

Use of mCherry Red Fluorescent Protein for Studies of Protein Localization and Gene Expression in *Clostridium difficile*

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Fluorescent proteins are powerful reporters in biology, but most require O₂ for chromophore maturation, making them inherently difficult to use in anaerobic bacteria. *Clostridium difficile*, a strict anaerobe with a genomic GC content of only 29%, is the leading cause of hospital-acquired diarrhea in developed countries, and new methods for studying this pathogen are sorely needed. We recently demonstrated that a cyan fluorescent protein called CFP_{opt} that has been codon optimized for production in low-GC bacteria can be used to study protein localization in *C. difficile* provided the cells are fixed prior to exposure to air. We describe here a codon-optimized variant of mCherry (mCherryOpt) that exhibits faster acquisition of fluorescence and a better signal-to-noise ratio than CFP_{opt}. We utilized mCherryOpt to construct plasmids for studying protein localization (pRAN473) and gene expression (pDSW1728) in *C. difficile*. Plasmid pRAN473 is an mCherryOpt fusion vector with a tetracycline-inducible promoter. To document its biological utility, we demonstrated septal localization of two cell division proteins, MldA and ZapA. Plasmid pDSW1728 is designed for cloning a promoter of interest upstream of mCherryOpt. As proof of principle, we studied the expression of the *pdaV* operon, which is required for lysozyme resistance. In confirmation and extension of previous reports, we found that expression of the *pdaV* operon requires the alternative sigma factor σ^V and that induction by lysozyme is dose dependent and uniform across the population of lysozyme-treated cells.

Clostridium difficile is a low-GC, spore-forming bacterium that is burdening the health care systems of developed countries (1–3). While genetic techniques to study *C. difficile* are becoming increasingly available, the repertoire of tools remains limited. This is due in part to the strictly anaerobic environment required to grow *C. difficile*.

Green fluorescent protein (GFP) can be produced in cells grown anaerobically, but it is unable to fluoresce because chromophore maturation requires O₂ for dehydration reactions that introduce double bonds into amino acids (4). Nevertheless, GFP produced in an anaerobic environment can mature and fluoresce upon subsequent exposure to air (4, 5). We recently took advantage of this observation to show that GFP can be used to localize cell division proteins in anaerobically grown *Escherichia coli* (6). Similarly, we showed that a derivative of cyan fluorescent protein named CFP_{opt} (because it has been codon optimized for low-GC bacteria) can be used to localize cell division proteins in anaerobically grown *C. difficile* (6). In both organisms, it was necessary to fix cells anaerobically to preserve their architecture and then expose them to air overnight to allow chromophore maturation, which required many hours. Fixation was necessary in the case of *E. coli* to ensure that the localization observed reflected anaerobic conditions rather than subsequent adaptation to air. In the case of *C. difficile*, fixation was necessary because cells lyse soon after transfer to air. Moreover, O₂ poisons energy metabolism in *C. difficile* and the divisome disassembles quickly (<2 min) when cells become depleted of energy (7–9).

One limitation of GFP and CFP for work in *C. difficile* is that the organism has considerable intrinsic green and blue autofluorescence. In contrast, there is virtually no red autofluorescence. We characterize here a codon-optimized allele of the gene encoding red fluorescent protein mCherry that we call “mCherryOpt.” An alternative red fluorescent protein, tdTOMATO, has been used with limited success as a reporter in *C. difficile*; however, requirements for its use have not been extensively investigated

(10). We focused our efforts on mCherry because it is half the size and folds much faster than tdTOMATO, although it is reported to be less bright (11). We show here that mCherryOpt produced in *C. difficile* is fully fluorescent within 2 h of exposure to air and that interference from intrinsic background fluorescence is negligible. We also describe plasmids that facilitate using mCherryOpt as a reporter of protein localization and gene expression in *C. difficile*. We hope that the availability of these plasmids and mCherryOpt will promote studies of the basic biology of *C. difficile* and other low-GC Gram-positive bacteria.

