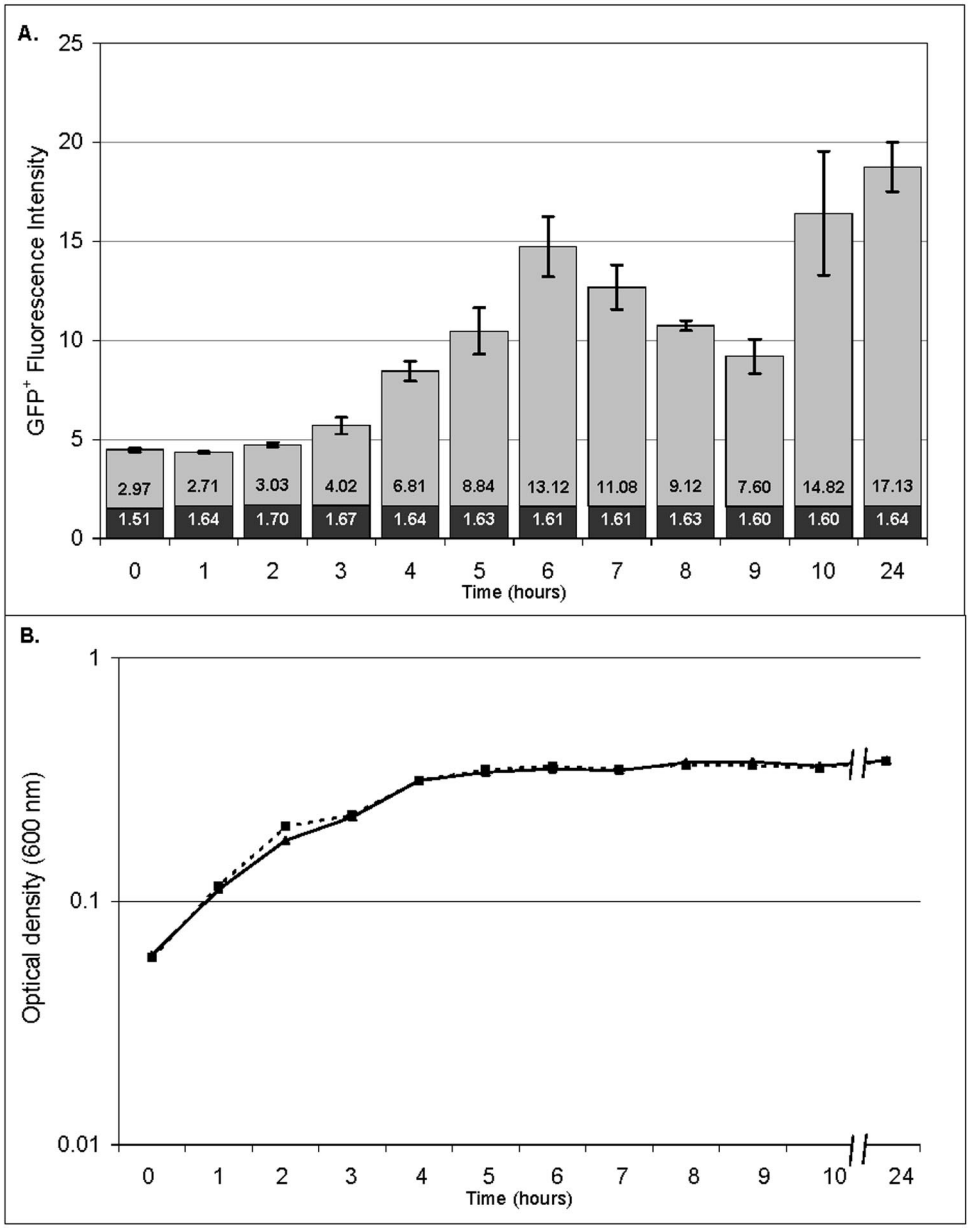

FIG. 4. Virulence gene induction in vitro. *Salmonella* serovar Typhimurium strains JH3009 (*ssaG-gfp*$^+$) and JH3008 (promoterless *gfp*$^+$) were grown overnight in LB broth. Both strains were subsequently diluted 50-fold in MM5.8 and were grown for 24 h. Time zero corresponded to the beginning of incubation in MM5.8. Samples were collected every hour from time zero until 10 h and once after 24 h, immediately fixed in 4% formalin, and analyzed by flow cytometry. (A) Fluorescence intensity of *Salmonella* strain JH3009 (grey bars) determined in triplicate. The fluorescence intensity of the negative control JH3008 strain is also indicated for each time point (solid bars). The values in the bars are the median fluorescence intensities of all individual bacteria in the populations. The error bars indicate the standard deviations. (B) Corresponding growth curve. Dashed line, strain JH3008; solid line, strain JH3009.

