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Broad-Host-Range Plasmids for Red Fluorescent Protein Labeling of Gram-Negative Bacteria for Use in the Zebrafish Model System▽†

John T. Singer,[1]* Ryan T. Phennicie,[1] Matthew J. Sullivan,[1]‡ Laura A. Porter,[1]§ Valerie J. Shaffer,[1]¶ and Carol H. Kim[1,2]

Department of Molecular and Biomedical Sciences, 5735 Hitchner Hall, University of Maine, Orono, Maine 04469,[1] and Graduate School for Biomedical Sciences, ESRB/Barrows Hall, University of Maine, Orono, Maine 04469[2]

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To observe real-time interactions between green fluorescent protein-labeled immune cells and invading bacteria in the zebrafish (*Danio rerio*), a series of plasmids was constructed for the red fluorescent protein (RFP) labeling of a variety of fish and human pathogens. The aim of this study was to create a collection of plasmids that would express RFP pigments both constitutively and under *tac* promoter regulation and that would be nontoxic and broadly transmissible to a variety of Gram-negative bacteria. DNA fragments encoding the RFP dimeric (d), monomeric (m), and tandem dimeric (td) derivatives d-Tomato, td-Tomato, m-Orange, and m-Cherry were cloned into the IncQ-based vector pMMB66EH in *Escherichia coli*. Plasmids were mobilized into recipient strains by conjugal mating. Pigment production was inducible in *Escherichia coli*, *Pseudomonas aeruginosa*, *Edwardsiella tarda*, and *Vibrio* (*Listonella*) *anguillarum* strains by isopropyl-β-D-thiogalactopyranoside (IPTG) treatment. A spontaneous mutant exconjugant of *P. aeruginosa* PA14 was isolated that expressed td-Tomato constitutively. Complementation analysis revealed that the constitutive phenotype likely was due to a mutation in *lacI*^{q} carried on pMMB66EH. DNA sequence analysis confirmed the presence of five transitions, four transversions, and a 2-bp addition within a 14-bp region of *lacI*. Vector DNA was purified from this constitutive mutant, and structural DNA sequences for RFP pigments were cloned into the constitutive vector. Exconjugants of *P. aeruginosa*, *E. tarda*, and *V. anguillarum* expressed all pigments in an IPTG-independent fashion. Results from zebrafish infectivity studies indicate that RFP-labeled pathogens will be useful for the study of real-time interactions between host cells of the innate immune system and the infecting pathogen.

The use of fluorescent proteins as probes has proven to be a vital tool in elucidating spatial and temporal patterns of gene expression, protein localization, and protein-protein interactions *in vivo*. In addition to well-known contributions to cell biology and biochemistry, the use of fluorescent proteins to label bacteria has provided insight into complex host-pathogen interactions and routes of entry (14, 23, 29).

The zebrafish has become an attractive model for *in vivo* infection studies. As an optically clear organism at the embryo and larval developmental stages, zebrafish are amenable to visualization during challenge with fluorescently labeled pathogens. In addition, fertilization in the zebrafish occurs *ex utero*, thereby simplifying infection by either static immersion or microinjection. The visualization of infection *in situ* can be conducted on the whole embryo using confocal or wide-field epifluorescence microscopy, an approach not possible in other model systems. To date, several zebrafish infection models have been established employing both fish and mammalian pathogens, including *Edwardsiella tarda* (21), *E. ictaluri* (14, 23), *Staphylococcus aureus* (16), *Streptococcus pyogenes* and *S. iniae* (18), and *Pseudomonas aeruginosa* (4). Illustrating the value of fluorescently labeled bacteria in studies of microbial pathogenesis, Van der Sar and colleagues established the zebrafish as a model for *Salmonella enterica* serovar Typhimurium infections using DsRed-labeled bacteria (30). Similarly, O'Toole and colleagues made use of a green fluorescent protein (GFP)-labeled strain of *Vibrio* (*Listonella*) *anguillarum* to elucidate the natural route of invasion of this fish pathogen (20). However, obstacles to the construction of fluorescently labeled microbes have prevented many from taking advantage of this technique to permit the real-time *in situ* analysis of infection. These obstacles include the nontransformable nature of many pathogens and the toxicity of RFP expression from high-copy-number plasmids.

Recently, two transgenic lines of zebrafish that express GFP in cells of the myeloid lineage have been developed. Lawson et al. described a transgenic (Tg) zebrafish that utilizes the protooncogene *fli1* promoter to drive the expression of enhanced GFP (EGFP) (15). The *fli1* gene is preferentially expressed in endothelial and hematopoietic cells. Intended for the study of vascular development, Tg(*fli1*::EGFP) zebrafish not only have fluorescent endothelial cells but also macrophages (15), making this zebrafish line attractive to those who study innate immunity. It has been shown that zebrafish macrophages possess strong phagocytic properties and migrate to sites of bacterial infection (13). In 2006, Renshaw and his colleagues engineered a novel Tg(*mpo*::GFP) zebrafish that expresses GFP under the control of the neutrophil-specific myeloperoxidase promoter (22). In this study, they characterized the GFP-la-

* Corresponding author. Mailing address: Department of Molecular and Biomedical Sciences, 5735 Hitchner Hall, University of Maine, Orono, ME 04469. Phone: (207) 581-2808. Fax: (207) 581-2801. E-mail: jsinger@maine.edu.

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‡ Present address: Quincy College, Quincy, MA 02171.

§ Present address: Department of Microbiology, University of Virginia, Charlottesville, VA 22908.

¶ Present address: The Jackson Laboratory, Bar Harbor, ME 04609.

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beled neutrophils, noting their capability of chemotaxis at wound sites (22). Given the recent progress in zebrafish genetics with regard to these GFP-expressing transgenic lines, there was a clear need for a simple method for the RFP labeling of Gram-negative bacteria. Such a method would facilitate further studies of microbial pathogenesis in the zebrafish and could further elucidate phagocyte (GFP labeled)-pathogen (RFP labeled) interactions, kinetics of bacterial clearance, and mechanisms of phagocyte migration to sites of bacterial infection.