MATERIALS AND METHODS

Strains, media, and growth conditions. Bacterial strains are listed in Table 1. All *C. difficile* strains are derived from the erythromycin-sensitive JIR8094 isolate, which is in turn derived from the 630 sequenced strain (12, 13). *E. coli* OmniMAX 2 T1^R and XL1-Blue were used for cloning, and HB101/pRK24 was used for conjugations. Tryptone yeast extract (TY) medium consisted of 3% tryptone, 2% yeast extract, and 0.1% L-cysteine, plus 2% agar for plates. Luria-Bertani (LB) medium contained 10% tryptone, 5% yeast extract, and 1% NaCl, plus 1.5% agar for plates. *C. difficile* was grown in TY medium supplemented as needed with thiamphenicol

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TABLE 1 Strains used in this study

Strain	Genotype and/or description ^a	Source or reference
<i>E. coli</i>		
OmniMAX-2 T1 ^R	F' [<i>proAB</i> ⁺ <i>lacI</i> ^q <i>lacZ</i> ΔM15 Tn10(Tet ^r) Δ(<i>ccdAB</i>)] <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> ΔM15 Δ(<i>lacZYA-argF</i>)U169 <i>endA1 recA1 supE44 thi-1 gyrA96 relA1 tonA panD</i>	Invitrogen
XL1-Blue	<i>endA1 gyrA96</i> (Nal ^r) <i>thi-1 recA1 relA1 lac glnV44</i> [F' <i>proAB</i> ⁺ <i>lacI</i> ^q Δ(<i>lacZ</i>)M15] <i>hsdR17</i> (r _K ⁻ m _K ⁺) Tn10(Tet ^r)	Agilent
HB101/pRK24	F ⁻ <i>mcrB mrr hsdS20</i> (r _B ⁻ m _B ⁻) <i>recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20</i>	47
EC3272	XL1-Blue/pDSW1728 (P _{tet} :: <i>mCherryOpt cat</i>)	This study
RAN473	OmniMAX/pRAN473 (P _{tet} :: <i>mCherryOpt</i> -MCS <i>cat</i>)	This study
<i>C. difficile</i>		
JIR8094	Spontaneous Em ^s derivative of strain 630	12
TCD20	<i>csfV</i> mutant	35
RAN346	JIR8094/pRAN334 (P _{tet} :: <i>cfp_{opt} cat</i>)	6
RAN347	JIR8094/pRAN332 (P _{tet} :: <i>gfpmut2 cat</i>)	This study
RAN429	JIR8094/pRAN416 (P _{tet} :: <i>mldA cat</i>)	This study
RAN433	JIR8094/pRPF185 (P _{tet} :: <i>gusA cat</i>)	6
RAN445	JIR8094/pDSW1728 (P _{tet} :: <i>mCherryOpt cat</i>)	This study
RAN538	JIR8094/pRAN534 (P _{tet} :: <i>mCherryOpt-zapA_{Cd} cat</i>)	This study
RAN539	JIR8094/pRAN535 (P _{tet} :: <i>mCherryOpt-mldA cat</i>)	This study
RAN829	JIR8094/pRAN738 (P _{pdaV} :: <i>mCherryOpt cat</i>)	This study
RAN836	TCD20/pRAN738 (P _{pdaV} :: <i>mCherryOpt cat</i>)	This study

^a Nal^r, nalidixic acid resistant; Tet^r, tetracycline resistant; Em^s, erythromycin sensitive.

(Thi) at 10 μg/ml, kanamycin at 50 μg/ml, or cefoxitin at 16 μg/ml. Genes under the control of the P_{tet} promoter were induced with anhydrotetracycline hydrochloride (aTet; Sigma, St. Louis, MO). *C. difficile* strains were maintained at 37°C in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) in an atmosphere of 10% H₂, 5% CO₂, and 85% N₂. *E. coli* strains were grown at 37°C in LB medium supplemented as needed with ampicillin at 200 μg/ml or chloramphenicol at 20 μg/ml.

Plasmid and strain construction. The oligonucleotide primers used in the present study are listed in Table 2 and were synthesized by Integrated DNA Technologies (Coralville, IA). All plasmids are listed in Table 3. Newly constructed plasmids for the present study were derived from pRPF185, which has a tetracycline-inducible promoter upstream of the

gusA gene (14). To replace *gusA* with *gfpmut2*, primers RP157 and RP158 were used to amplify *gfpmut2* from pGFPmut2 (15) by PCR. The resulting PCR product was digested with SacI and BamHI and ligated into the same sites of pRPF185 to generate pRAN332. *mCherryOpt* was synthesized by GeneArt (Life Technologies, Grand Island, NY) and delivered in a high-copy-number plasmid named pMA-T-*mCherryOpt*. This plasmid was digested with SacI and BamHI, and the 735-bp fragment encoding *mCherryOpt* was ligated into SacI/BamHI-digested pRPF185 to generate pDSW1728. To create a derivative with an in-frame multiple-cloning site (MCS) suitable for making gene fusions, *mCherryOpt* was amplified by PCR with pDSW1728 as the template and primers RP204 and RP203, the latter of which encodes the MCS. The resulting PCR product was digested