The approach summarized in Fig. 1 allowed successful construction and integration of *gfp*$^+$ fusions, and it has proved to be a rapid and flexible method for generating single-copy chromosomal *gfp*$^+$ fusions in *Salmonella*.

Single-copy *gfp*$^+$ fusion allows detection of in vitro induction of virulence gene expression. Understanding the host-pathogen interaction requires monitoring of virulence gene expression during infection of mammalian cells and animal models, as well as in vitro. We first verified the single-copy *ssaG-gfp*$^+$ fusion by monitoring expression during growth of *Salmonella* serovar Typhimurium under inducing conditions in vitro. The *ssaG* gene encodes a component of the SPI2 type III secretion system, which is highly induced during macrophage infection (18, 62), when *Salmonella* faces an acidic pH combined with low levels of phosphate and magnesium (14, 47). We chose an acidic minimal medium (MM5.8) (30) to reproduce some of these conditions. The negative control *Salmonella* strain JH3008 (promoterless *gfp*$^+$) did not express GFP$^+$ and had a

constant fluorescence intensity of about 1.6 (Fig. 4). This confirmed that the transcriptional terminator T1 included in our constructs efficiently prevented transcriptional readthrough. Induction of *ssaG* from strain JH3009 was detected after 3 h of growth, corresponding to the mid-exponential growth phase. Expression of *ssaG-gfp*$^+$ increased eightfold at 6 h, when the bacteria entered the stationary growth phase. This is in agreement with the previously described pattern of expression of SPI2 transcriptional fusions to the luciferase gene, as monitored in acidic medium (3). Figure 4 shows that *ssaG-gfp*$^+$ was then switched off, since GFP$^+$ fluorescence decreased twofold between 7 and 9 h. A second induction of *ssaG-gfp*$^+$ expression was shown by the doubling of fluorescence intensity between 9 and 10 h, which was maintained at 24 h. Monitoring of *ssaG-gfp*$^+$ expression showed that increases as well as decreases in GFP$^+$ fluorescence intensity could be measured through time (Fig. 4), confirming that single-copy fusions are a valuable tool for looking at growth phase-dependent gene expression and for performing time course experiments.

Single-copy *gfp*$^+$ fusion reveals variation in gene expression between individual bacterial cells. The combination of single-copy *gfp*$^+$ fusions and the ability to measure fluorescence in individual bacterial cells offers a powerful system for searching for different levels of gene expression in bacteria within a population. We monitored expression of the *prgH-gfp*$^+$ single-copy fusion in *Salmonella* strain JH3010. The *prgH* gene encodes a basal component of the needle complex of the SPI1 type III secretion machinery. SPI1 genes are induced by the high osmolarity, low oxygen levels, and short-chain fatty acids thought to be present in the ileum of the digestive tract (19, 31). Figure 5 shows that the *prgH-gfp*$^+$ fusion was induced 12-fold after 4 h of growth only in the presence of salt (LO medium containing 0.3 M NaCl). The salt-dependent induction of *prgH-gfp*$^+$ increased to 18-fold at 6 h and remained at 17-fold at 8, 12, and 24 h. This suggests that *prgH* is induced soon after salt addition (<4 h, mid-exponential phase) and that expression of this gene is dramatically reduced after 6 h (from the late exponential phase to the stationary phase) since no more GFP$^+$ accumulates in each bacterial cell. Concomitantly, the experiment revealed that there was significant differential gene expression within the bacterial population (Fig. 5). The sequential flow cytometric analysis showed for the first time that *prgH-gfp*$^+$ is not induced in every bacterial cell. Only 53% of the population showed *prgH-gfp*$^+$ induction at 4 h, and the percentage decreased to approximately 22% from 8 h until the end of the experiment. To ensure that the single-copy *prgH-gfp*$^+$ fusion was stable on the chromosome and had not been lost from any of the cells, we screened the bacteria from the 24-h culture for Cm^r resistance. All of the 200 *Salmonella* colonies tested were Cm^r, showing that every bacterial cell still carried *prgH-gfp*$^+$. This observation of differential expression of an SPI1 gene is completely novel, and the effect of this expression on the ability of *Salmonella* to succeed during infection merits further investigation.