For the purposes of this study, we employed dimeric (d) and monomeric (m) red fluorescent protein variants d-Tomato, td-Tomato, m-Orange, and m-Cherry, which were constructed by Shaner et al. (25). These variants were chosen based on combinations of their high relative brightness and decreased RFP maturation times compared to those of the original RFPs (25). We describe here a simple method for the RFP labeling of Gram-negative bacteria.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used are listed in Table 1. *Escherichia coli* HB101 (3) was used as a cloning host and conjugal donor for the mobilization of pMMB66EH derivatives into other Gram-negative bacteria. Spontaneous rifampin (Rif; rifampicin)-resistant mutants of *V. anguillarum*, *E. tarda*, and *P. aeruginosa*, selected on L agar plates containing rifampin (100 μg/ml), were used as recipients in conjugal matings with *E. coli* HB101 donors.

Plasmid pMMB66EH is a broad-host-range, mobilizable, RSF1010-based *tac* expression vector (1, 12) that was used for the cloning of RFP genes. It carries a hybrid *tac* promoter with sequences upstream of −20 from *trp* and downstream of −20 from *lacUV5* (8). Expression from *tac* under noninducing conditions is completely blocked by a copy of *lacI*^{q} carried by the plasmid (1). The plasmid carries a polylinker downstream from *tac* that contains restriction endonuclease recognition sites for EcoRI, SmaI, BamHI, SalI, PstI, and HindIII (12).

Plasmids pRSETB d-Tomato, pRSETB td-Tomato (td, tandem dimer), pRSETB m-Orange, and pRSETB m-Cherry were described previously (25) and served as sources of RFP genes. The general structure of the pigment-coding region in these plasmids is shown in Fig. 1. All pRSETB plasmids contained pigment gene-coding sequences on 0.85- to 1.6-kb XbaI-HindIII fragments (Fig. 1), and each also contains a BamHI recognition site immediately upstream of the ATG start site for the pigment (25).

Media and growth conditions. *E. coli* strains and *P. aeruginosa* strains were cultured in L broth (17) with shaking at 37°C. *V. anguillarum* strains were grown with shaking in L broth containing 2% NaCl at 26°C (27). *E. tarda* was grown with shaking in L broth at 26°C. Solid medium contained 1.6% agar. Where required, ampicillin was added to media at the following final concentrations (in micrograms per milliliter): 100 (*E. coli* and *E. tarda*), 300 (serotype O1 *V. anguillarum*), 600 (serotype O2β *V. anguillarum*), 750 (*P. aeruginosa* PA14), and 1,250 (*P. aeruginosa* PA01). Kanamycin was used at 25 μg/ml, and rifampin (100 μg/ml) was used to counterselect against donor strains following conjugal matings. IPTG was used at a concentration of 0.4 mM. For the selective isolation of *P. aeruginosa* during bacterial burden studies and for plasmid stability studies using zebrafish embryos, cells were plated on cetrimide selective agar (Acumedia, Lansing, MI).

Nucleic acid manipulations. Plasmid DNA preparations were isolated from 3-ml broth cultures by the alkaline lysis method (2, 24). DNA digests were performed in TA buffer (19). DNA fragments were separated in 0.8% agarose gels in TAE buffer (40 mM Tris, 20 mM acetic acid, 2 mM EDTA), stained with ethidium bromide, and photographed over a UV transilluminator. Restriction endonuclease-generated DNA fragments were purified using the QIAquick gel extraction kit (Qiagen). Vector DNA and fragments to be ligated were combined in equimolar ratios in 20-μl ligation mixes containing 1 μg of DNA. Ligations were performed in TA buffer at 13°C for 12 to 18 h with 1 U of T4 DNA ligase and 200 μM ATP. Competent cells of *E. coli* were prepared and transformed according to Cohen et al. (5).

Construction of pMMB66EH-RFP derivatives. pMMB66EH was digested with EcoRI, and the 3-prime recesses were filled in using the Klenow fragment of DNA polymerase I. The flush-ended vector was digested with HindIII, and agarose gel electrophoresis was used to purify vector DNA containing one flush end and a HindIII half site. Pigment gene inserts from pRSETB plasmids were isolated similarly. Plasmids were digested with XbaI, made flush ended with Klenow, and digested with HindIII, and pigment-gene-containing DNA fragments with one flush end and a HindIII half site were purified from agarose gels. Ligations were performed, and a portion of the ligation mix was used to transform *E. coli* HB101. Following overnight incubation at 37°C, Ap^r transformants were tested for pigment production on L agar-ampicillin plates containing IPTG.

Conjugal matings. The restricted-host-range ColE1 plasmid pRK2013 (Km^r) (11) was used to mobilize pMMB66EH derivatives during conjugal matings. Donor and recipient cell cultures were grown overnight with antibiotic selection and harvested by centrifugation, and cells were resuspended in fresh growth medium minus antibiotic. Donor and recipient cultures were mixed (10 A_{600} units of each) and filtered through 47-mm-diameter and 0.45-μm-pore-size membrane filters. Filters were incubated for 16 to 18 h at 26 or 37°C on the surface of L agar plates containing 1% NaCl. Cells were resuspended and diluted in L broth–1% NaCl, and dilutions were plated onto L agar plates (0.5 or 2% NaCl) supplemented with rifampin and ampicillin to select the desired exconjugants.

Plasmid stability in culture. The stability of pigment production was tested under nonselective culture conditions to determine if plasmids were maintained at frequencies that would enable their use for zebrafish infectivity studies. Five milliliters of L broth or L broth–2% NaCl was inoculated with ice crystals from frozen stocks of plasmid-containing cultures of *E. tarda* (EtR1), *P. aeruginosa* PA14, *V. anguillarum* 775, and *V. anguillarum* NVI5812, and cultures were grown overnight to saturation (A_{600} of 5 to 6). Serial 10-fold dilutions were prepared and plated on L agar or L agar–2% NaCl plates with and without ampicillin selection. Pigment production by cells harboring IPTG-inducible plasmids was verified by picking colonies to IPTG-containing medium. Following incubation, the percentages of Ap^s pigment-free colonies and Ap^r pink colonies were determined as an estimate of plasmid instability.