TABLE 2 Oligonucleotide primers used in this study

Oligonucleotide	Purpose	Sequence (5'-3') ^a
RP157	Cloning <i>gfpmut2</i> into pRPF185	GGGGAGCTCCTGCAGTAAAGGAGAAAAATTTATGAGTAAAGGAGAAGAA CTTTCACTGG
RP158	Cloning <i>gfpmut2</i> into pRPF185	CCCGGATCCTTATTTGTATAGTTCATCCATGCCATGTG
RP176	Cloning <i>zapA</i> into pRAN357	AAAGCATGCATGAACAAAGTAATGGTAAAATCCATGG
RP177	Cloning <i>zapA</i> into pRAN357	TTTGGCGCGCCTTATCCACATTTTTCATCATTATTTAACC
RP204	Cloning <i>mCherryOpt</i> with MCS into pRPF185	GGGGAGCTCCTGCAGTAAAGGAGAAAAATTTATGG
RP203	Cloning <i>mCherryOpt</i> with MCS into pRPF185	GGCGGATCCGGCGCGCCTCAGCTGTTTAAATTAAGTCGACGCATGCGTT CATTATATAATTCATCCATACCTCC
RP206	<i>mCherryOpt</i> sequencing primer	CAAATTCATGTCCATTAACAGATCCTTCC
RP207	<i>mCherryOpt</i> sequencing primer	GATATAATGTTAATATTAATTAAGATATAAC
RP306	Cloning <i>pdaV</i> promoter in front of <i>mCherryOpt</i>	AAAAGCTAGCATGTGGCAAATAGTTGTTTGGCTATTTATTATTG
RP307	Cloning <i>pdaV</i> promoter in front of <i>mCherryOpt</i>	TTTGAGCTCTACATTATATTTTGTAGTATTTTATCCAAAAATTTACAC
TEQ009	qPCR primer for <i>rpoB</i>	AAGAGCTGGATTTCGAAGTGCCTGA
TEQ010	qPCR primer for <i>rpoB</i>	ACCGATATTTGGTCCCCTCTGGAGT
RP314	qPCR primer for <i>catR</i>	GAAGGTTGACCAACGGTATCAT
RP315	qPCR primer for <i>catR</i>	CGCAACGGTATGGAAACAATC
RP316	qPCR primer for <i>mldA</i>	AGTGGTTATTGTTGGTGTAGGA
RP317	qPCR primer for <i>mldA</i>	GCTTGTGCTGAGTTGATGA
RP376	qPCR primer for <i>pdaV</i>	TGTTTCGCGTCAGCTCTTT
RP377	qPCR primer for <i>pdaV</i>	ACTTGGCCCTTTACTAACTTCT
RP378	qPCR primer for <i>mCherryOpt</i>	AGAAGGAGAAGGAGAAGGAAGA
RP379	qPCR primer for <i>mCherryOpt</i>	AATGGTAATGGACCACCTTTAGT

^a Restriction sites are underlined.

TABLE 3 Plasmids used in this study

Plasmid	Relevant features	Source or reference
pGFPmut2	<i>gfpmut2</i>	15
pMA-T-mCherryOpt	Synthesized <i>mCherryOpt</i>	This study
pRPF185	<i>E. coli-C. difficile</i> shuttle vector with tetracycline-inducible promoter; P _{tet} :: <i>gusA cat CD6ori RP4oriT-traJ pMB1ori</i>	14
pDSW1728	P _{tet} :: <i>mCherryOpt cat</i>	This study
pRAN332	P _{tet} :: <i>gfpmut2 cat</i>	This study
pRAN334	P _{tet} :: <i>cfp_{opt} cat</i>	6
pRAN357	P _{tet} :: <i>cfp_{opt}-MCS cat</i>	6
pRAN409	P _{tet} :: <i>cfp_{opt}-zapA_{Cd}</i>	This study
pRAN410	P _{tet} :: <i>cfp_{opt}-mldA</i>	6
pRAN416	P _{tet} :: <i>mldA</i>	6
pRAN473	P _{tet} :: <i>mCherryOpt-MCS cat</i>	This study
pRAN534	P _{tet} :: <i>mCherryOpt-zapA_{Cd} cat</i>	This study
pRAN535	P _{tet} :: <i>mCherryOpt-mldA cat</i>	This study
pRAN738	P _{pdaV} :: <i>mCherryOpt cat</i>	This study