Monitoring virulence gene expression in complex environments. To determine the utility of single-copy *gfp*$^+$ fusions in complex environments, we monitored GFP$^+$ expression in bacteria following infection of mammalian cells. We detected and quantified *ssaG-gfp*$^+$ expression in individual bacteria released from infected J774-A.1 macrophage-like cells. Data that were generated from a plasmid-borne *ssaG-gfpmut3* fusion (originally referred to as *ssaH*) showed that *ssaG* was highly expressed 6 h after macrophage infection (62). We infected J774-A.1 murine macrophages with opsonized strains JH3009, JH3016, and JH3008 carrying single-copy *ssaG-gfp*$^+$ and *rpsM-gfp*$^+$ fusions and a promoterless *gfp*$^+$ gene, respectively. The *rpsM* gene had previously been reported to be expressed at similarly high levels in LB medium and in macrophages (61), suggesting it would be an appropriate positive control.

For flow cytometric analysis, detection of multiple fluorescent colors requires adjustment of settings to ensure that each fluorescent signal does not spill over into a second signal and to avoid false-positive data. This adjustment, referred to as compensation, required the use of comparable positive and negative control strains, JH3016 and JH3008. The analysis was performed with bacteria that either were released from mammalian cells after 6 h or remained outside the macrophages following the initial incubation (see Materials and Methods). To be able to detect all bacteria, we used an anti-*Salmonella* antibody to distinguish *Salmonella* cells from host cell debris. This crucial part of the protocol allowed us to observe all bacteria that either expressed or did not express GFP$^+$. Figure 6A shows that extracellular JH3009 *Salmonella* cells did not express the *ssaG* fusion and exhibited levels of fluorescence similar to those of the control JH3008 extracellular bacteria. As expected, the JH3016 strain expressed the *rpsM-gfp*$^+$ fusion outside the macrophages with 40-fold more GFP$^+$ fluorescence than the negative control (Fig. 6B). Figure 6C clearly shows the novel finding that the single-copy *ssaG-gfp*$^+$ fusion was induced eightfold in all bacterial cells when *Salmonella* was internalized within the macrophages. This increase in *ssaG* expression is consistent with the increase observed at the RNA level by DNA microarray analysis (18) and confirms that reporter genes can accurately reflect the level of bacterial gene transcription within mammalian cells. No differential expression of *ssaG-gfp*$^+$ was observed within the intracellular bacterial population. Interestingly, the fluorescence of strain JH3016, which expressed the *rpsM-gfp*$^+$ fusion, decreased fivefold 6 h after phagocytosis and was only eightfold higher than the fluorescence of the negative control strain JH3008 (Fig. 6D), suggesting that a high level of expression of the small S13 ribosomal protein is no longer required once the bacteria are inside a *Salmonella*-containing vacuole. This is consistent with the threefold decrease in *rpsM* expression observed at the RNA level (18). Figures 6C and D also show that the intrinsic green autofluorescence of *Salmonella* cells increased within macrophages. Indeed, the relative fluorescence intensity of JH3008 (promoterless *gfp*$^+$) doubled from the extracellular location to the intraphagosomal location (Fig. 6B and D). This observation shows that it is not sufficient simply to compare extracellular bacteria with intracellular bacteria, ignoring the variation in the level of bacterial autofluorescence with cellular location. A simplistic comparison would have suggested that *ssaG-gfp*$^+$ expression from strain JH3009 was induced more than 16-fold inside macrophages compared to the level of expression in extracellular bacteria. Because the level of autofluorescence of the promoterless control strain JH3008 more than doubled intracellularly, the true level of *ssaG* induction was eightfold. This observation shows that choosing an appropriate negative control is crucial when *gfp*$^+$ fusions are