DNA sequencing. Automated DNA sequencing was performed using an ABI 3730 sequencer and the ABI Prism BigDye Terminator v. 3.1 cycle sequencing kit (Applied Biosystems). A 1,220-bp fragment containing the *lacI*^{q} gene was sequenced from plasmids pMMB66EH and p66TD*C*1 using the *lacI*^{q} forward primer 5′-GGCATTTACGTTGACACC-3′ and *lacI*^{q} reverse primer 5′-CTTTC CTGGCTTTGCTTCC-3′. The fragment sequenced included *lacI*^{q} and approximately 50 bp upstream and 90 bp downstream of *lacI*^{q}.

Zebrafish care and maintenance. Transgenic *fli1*::EGFP zebrafish (15) were obtained from Nathan Lawson (NIH, Bethesda, MD). Transgenic *mpo*::GFP zebrafish (22) were obtained from Stephen Renshaw (University of Sheffield, Sheffield, United Kingdom). Wild-type AB, Tg(*fli1*::EGFP), and Tg(*mpo*::GFP) zebrafish were housed at the University of Maine Zebrafish Facility in recirculating systems (Aquatic Habitats, Apopka, FL). Water temperature was maintained at 28°C with a total system flow rate of 150 liter/min. Zebrafish were maintained in accordance with University of Maine Institutional Animal Care and Use Committee guidelines. Adult zebrafish (>3 months old) were used for breeding embryos, which were reared at 28°C in petri dishes containing 60 ml of egg water (60 mg of Instant Ocean/liter of water). Until 48 h postfertilization (hpf), egg water was supplemented with methylene blue (0.3 mg/liter) to prevent fungal growth. Egg water was changed daily. For infection and challenge studies, embryos were transferred to and held in embryo-rearing medium (137 mM NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$).

Infection of zebrafish embryos. Bacterial cultures were grown for 12 h as described above and harvested by centrifugation, and cells were washed twice with Dulbecco's phosphate-buffered saline (PBS). Culture density was determined spectrophotometrically and was adjusted to 10^7 CFU/ml of PBS. Zebrafish embryos (48 hpf) were manually dechorionated and injected as described by Clatworthy et al. (4). Five nanoliters of culture was injected (50 CFU/embryo) into the duct of Cuvier using an MPPI-2 pressure microinjection system (Applied Scientific Instrumentation, Eugene, OR). As described previously (4), the duct of Cuvier (the yolk sac circulation valley) collects venous blood from the tail and trunk and returns it to the embryonic heart. Bacteria injected into the duct of Cuvier are introduced immediately into the embryonic circulatory system, and relative to the axial vein, the duct of Cuvier is a much larger and more easily injected target in embryos (4). Negative controls for infection studies included groups of PBS-injected embryos and embryos injected with heat-killed bacterial cultures. At 6 h postinfection (hpi), embryos were anesthetized (200 mg Tris-buffered tricane methanesulfonate/liter) and subjected to image analysis using an Olympus IX-81 inverted epifluorescence microscope equipped with a Hammatsu ORCA-ER charge-coupled-device (CCD) camera and IPLab software (BD Biosciences, Rockville, MD). Confocal microscopy was performed with an Olympus

TABLE 1. Bacterial strains and plasmids

Name	Relevant characteristic(s)	Source or reference(s)
Bacterial strains		
Escherichia coli HB101	Rif^s; cloning host and conjugal donor	3
Edwardsiella tarda	Ap MIC, <100 μg/ml	21
Edwardsiella tarda ETR1	Spontaneous Rif^r mutant of *E. tarda,* conjugal recipient for RFP plasmids	This work
Pseudomonas aeruginosa PA01	Ap MIC, 1,050 μg/ml	George O'Toole, Dartmouth Medical School
Pseudomonas aeruginosa PA01R1	Spontaneous Rif^r mutant of PAO1, conjugal recipient for RFP plasmids	This work
Pseudomonas aeruginosa PA14	Ap MIC, 600 μg/ml; Rif^r conjugal recipient for RFP plasmids	This work
Vibrio (*Listonella*) *anguillarum* 775(pJM1)	Serotype O1 marine fish pathogen; Ap MIC, 200 μg/ml	7
Vibrio (*Listonella*) *anguillarum* 775R1(pJM1)	Spontaneous Rif^r mutant of 775(pJM1), conjugal recipient for RFP plasmids	26
Vibrio (*Listonella*) *anguillarum* H775-3	pJM1-cured derivative of 775(pJM1)	6
Vibrio (*Listonella*) *anguillarum* H775-3R1	Spontaneous Rif^r mutant of H775-3, conjugal recipient for RFP plasmids	26
Vibrio (*Listonella*) *anguillarum* NVI5812	Serotype O2β cod pathogen; Ap MIC, 400 μg/ml	Una McCarthy, University of Maine
Vibrio (*Listonella*) *anguillarum* NVI5812R1	Spontaneous Rif^r mutant of NVI5812, conjugal recipient for RFP plasmids	This work
Plasmids		
pMMB66EH	8.8-kb Ap^r moderate-copy IncQ-based vector, mobilizable, *lacI*$^{q+}$, IPTG-inducible p*tac*	1, 12
pUC4-K	Source of 1.5-kb BamHI *kan* DNA fragment	31
p66-Km1	Carries 1.5-kb BamHI *kan* fragment from pUC4-K cloned into pMMB66EH; Ap^r Km^r, *lacI*$^{q+}$	This work
pRK2013	20-kb Km^r ColE1-based restricted-host-range helper plasmid, mobilizes pMMB66EH derivatives	9, 11
pRSETB d-Tomato	Source of 852-bp XbaI-HindIII d-Tomato DNA	25
pRSETB td-Tomato	Source of 1,578-bp XbaI-HindIII td-Tomato DNA	25
pRSETB m-Orange	Source of 858-bp XbaI-HindIII m-Orange DNA	25
pRSETB m-Cherry	Source of 848-bp XbaI-HindIII m-Cherry DNA	25
p66T1	pMMB66EH-based d-Tomato construct; Ap^r; encodes IPTG-inducible pigment production	This work
p66TD1	pMMB66EH-based td-Tomato construct; Ap^r; encodes IPTG-inducible pigment production	This work
p66M1	pMMB66EH-based m-Orange construct; Ap^r; encodes IPTG-inducible pigment production	This work
p66MC1	pMMB66EH-based m-Cherry construct, Ap^r; encodes IPTG-inducible pigment production	This work
p66TD*C*1	pMMB66EH-based td-Tomato construct; Ap^r; carries spontaneous *lacI* mutations resulting in constitutive pigment production	This work
p67T1	p66TD*C*1 BamHI-HindIII lacI$^-$ vector fragment carrying 718-bp BamHI-HindIII d-Tomato DNA; Ap^r; constitutive pigment production	This work
p67TD1	Like p67T1, but carrying 1,444-bp BamHI-HindIII td-Tomato DNA	This work
p67M1	Like p67T1, but carrying 724-bp BamHI-HindIII m-Orange DNA	This work
p67MC1	Like p67T1, but carrying 714-bp BamHI-HindIII m-Cherry DNA	This work