with SacI and BamHI and then ligated into the same sites of pRPF185 to create pRAN473. PCR was used to amplify a *zapA* homolog (*cd0701*) from the JIR8094 chromosome with primers RP176 and RP177. The resulting PCR fragment was digested with SphI and AscI and then ligated into pRAN357 (6) to create pRAN409 (P_{tet}::*cfp_{opt}-zapA_{Cd}*). Digestion of pRAN409 with SphI and AscI yielded a *zapA* restriction fragment that was ligated into SphI/AscI-digested pRAN473 to create pRAN534 (P_{tet}::*mCherryOpt-zapA_{Cd}*). To create pRAN535 (P_{tet}::*mCherryOpt-mldA*), *mldA* was moved as a SphI/AscI restriction fragment from pRAN410 (P_{tet}::*cfp_{opt}-mldA*) (6) into pRAN473. To construct pRAN738, the *pdaV* promoter was amplified from JIR8094 chromosomal DNA using the primers RP306 and RP307. The PCR product was digested with NheI and SacI and ligated into pRPF185 digested with the same enzymes. The resulting plasmid carries sequences extending from positions -320 to -29 with respect to the A of the ATG start codon of *pdaV*. All plasmids were introduced into *C. difficile* strains by conjugation from strain HB101/pRK24 and selecting for thiamphenicol resistance (16, 17).

Bioinformatics. Codon usage was analyzed with the Codon Usage Database at <http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=272563> (18). *ZapA* sequences were aligned using Clustal Omega (19).

Comparison of mCherryOpt, CFP_{opt} and GFPmut2. To compare different fluorescent proteins, *C. difficile* JIR8094 containing various plasmids was grown overnight in TY with 10 µg of Thi/ml. The next morning, these cultures were diluted 1:100 into the same medium containing aTet at 400 ng/ml to induce expression of plasmid-borne genes. Cultures were grown to an optical density at 600 nm (OD₆₀₀) of 0.6, and then cells from 500 µl of culture were fixed as described below, washed, suspended in 30 µl of phosphate-buffered saline (PBS), and processed for microscopy as described previously (6, 20). For convenience, fluorescence was typically allowed to develop during an overnight (~16 h) exposure to air, although much shorter incubations are sufficient for mCherryOpt.

Kinetics of fluorescence acquisition. To determine the rate of mCherryOpt maturation, cultures were grown and processed as described above except that, as a control, an aliquot of cells was processed without fixation. Fluorescence was monitored by microscopy or using an Infinite M200 Pro plate reader (Tecan, Research Triangle Park, NC). To monitor fluorescence in the plate reader, 20 µl of cells in PBS was added to 180 µl of PBS and transferred to the well of a flat-bottom 96-well microtiter plate (AS Plate-PS-96-F-C; AG Advangene, IL). Fluorescence was recorded at 15-min intervals (excitation, 554 nm; emission, 610 nm; gain setting, 100) with a 10-s shake and a 20-s pause immediately prior to each reading. The cell density (OD₆₀₀) was recorded at the start and end of the experiment to correct for cell number but not more often in order to minimize the bleaching of mCherryOpt.

Protein localization. For studies of protein localization, we recommend inducing at a variety of aTet concentrations for a variety of times

because each fusion protein is unique. We observed convincing septal localization of mCherryOpt fusions to MldA and ZapA using the simple induction protocol described above wherein an overnight culture is diluted 1:100 into TY containing aTet and grown to mid-log phase before fixation. However, the micrographs shown in the present study were obtained by diluting overnight cultures 1:100 into TY containing a low level of aTet (20 ng/ml) and grown to an OD₆₀₀ of 0.3. A higher concentration of aTet (80 to 160 ng/ml; it varied from day to day) was then added to boost the expression of plasmid-borne gene fusions. After 1 h, the cells were fixed and processed for microscopy as described below and elsewhere (6).