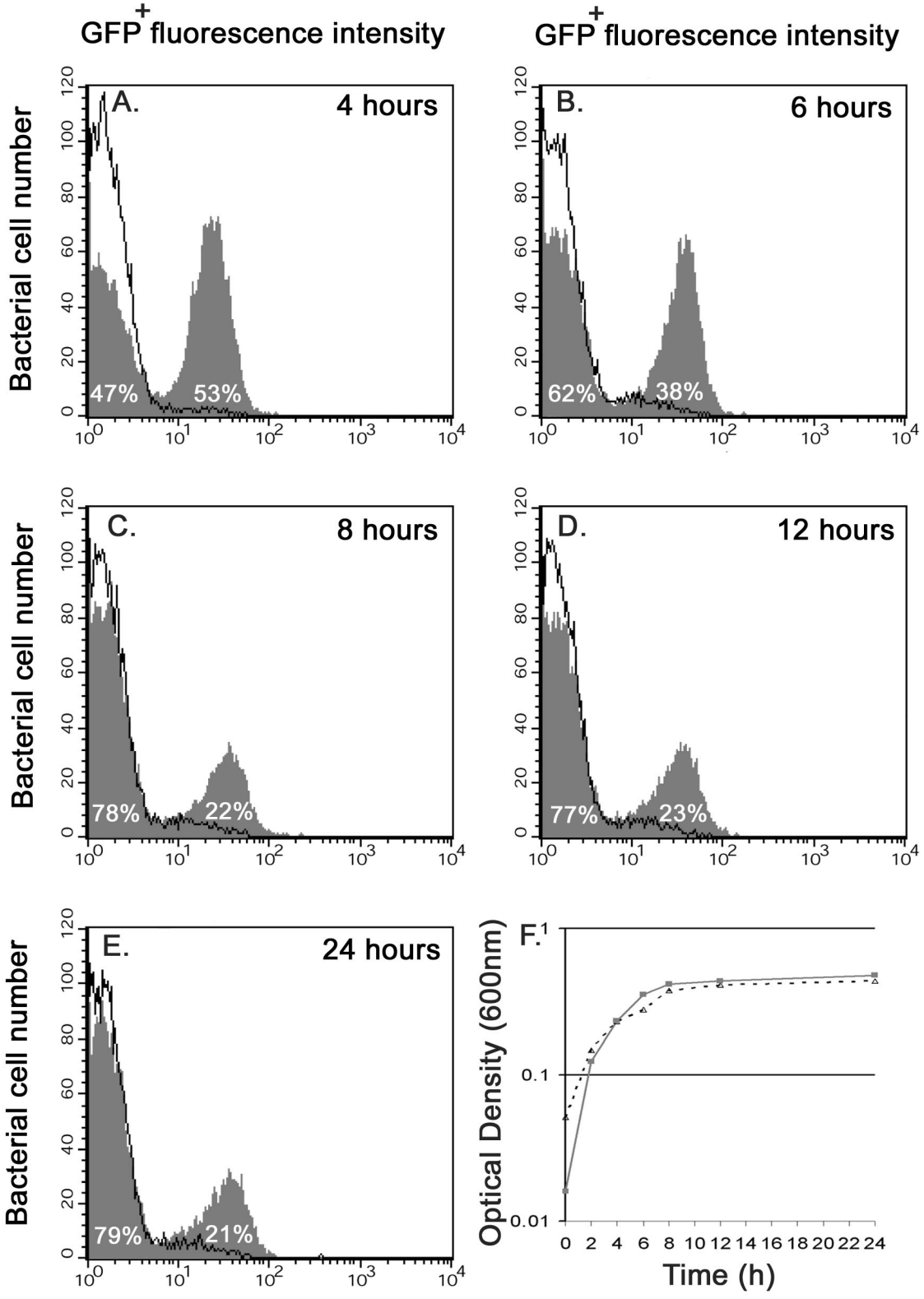

FIG. 5. Differential expression of a promoter within a genetically identical population. *Salmonella* serovar Typhimurium strain JH3010 (carrying *prgH-gfp*$^+$) was grown overnight in LO medium containing no salt. A culture containing 10^4 bacteria per ml (final concentration) was then grown in either LO medium (solid line) or LO medium containing 0.3 M NaCl (shaded graph). Samples were collected after 4 h (A), 6 h (B), 8 h (C), 12 h (D), or 24 h (E) of growth with or without salt (F) (dotted line, LO medium; solid line, LO medium containing 0.3 M NaCl), fixed in 4% formalin, and analyzed by flow cytometry. The level of induction was calculated by comparing the fluorescence for the most fluorescent peak at each time point with the fluorescence at the same time obtained when strain JH3010 was grown in LO medium. The percentage of the population in each fluorescence peak is indicated on the graph. To ensure that the differential expression of the *prgH-gfp*$^+$ fusion was genuine, the experiment was repeated 25 times, and the data from a single representative experiment are shown.