IX-81 microscope equipped with an Olympus FV-1000 laser-scanning confocal using the Olympus FV-10 software package.

Plasmid stability in injected embryos. *P. aeruginosa* PA14(p67T1) was cultured and injected into zebrafish embryos (50 CFU/embryo) as described above. Three independent replicates were performed with 20 embryos per replicate. Infected embryos were incubated at 28°C in embryo-rearing medium for 8 h. Embryos were harvested, washed in sterile PBS, and homogenized in 1 ml of L broth. Serial 10-fold dilutions were plated on cetrimide selective agar. Plates

XbaI–23 bp–Shine Dalgarno–7 bp–ATG–9 bp–6 His–62 bp–BamHI–ATG–Pigment Coding–Stop–HindIII

FIG. 1. General structure of pigment-coding regions in pRSETB plasmids carrying d-Tomato, td-Tomato, m-Orange, and m-Cherry DNAs. All pigment DNAs were carried on XbaI-HindIII fragments, and structural pigment sequences could be isolated only on smaller BamHI-HindIII fragments. Sequences upstream of the ATG start site for pigment production contained a ribosome binding site, 6-His codons, and a 62-bp region encoding an antigenic epitope for immunological detection (25).

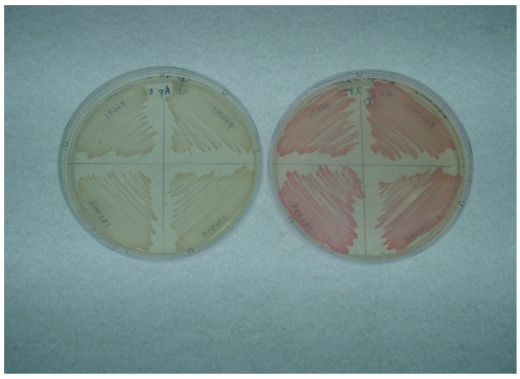

FIG. 2. *E. coli* HB101 strains carrying IPTG-inducible pMMB66EH derivatives expressing RFPs. Strains were streaked on L agar plates containing ampicillin. The plate on the right contained 0.4 mM IPTG. Plasmids carried by each strain are the following (clockwise from the top): p66MC1 (m-Cherry), p66M1 (m-Orange), p66TD1 (td-Tomato), and p66T1 (d-Tomato).

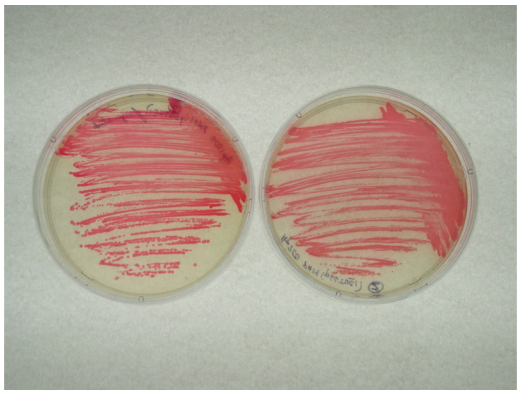

FIG. 3. *P. aeruginosa* PA01(p66TD*C*1) and PA14(p66TD*C*1) strains carrying a constitutive variant of p66TD1. Strains were streaked on L agar plates containing ampicillin only. The plate on the left shows pigment production by PA01(p66TD*C*1), and that on the right shows pigment production by PA14(p66TD*C*1).

were incubated at 37°C for 24 h, and plasmid-free colorless colonies were scored against results for red plasmid-containing colonies.

Bacterial burden studies. *P. aeruginosa* PA14 and *P. aeruginosa* PA14(p67T1) were cultured and injected into 20 zebrafish embryos (50 CFU/embryo) in four replicates as described above. After an 8-h incubation at 28°C in embryo-rearing medium, embryos were harvested, washed, and homogenized, and serial 10-fold dilutions were plated in duplicate on cetrimide selective agar. Plates were incubated at 37°C for 24 h, and mean titers of *P. aeruginosa* PA14 and *P. aeruginosa* PA14(p67T1) were determined. Standard errors of the means (SE) were calculated, and the Student's *t* test was used to determine statistical significance between mean titers.

Nucleotide sequence accession number. The GenBank nucleotide sequence accession number for the 1,222-bp *lacI* region sequence of p66TD*C*1 is HM020673.

RESULTS

Characterization of plasmids directing IPTG-inducible pigment synthesis. IPTG-inducible pigment production was observed in all Ap^r transformants of HB101. Plasmid DNA was characterized by digestion with BamHI and HindIII to verify that plasmids carried single inserts of the expected size. Plasmid p66T1 carried a diagnostic 718-bp d-Tomato insert, p66TD1 carried the expected 1,444-bp td-Tomato fragment, p66M1 carried a 724-bp m-Orange insert, and p66MC1 contained a 714-bp m-Cherry insert. Figure 2 shows *E. coli* HB101 transformants plated in the presence and absence of IPTG.