Quantifying expression of P_{pdaV}::mCherryOpt. To monitor the induction of P_{pdaV}::*mCherryOpt* by lysozyme, overnight cultures grown in TY containing 10 µg of Thi/ml were diluted 1:100 into the same medium. To determine the effect of a 4-h induction, lysozyme (or water, as a control) was added when the OD₆₀₀ reached 0.05, and incubation was continued for 4 h until the OD₆₀₀ reached 0.8. To determine the effect of a 1-h induction, lysozyme (or water) was added at an OD₆₀₀ of 0.4, and incubation was continued for 1 h until the OD₆₀₀ reached 0.8. An aliquot of each culture was transferred to ethanol-acetone, and the mRNA levels were determined by quantitative reverse transcription-PCR (qRT-PCR) as described previously (6, 17) using Superscript III and Sybr green master mix (Life Technologies, Carlsbad, CA) with the following gene-specific primer pairs: *ropB*, TEQ009 and TEQ010; *pdaV*, RP376 and RP377; and *mCherryOpt*, RP378 and RP379. A second aliquot of each culture was fixed with paraformaldehyde-glutaraldehyde, and mCherryOpt fluorescence was measured using a Tecan plate reader as described above.

Fixation protocol. Cells were fixed for microscopy by adapting procedures developed for *Bacillus subtilis* (20). A 16% (wt/vol) paraformaldehyde aqueous solution (methanol-free; catalog no. AA433689M) was obtained from Alfa Aesar (Ward Hill, MA). A 25% glutaraldehyde aqueous solution (catalog no. 16220) was obtained from Electron Microscopy Services (Hatfield, PA). A 5× fixation cocktail was prepared fresh each day and consisted (per sample) of 20 µl of 1 M NaPO₄ buffer (pH 7.4), 100 µl of 16% paraformaldehyde, and 4 µl of 25% glutaraldehyde. The cocktail was transferred into the anaerobic chamber immediately prior to use, and 120-µl aliquots were dispensed to 1.5-ml microcentrifuge tubes that had been in the chamber for at least 24 h. A 500-µl aliquot of cells (OD₆₀₀ ~ 0.6) in growth medium was added directly to a microcentrifuge tube containing 120 µl of the fixation cocktail, mixed by pipetting up and down three times, and then allowed to sit for 30 min, at which time the sample was removed from the chamber and incubated on ice for 60 min. Fixed cells were washed three times with PBS, resuspended in 30 µl of PBS, and left in the dark at room temperature to allow chromophore maturation. Because the fixation step was typically not completed until late in the afternoon, fluorescence microscopy was usually not performed until the

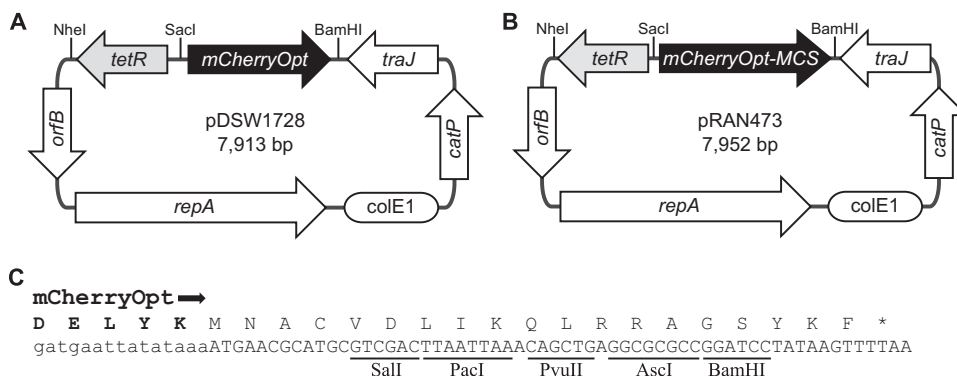


FIG 1 Genetic maps of *C. difficile* mCherryOpt plasmids. (A) pDSW1728, for studies of gene expression. Expression of *mCherryOpt* is under P_{tet} control, but the promoter-regulatory region can be replaced using the *NheI* and *SacI* restriction sites. (B) pRAN473, for studies of protein localization. (C) Multicloning site (MCS) in pRAN473. The last five amino acids derived from mCherryOpt are shown in boldface above the DNA sequence. Both plasmids are derivatives of pRPF185. Features depicted: *mCherryOpt* or *mCherryOpt-MCS* encodes mCherryOpt protein codon optimized for expression in low-GC bacteria; *tetR* encodes the Tet repressor from Tn10; *orfB* and *repA*, the replication region; ColE1 ori, replication region of the *E. coli* plasmid pBR322 modified for higher copy number; *catP*, the chloramphenicol acetyltransferase gene from *Clostridium perfringens*, conferring resistance to thiamphenicol in *C. difficile* or chloramphenicol in *E. coli*; *traJ*, encodes a conjugation transfer protein from plasmid RP4.