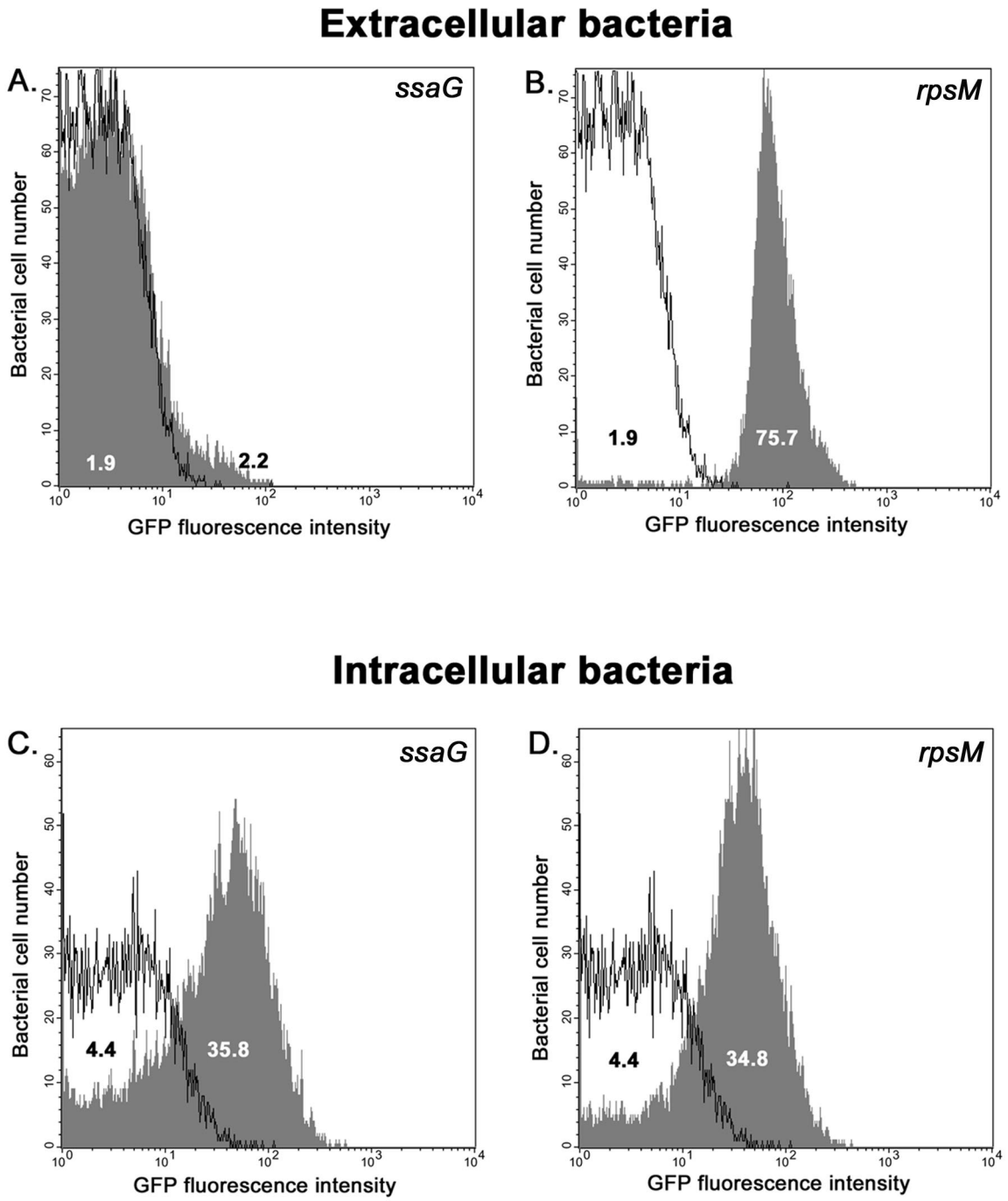

FIG. 6. Single-copy SPI2 gene fusion accurately reports gene expression in mammalian cells. J774-A.1 murine macrophages were infected with *Salmonella* serovar Typhimurium strains JH3008, JH3009, and JH3016 harboring a promoterless *gfp*$^+$ gene, an *ssaG-gfp*$^+$ fusion, and an *rpsM-gfp*$^+$ fusion, respectively. Six hours after infection, intracellular bacteria were released under hypotonic conditions and immediately fixed in 4% formalin. *Salmonella* cells were then labeled with a specific antibody, and their fluorescence was measured by flow cytometry. The results shown represent the GFP$^+$ fluorescence intensity of extracellular or intracellular bacteria identified as *Salmonella* cells by antibody labeling. Each panel shows an overlay of the GFP$^+$ fluorescence of either JH3009 or JH3016 (shaded graph) with the GFP$^+$ fluorescence of JH3008 (solid line), which was used as negative control. (A and B) Levels of expression of *ssaG-gfp*$^+$ or *rpsM-gfp*$^+$ in extracellular *Salmonella* cells. (C and D) Fluorescence in bacteria of *ssaG-gfp*$^+$ or *rpsM-gfp*$^+$ released from inside macrophages at 6 h postinfection. The values indicate the median GFP fluorescence intensity of all individual bacteria in a population.

used to study in vivo gene expression. The results presented in Fig. 6 validate the use of our system for monitoring induction of virulence gene expression in the complex environment of infected mammalian cells.