Mobilization of plasmids into *E. tarda*, *Pseudomonas*, and *V. anguillarum*. Plasmids p66T1, p66TD1, p66M1, and p66MC1 were transformed into *E. coli* HB101(pRK2013), and Ap^r Km^r transformants were used as donors in conjugal matings. Exconjugants of *E. tarda*, *P. aeruginosa*, and *V. anguillarum* were selected by plating serial 10-fold dilutions onto L agar plates supplemented with rifampin and ampicillin at concentrations that were 100 to 150 μg/ml above their MICs for plasmidless recipient strains. Rif^r Ap^r exconjugants of *E. tarda*, *P. aeruginosa* PA01 and PA14, and serotypes O1 and O2β of *V. anguillarum* were obtained at frequencies of 0.2 to 0.8/recipient plated. Rif^r Ap^r exconjugants were screened on medium containing IPTG to verify pigment production. Results were similar to those shown for *E. coli* in Fig. 2, and all exconjugants, with one exception, produced pigment only in the presence of IPTG.

Isolation of a p66TD1-containing exconjugant that directed constitutive expression of td-Tomato. When *E. coli* HB101 (pRK2013, p66TD1) was mated with *P. aeruginosa* PA14, a single deeply pigmented exconjugant colony was detected among nonpigmented colonies on selection plates containing rifampin and ampicillin (750 μg/ml). All other exconjugant colonies on the plate were pigmentless and were IPTG inducible when tested. A BIOLOG bacterial identification analysis on the pigmented exconjugant revealed 100% identity with *P. aeruginosa*.

Plasmid DNA was purified from this pigmented exconjugant and was transformed into *E. coli* HB101 for further characterization. Ap^r transformants of HB101 were pigmented even in the absence of IPTG. Restriction endonuclease digestions with BamHI and HindIII were performed to verify that the expected 1,444-bp td-Tomato insert was present. Restriction endonuclease fragmentation patterns instead revealed the presence of a smaller fragment that was indistinguishable in size from the single 718-bp d-Tomato insert carried by p66T1. It appeared that a recombination event had occurred between the tandem copies of the d-Tomato coding region, resulting in what was essentially a single copy of the d-Tomato insert. This plasmid was designated p66TD*C*1 to denote constitutive pigment expression. Figure 3 shows pigment production by exconjugants of *P. aeruginosa* PA01(p66TD*C*1) and PA14 (p66TD*C*1) in the absence of IPTG.

Characterization of p66TDC1. Two explanations that might account for constitutive pigment expression included the possibility of a spontaneous mutation in *lacI*^{q} carried on p66TD*C*1 or an operator-constitutive mutation in *tac.* If an operator-constitutive mutation in *tac* was responsible, introducing a second functional copy of *lacI*^{q} into the cell would have little or no effect on pigment production. Alternatively, if a spontaneous mutation in *lacI*^{q} occurred in p66TD*C*1, introducing a second functional copy of *lacI*^{q} would restore IPTG inducibility. These possibilities were tested by complementation analysis with *E. coli* HB101 (Table 2). To provide a second copy of *lacI*^{q}, p66-*kan* was constructed by inserting a kanamycin resistance

TABLE 2. Complementation of d-Tomato pigment production directed by p66TD*C*1

Strain	Pigment	
	−IPTG	+IPTG
HB101(p66T1)	Colorless	Pink
HB101(p66T1, p66-*kan*)	Colorless	Light pink
HB101(p66TD*C*1)	Pink	Deep pink
HB101(p66TD*C*1, p66-*kan*)	Colorless	Pink

determinant from plasmid pUC4-K into the BamHI site of pMMB66EH. Transformants of HB101 carrying dual plasmids were selected on L agar plates containing ampicillin and kanamycin.

Results shown in Table 2 demonstrate that *lacI*^{q} on p66-*kan* was able to partially repress pigment production directed by the *lacI*$^{q+}$ plasmid p66T1, even in the presence of 0.4 mM IPTG. Thus, two functioning copies of *lacI*^{q} resulted in the partial repression of *tac*, even in the presence of IPTG in HB101(p66T1, p66-*kan*). Table 2 also shows that constitutive pigment production by cells of HB101(p66TD*C*1) was increased to visibly higher levels by the treatment of cells with IPTG. This result is consistent with the presence of a chromosomal copy of *lacI* in *E. coli* HB101 (3). Further, in *lac*$^-$ strains of *P. aeruginosa* (p66TD*C*1) and *Vibrio anguillarum* (p66TD*C*1), levels of pigment production in the presence and absence of IPTG were indistinguishable (not shown). In HB101(p66TD*C*1, p66-*kan*), *lacI*^{q} carried on p66-*kan* was able to restore IPTG-inducible pigment production to levels that were indistinguishable from those observed with HB101(p66T1). Results in Table 2 indicated that a spontaneous mutation in *lacI*^{q} carried on p66TD*C*1 was responsible for constitutive pigment expression.

DNA sequencing of *lacI*^{q}. To further characterize constitutive pigment expression, the *lacI* region of p66TD*C*1 was sequenced and compared to the *lacI* carried by pMMB66EH. Results are shown in Fig. 4. The DNA sequence of *lacI* from pMMB66EH was as previously reported for *lacI* in *E. coli* (10), but rather dramatic changes had occurred between nucleotide positions 729 and 740 of *lacI* carried by p66TD*C*1. Figure 4 shows that a series of five transition mutations and four transversion mutations occurred from nucleotide positions 729 to 740, followed by a 2-bp insertion at nucleotide position 742. The substitution mutations would alter the amino acid sequence beginning with residue 244, and the frameshift mutation would result in a nonsense codon being encountered at what would normally be amino acid residue 274. Thus, it is likely that the LacI peptide encoded by p66TD*C*1 contains only

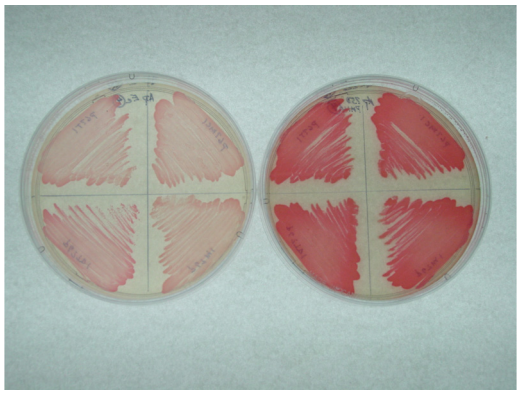

FIG. 5. *E. coli* HB101 (*lacI*$^+$) and *P. aeruginosa* PA14 (*lacI*$^-$) carrying constitutive p67 derivatives expressing RFPs. Strains were streaked on L agar plates containing ampicillin. *E. coli* HB101 is on the left and *P. aeruginosa* PA14 is on the right. Plasmids carried by each strain are the following (clockwise from the top): p67MC1 (m-Cherry), p67M1 (m-Orange), p67TD1 (td-Tomato), and p67T1 (d-Tomato).