next morning but, as shown below, exposure to air for 2 h appears to be sufficient for mCherryOpt to reach maximum fluorescence. Shorter fixation times (e.g., 15 min at room temperature plus 30 min on ice) and removal of the samples from the anaerobic chamber immediately after mixing with the fixation cocktail yielded similar results (not shown).

Microscopy. For imaging, the cells were immobilized on thin agarose pads. Phase-contrast and fluorescence micrographs were recorded on an Olympus BX60 microscope equipped with a $\times 100$ UPlanApo objective (numerical aperture, 1.35). Images were captured using a black-and-white Spot 2-cooled charge-coupled device camera (Diagnostic Instruments, Sterling Heights, MI) with a KAF1400E chip (class 2), a Uniblitz shutter, and a personal computer with Image-Pro software version 4.1 (Media Cybernetics, Silver Spring, MD). Filter sets for fluorescence imaging were from Chroma Technology Corp. (Burlington, VT). The GFP filter set (catalog no. 41017) comprised a 450- to 490-nm excitation filter, a 495-nm dichroic mirror (long pass), and a 500- to 550-nm emission filter. The CFP_{opt} filter (catalog no. 31044v2) comprised a 426- to 446-nm excitation filter, a 455-nm dichroic mirror (long pass), and a 460- to 500-nm emission filter (band pass). For mCherryOpt the filter set (catalog no. 41004) comprised a 538- to 582-nm excitation filter, a 595-nm dichroic mirror (long pass), and a 582- to 682-nm emission filter.

Fluorescence micrographs were captured using 3-s exposure times and identical display range settings in all cases, so the images can be compared directly. Black level subtraction and chip defect correction were “on”; all other processing options were “off.” To ensure comparability of fluorescence images, the display range option was adjusted identically for all images. Micrographs were cropped, and figures were assembled in Adobe Illustrator (Adobe Systems, Inc., San Jose, CA).

Plasmid copy number. Real-time qRT-PCR experiments were performed as previously described (17) using Sybr green master mix (Applied Biosystems) and the following gene-specific primer pairs: *rhoB*, TEQ009 and TEQ010; *catR*, RP314 and RP315; and *mldA*, RP316 and RP317. *C. difficile* chromosomal DNA was harvested from cells grown to mid-log phase. Cells were lysed as described previously (17), except that the lysozyme step was omitted. The DNA was extracted using a phenol-chloroform protocol (21).

Plasmid stability. Plasmid maintenance was determined using a protocol similar to that described by Heap et al. (22). Briefly, JIR8094 containing various plasmids was grown overnight in TY medium with Thi at 10 $\mu\text{g}/\text{ml}$. Residual Thi was removed by pelleting cells from 1 ml of the culture and suspending the pellet in 1 ml of TY medium without antibiotic. Washed cells were diluted 1:100 into TY medium without antibiotic every 12 h for 3.5 days, for a total of seven passages, at which point dilu-

tions of the cultures were plated on TY without antibiotic. At least 50 colonies were patched onto TY medium $\pm 10 \mu\text{g Thi}/\text{ml}$ to determine the fraction of cells that contained the plasmid. Strain JIR8094 (Thi^s) was patched as a negative control. Assuming cultures return to maximum cell density in 12 h, there are 6.64 doublings per 12-h growth period (i.e., $2^{6.64} = 100$), which corresponds to 46 generations after seven passages. Segregational stability per generation was calculated using $R^{1/n}$, where n is the number of generations, and R is the fraction of Thi^r colonies.

Nucleotide sequence accession number. The DNA sequence of codon-optimized mCherry (*mCherryOpt*) has been submitted to GenBank and is available under the accession number [KM983420](#).