This study confirmed that single-copy GFP$^+$ fusions allow low levels of gene expression to be quantified in individual bacterial cells as accurately as has been possible in entire bacterial populations with *lacZ* fusions. For the first time, the

combination of a single-copy transcriptional fusion with flow cytometric analysis revealed different levels of expression of a virulence promoter.

The ability to record the level of variation in the expression of a particular gene in genetically identical populations is particularly important because of the unexplained phenotypic heterogeneity that has been reported previously for bacterial populations (5, 32, 55). Flow cytometry and cell sorting have been used to measure the variation in several phenotypic parameters in bacterial cells (39). Heterogeneity has only begun to be studied at the level of gene expression in the last decade; Mulec et al. (38) showed that induction of a plasmid-borne *cka-gfp* fusion occurred in only 3% of *E. coli* cells, and Siegele and Hu (52) observed variations in the level of expression of an *araBAD*-GFP transcriptional fusion in different bacterial cells. Other techniques, such as in situ PCR, have been used to detect qualitative differences in mRNA levels between individual bacterial cells (59). However, the study of variations in gene expression within bacterial populations has been hampered by the paucity of techniques to measure the levels of promoter activity within individual bacteria. Recently, a robust mathematical modeling study based on single-copy *gfp* fusions in *Bacillus subtilis* showed that phenotypic variations commonly observed between bacterial cells of a genetically identical population are strongly linked to translational rather than transcriptional efficiency (41). This confirms that transcriptional fusions can be relied upon to show real variations in gene expression rather than phenotypic noise. The single-copy *gfp* fusions described here permit the study of virulence gene expression in individual bacterial cells during infection of mammalian cells.

In the last two decades, the technology for construction of single-copy reporter gene fusions has been in constant development and has relied upon the site-specific recombination systems of various phages, transposons, and suicide vectors, as summarized by Slauch and Silhavy in 1991 (54) and by Hand and Silhavy more recently (23). In a large number of studies the workers have successfully used these methods to study and dissect regulatory pathways (21, 58). However, many of the approaches have been problematic, because integration has been restricted to one specific site on the chromosome or there has been genetic instability. The recently developed Lambda Red system has revolutionized recombinant genetics in enteric bacteria. This system provides scientists with an excellent tool for stable insertion of DNA fragments anywhere in the bacterial chromosome (13). This system has recently been used for construction of single-copy *lacZ* fusions in the chromosome of *Salmonella* serovar Typhimurium (16). Ellermeier et al. used FLP/FRT-mediated site-specific recombination events to incorporate a promoterless *lacZ* gene at the site of a mutated gene of interest, which had previously been knocked out by using the Lambda Red system. However, this approach initially involves creation of a gene knockout. Because no wild-type copy of the gene remains, the resulting strain might show attenuated virulence or phenotypes might be affected. Our system is also based on the Lambda Red recombination method but has the advantage of leaving an intact copy of the gene of interest in its original site, as well as the benefit of generating single-copy gfp^+ gene fusions in a chromosomal location that is known to have no apparent effect on *Salmonella* virulence. The insertion locus can also be varied without a requirement for a supplementary cloning step, which makes this tool an adaptable system that is applicable to a large number of gram-negative bacteria. The approach described here promises to provide answers to key biological questions concerning the pattern of bacterial gene expression within populations, both in vitro and during the process of infection itself.

ACKNOWLEDGMENTS

We thank Sofia Eriksson for sharing her expertise with *Salmonella* infection of macrophages, Martin Goldberg for useful technical advice, and Roy Bongaerts for critical evaluation of the manuscript. We are grateful to Chris Higgins and Julie Sidebotham for their input at the early stages of this work. We thank Mikael Niederweis for providing us with the pWH1012gfp+ plasmid.

This work was originally supported by Wellcome Trust Programme grant 045490. Isabelle Hautefort was initially supported by a Training and Mobility of Researchers fellowship from the European Union (contract number ERBFMRXCT9), and the work was subsequently supported by the BBSRC.

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