243 of the normal 360 amino acids and is lacking 24% of the peptide at the carboxy terminus.

Plasmids that direct constitutive RFP expression. Plasmid p66TD*C*1 was digested with BamHI and HindIII, and the large vector fragment that drove constitutive pigment expression was purified from agarose gels. pRSETB derivatives carrying coding sequences for d-Tomato, td-Tomato, m-Orange, and m-Cherry were digested with BamHI and HindIII, and the 0.7- to 1.4-kb fragments containing only structural gene RFP sequences were purified from agarose gels. Following ligation, transformants were selected on L agar plates containing ampicillin, and as expected, all transformants were pigmented. Plasmids from these transformants were designated p67T1 (d-Tomato), p67TD1 (td-Tomato), p67M1 (m-Orange), and p67MC1 (m-Cherry) to distinguish them from the IPTG-inducible p66 series of plasmids.

Mobilization of plasmids into *E. tarda*, *Pseudomonas*, and *V. anguillarum*. The plasmids described above were isolated and transformed into *E. coli* HB101(pRK2013), and Ap^r Km^r transformants were used as conjugal donors as described above. Exconjugants were selected on L agar plates containing rifampin and ampicillin at 100 to 150 μg/ml above the MICs for recipient strains. Rif^r Ap^r exconjugants of *E. tarda*, *P. aeruginosa* PA01 and PA14, and both serotypes of *V. anguillarum* were obtained, and all exconjugants were pigmented. Figure 5 shows typical pigment production in *lacI*$^+$ *E. coli* HB101 transformants and *lacI*$^-$ exconjugants of *P. aeruginosa* PA14. The

```
                730         740         750         760         770
                 |           |           |           |           |
                 P   T   A   M   L   V   A   N   D   Q   M   A   L   G   A   M
pMMB66EH         CCA CTG CGA TGC TGG TTG CCA ACG ATC AGA TGG CGC TGG GCG CAA TGC
                     *   *** *   *** **
p66TDC1          CCA CTA CCG AGA TAT CCG CAC CAA CGA TCA GAT GGC GCT GGG CGC AAT
                 P   T   T   E   I   S   A   P   T   I   R   W   R   W   A   Q
```

FIG. 4. DNA sequence of *lacI*^{q} carried by plasmids pMMB66EH and p66TD*C*1. A 1,220-bp region containing the *lacI* genes of pMMB66EH and p66TD*C*1 was subjected to automated DNA sequencing. A portion of the sequence from nucleotide (nt) 724 to 771 is shown above. Numbers above the sequence denote nucleotide positions. Letters above and below the sequences indicate amino acid assignments. Single-base-pair substitutions are indicated by an asterisk. The 2-bp insertion at nt 742 in p66TD*C*1 *lacI* is underlined.

TABLE 3. Stability of RFP-expressing plasmids under nonselective conditions

Host strain	Plasmid loss (%) from overnight cultures				
	p66T1	p66TD1	p67T1	p67M1	p67MC1
EtR1	1	0	49	25	0
PA14	5	97	19	15	10
775R1	0	1	1	1	3
NVI5812R1	5	2	6	2	2

differences in levels of RFP expression are substantial. Similar results were obtained with *E. tarda* and *V. anguillarum* strains (not shown).

Plasmid stability. Plasmid stability was tested under nonselective culture conditions to determine if plasmids would be stable enough to be used for zebrafish infectivity studies. Results presented in Table 3 show that plasmid-free (Ap^s) pigmentless colonies of *E. tarda* arose from p66T1- and p66TD1-containing cultures at frequencies of 1% or less in overnight cultures. However, p67T1 (constitutive production of d-Tomato) was highly unstable in *E. tarda.* After overnight culturing in L broth, only about 50% of the cells plated were able to form colonies on medium containing ampicillin. In repeated trials using a single Ap^r pigmented colony of *E. tarda*(p67T1) as the inoculum, the plasmid stability of p67T1 was not improved. The constitutive expression of m-Orange resulted in a 25% plasmid loss from overnight cultures of *E. tarda*(p67M1), while constitutive m-Cherry expression resulted in no detectable plasmid instability in cultures of *E. tarda*(p67M1). Even though p67M1 and p67MC1 were retained at higher frequencies in *E. tarda* than was p67T1, less than 10% of the Ap^r colonies that retained plasmids after overnight culturing produced visible pigment on plates. Taken together, these results suggest that the constitutive expression of RFP variants is toxic and not tolerated by *E. tarda* hosts.

In *P. aeruginosa* PA14, the constitutive expression of d-Tomato (p67T1), m-Orange (p67M1), and m-Cherry (p67MC1) resulted in 10 to 19% plasmid loss from overnight cultures in the absence of selection (Table 3), but 100% of the Ap^r colonies that retained plasmid DNA formed highly pigmented colonies on plates. All of the IPTG-inducible constructs (p66 series) also were stable in *P. aeruginosa* PA14 (5% or less plasmid loss), with one exception. Plasmid p66TD1 carries an IPTG-inducible tandem duplication of the d-Tomato coding sequence. Table 3 shows that p66TD1 was lost from 97% of the cells after overnight culturing in the absence of ampicillin. Colonies that arose on ampicillin-containing medium all produced high levels of pigment in response to IPTG. In contrast, p66T1, which is essentially identical to p66TD1 except that it carries only a single copy of the d-Tomato coding sequence, was lost from only about 5% of the cells after growth under nonselective conditions. These results suggest that *P. aeruginosa* PA14 does not tolerate direct tandem repeats of d-Tomato DNA, even under conditions where pigment expression is repressed. The transfer of p66 and p67 series plasmids to two serotypes of *V. anguillarum* resulted in essentially no observed instability (Table 3). Plasmids were retained by at least 94% of the cells plated, and all Ap^r colonies that formed were pigment producers.

Plasmid stability in zebrafish embryos. Plasmid stability also was tested for p67T1 using injected zebrafish embryos. *P. aeruginosa* PA14(p67T1) was cultured and injected (50 CFU/embryo) as described in the text, and after an 8-h incubation embryos were harvested and homogenized, and the titers of plasmid-free colorless colonies were determined and compared to those of red plasmid-containing colonies by plating on cetrimide agar. In three separate replicates, two, four, and two colorless colonies were obtained from samples that gave rise to 2,732, 2,964, and 1,993 pigmented colonies, respectively. Overall, plasmid p67T1 was retained by 99.9% of PA14 cells present at 8 hpi. This result, combined with reasonable stability under nonselective culture conditions, made p67T1 an attractive candidate for the RFP labeling of *P. aeruginosa* PA14 to be used in zebrafish innate immunity studies.

Bacterial burden in zebrafish embryos. To determine if *P. aeruginosa* PA14 and *P. aeruginosa* PA14(p67T1) behaved in a similar fashion in injected embryos, bacterial burden assays were performed in quadruplicate as described in the text. Mean titers ± standard errors of the means (SE) were calculated per ml of embryo lysate for four trials. Data were subjected to statistical analysis using the Student's *t* test to determine if mean titers of *P. aeruginosa* PA14 and *P. aeruginosa* PA14(p67T1) were significantly different at 8 hpi. The mean titer ± SE for *P. aeruginosa* PA14 was $1.7 \times 10^4 \pm 0.4 \times 10^4$ CFU/ml; that for embryos injected with *P. aeruginosa* PA14(p67T1) was $1.2 \times 10^4 \pm 0.5 \times 10^4$ CFU/ml. Differences in mean titers were not statistically significant at the $P \leq 0.05$ level, and the *P* value associated with the Student's *t* test was 0.405.

Infection of zebrafish embryos with *P. aeruginosa* PA14 (p67T1). Zebrafish embryos at 48 hpf were challenged as described above with 50 CFU of *P. aeruginosa* PA14(p67T1) by microinjection in the duct of Cuvier, resulting in a lethal disseminated infection typical of that characterized previously by Clatworthy et al. (4) for PA14. RFP-labeled bacteria initially colonized the yolk but then spread to other regions of the fish, including the pericardial sac and tissues of the head, by 6 hpi (Fig. 6A). Red fluorescence was visible in each colonized tissue type using wide-field epifluorescence microscopy at ×40 magnification or higher. Red fluorescence was not observed in control embryos injected with heat-killed bacteria. When embryos from Tg(*fli1*::EGFP) and Tg(*mpo*::GFP) zebrafish were challenged as described above, the same temporal pattern of infection was observed, as well as the migration of GFP-labeled macrophages and neutrophils to the sites of infection and the phagocytosis of RFP-labeled bacteria by GFP-labeled macrophages (Fig. 6B) and GFP-labeled neutrophils (Fig. 6C). These results suggest that RFP-labeled bacteria will provide a valuable tool for the study of host-pathogen interactions and innate immunity in the zebrafish system.

DISCUSSION

We have constructed a series of moderate-copy-number, mobilizable, broad-host-range plasmids that express RFP variants d-Tomato, td-Tomato, m-Orange, and m-Cherry both constitutively and under IPTG control in *E. coli.* These IncQ-based plasmids were mobilized by pRK2013 into *E. tarda*, *P. aeruginosa*, and *V. anguillarum* strains. While we were inter-

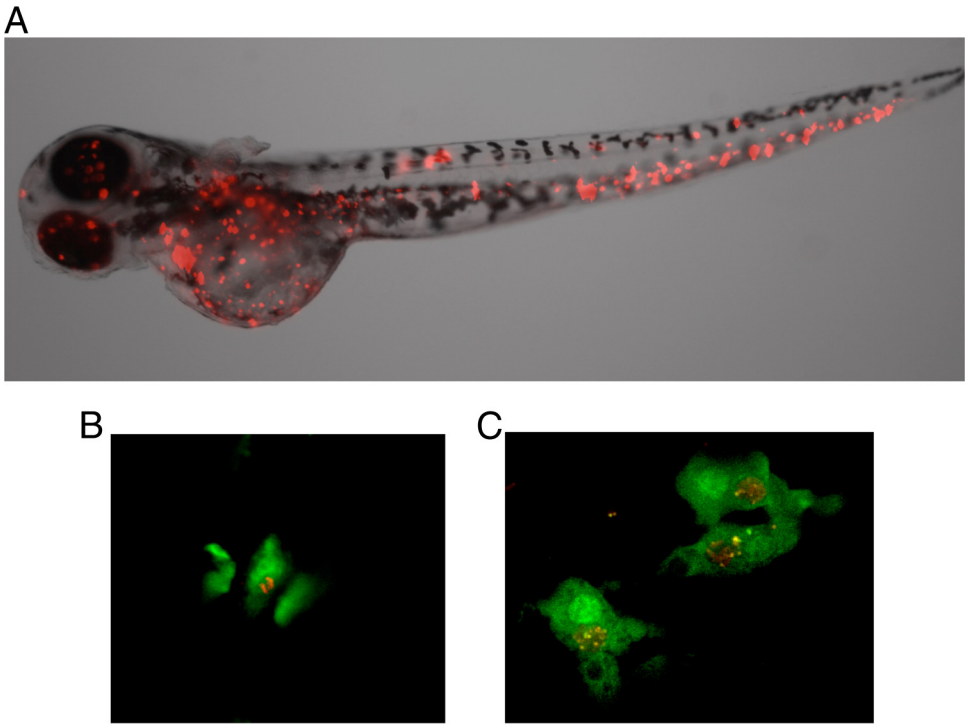

FIG. 6. Infection of zebrafish embryos (48 hpf) with *P. aeruginosa* PA14(p67T1) encoding d-Tomato. Embryos were microinjected with 50 CFU of *P. aeruginosa* PA14(p67T1) through the duct of Cuvier and imaged at 6 hpi. (A) Image analysis using an Olympus IX-81 inverted epifluorescence microscope equipped with a Hammatsu ORCA-ER CCD camera and IPLab software. Red fluorescence was observable at ×40 magnification (shown) or higher. Punctate fluorescence was observed in many tissues, including the vasculature, the yolk, and the pericardial sac. The image is an overlay of wide-field differential interference contrast (DIC) and fluorescence images. (B and C) Image analysis using an Olympus IX-81 and FV-1000 laser-scanning confocal microscope with Olympus FV-10 software. (B) Tg(*fli1*::EGFP) zebrafish express EGFP in macrophages (15) and were observed to contain phagocytosed *P. aeruginosa* PA14(p67T1) encoding d-Tomato. Three green fluorescent macrophages along the peripheral yolk are shown in association at ×1,000 magnification, with one containing RFP-labeled *P. aeruginosa* PA14(p67T1). (C) Tg(*mpo*::GFP) zebrafish express GFP in neutrophils (22) and were observed containing phagocytosed *P. aeruginosa* PA14(p67T1) encoding d-Tomato. The figure is at ×1,000 magnification. Filters providing excitation/emission at 543 nm/610 nm and 488 nm/510 nm were used for the detection of RFP and EGFP, respectively.

ested in developing RFP-labeled bacteria for use in studies of innate immunity in zebrafish, these plasmids also should permit the labeling of other Gram-negative bacteria. IncQ-based vectors have been used previously in *Acinetobacter* species (28) and other *Pseudomonas* species (12).

There are several advantages of using the plasmids described above for studies of infectivity. Biparental conjugal matings are used to introduce plasmids into recipient bacteria at plasmid transfer frequencies that approach 90%. Even under suboptimal conjugal mating conditions, due to large differences in optimal growth temperature between donor and recipient or different salt requirements, conjugal transfer frequencies do not fall below 20%. Thus, the need to develop transformation or electroporation procedures for organisms that may be poorly characterized is eliminated. The only requirement is the selection of a spontaneous Rif^r (or Nal^r) recipient strain to counterselect against the donor. The plasmids described above also can be used with bacteria that already exhibit significant levels of ampicillin resistance. We successfully used recipient bacteria that displayed ampicillin MICs greater than 1 mg/ml. This required that the MIC for each recipient strain be determined, and the concentration of ampicillin employed was 150 to 200 μg/ml above the MIC. An additional advantage of using the plasmids described above is that the vector portions of the p66 and p67 series of plasmids can be used for inserting additional RFP genes, such as m-Honeydew, m-Banana, m-Tangerine, and m-Strawberry, as described by Shaner et al. (25), as they all can be isolated on similarly sized BamHI-HindIII fragments.

Unlike GFP-labeled bacteria, the RFP-labeled bacteria constructed here form colonies that are brightly pigmented under normal room illumination. There is no need to perform phenotypic or molecular identification on colonies that arise from a moribund individual in an infectivity study, making bacterial load determinations on embryos or individual animals less laborious. A second advantage of using RFP-labeled instead of GFP-labeled bacteria is that the green background fluorescence of whole tissues, which is often encountered when trying to visualize GFP-labeled bacteria in tissue sections, is completely eliminated by the use of RFP-labeled bacteria. Lastly, the use of the constitutive d-Tomato construct p67T1 to monitor short-term infections in the zebrafish resulted in no significant differences in bacterial load between embryos injected with plasmidless and plasmid-containing RFP-producing pathogens.

While the advantages of using the plasmids reported here are considerable, there still are caveats associated with their use. The first deals with spontaneous plasmid loss in the absence of antibiotic selection. We observed that several constructs appeared to be unstable in the absence of antibiotic selection. The results presented in Table 3 indicate that insta-

bility is a function of both the bacterial host and the specific pigment being produced. For example, the constitutive production of d-Tomato and m-Orange by *E. tarda* lead to an apparent 49 and 25% curing of plasmids from overnight laboratory cultures in the absence of antibiotic selection. However, the IPTG-regulated constructs displayed virtually no instability. Only minor instability was observed with *V. anguillarum* hosts, regardless of whether pigment was under IPTG regulation or was being expressed constitutively. With Rec^+ *P. aeruginosa* PA14 backgrounds, gene duplication appeared to play a role in plasmid instability. Results in Table 3 show that plasmids containing a tandem dimer of the d-Tomato coding sequence were highly unstable, regardless of whether pigment production was constitutive or repressed in the absence of IPTG. Plasmids containing only a single d-Tomato coding region were far more stable, suggesting that homologous recombination across the tandem repeats of the d-Tomato coding sequence is responsible for instability. Plasmids such as p67T1 (d-Tomato), p67M1 (m-Orange), and p67MC1 (m-Cherry) were retained by 80 to 90% of cells after overnight culturing in the absence of selection, and all Ap^r colonies arising on selective media after plating were pigmented. In addition, p67T1 was retained by 99.9% of PA14 cells recovered 8 h after the infection of zebrafish embryos. This result represents an increase in plasmid stability above previously reported levels. Clatworthy et al. (4) observed a 26% plasmid loss from cells of PA14 carrying the constitutive GFP-expressing plasmid pSMC21 at just 7 hpi. Because most infectivity and innate immunity studies are conducted in zebrafish embryos for a duration of what is largely a matter of hours rather than days, the minor instability observed with plasmids such as p67T1 poses no obstacle to use in the zebrafish system.

The infection of zebrafish embryos at 48 hpf with *P. aeruginosa* PA14(p67T1) encoding d-Tomato was achieved by microinjection into the duct of Cuvier. The visualization of fluorescence in the pericardial sac and the head was possible using wide-field epifluorescence microscopy at a magnification of 40× or higher (Fig. 6A). In addition, green fluorescent macrophages from Tg(*fli1*::EGFP) embryos (Fig. 6B) and green fluorescent neutrophils from Tg(*mpo*::GFP) embryos (Fig. 6C) were observed to contain phagocytosed *P. aeruginosa* PA14 (p67T1), illustrating the value of using RFP-labeled strains for *in vivo* infection studies. The use of the RFP-labeled strains of bacteria described above provides a powerful tool for the characterization of infection and the innate immune response in the zebrafish model system.

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