RESULTS

mCherryOpt is superior to CFP in *C. difficile*. Many factors go into determining which fluorescent protein is “best” for any particular use (23). The monomeric red fluorescent protein mCherry has a generally favorable combination of brightness, photostability, and rapid maturation, but it has a relatively high GC content because it has been codon optimized for expression in mammalian cells (11, 23). The GC content of the *C. difficile* 630 chromosome is only 29% (13). Analysis of the DNA sequence of mCherry revealed that it is 62% GC and that >90% of the codons are not commonly used in endogenous *C. difficile* genes (see Table S1 in the supplemental material). Sastalla et al. showed that production of fluorescent proteins in low-GC Gram-positive bacteria is greatly improved by modifying the genes to make them more AT-rich (24). We therefore designed a synthetic *mCherry* gene that was codon optimized for expression in *C. difficile* (see Fig. S1 in the supplemental material). The modified gene, which we will refer to as *mCherryOpt*, is 30% GC, and only 1% of the codons are unfavorable for *C. difficile* (see Table S1 in the supplemental material).

We constructed pDSW1728 by cloning *mCherryOpt* into pRPF185 (14), an *E. coli*-*C. difficile* shuttle vector with a tetracycline-inducible promoter (Fig. 1). Exconjugants of *C. difficile* were bright red when grown in TY medium containing anhydrotetracycline (aTet) and visualized by fluorescence microscopy (Fig. 2). Half-maximal fluorescence intensity was obtained after ~ 1 h of exposure to air and did not require fixation (Fig. 3). However, there was extensive lysis in the unfixed samples (data not shown),

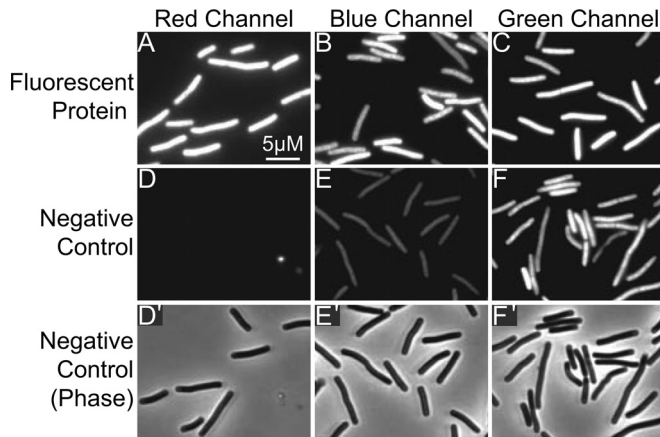


FIG 2 Comparison of fluorescent proteins and autofluorescence. The top row of micrographs shows *C. difficile* exconjugants producing mCherryOpt from pDSW1728 (A), CFP_{opt} from pRAN334 (B), or GFPmut2 from pRAN332 (C). Cells were photographed using filters appropriate for each protein as indicated above the images. The middle row shows *C. difficile* carrying pRPF185, which does not produce any fluorescent protein, photographed with the same filter sets (D, red; E, blue; F, green). The bottom row shows phase-contrast micrographs corresponding to images D to F.

so fixation is necessary for studies that require preservation of cellular architecture. Importantly, control cells carrying the empty vector pRPF185 had essentially no detectable fluorescence in the red channel. (The bright spots visible in Fig. 2D come from debris and were useful for aligning the fluorescence and phase images.) Although *C. difficile* strains producing CFP_{opt} or GFPmut2 were fluorescent in the blue and green channels, respectively, the corresponding pRPF185 controls had considerable background fluorescence, particularly in the green channel. Thus, at least in *C. difficile*, mCherryOpt has a significantly better signal-to-noise ratio than the other two fluorescent proteins.

Application of mCherryOpt to protein localization. We constructed pRAN473 by introducing an in-frame MCS at the 3' end of mCherryOpt to facilitate fusing mCherryOpt to the N terminus of proteins of interest (Fig. 1). As proof of principle, we fused mCherryOpt to MldA (locus tag CD2717), a recently described cell division protein found only in *C. difficile* and a few of its closest relatives (6). We reported previously that a CFP_{opt}-MldA fusion localized to the division site in ~35% of cells growing in TY (6). Similar results were obtained with mCherryOpt-MldA, with septal localization apparent in 41% ± 3% of the population (mean ± the standard deviation, *n* = three independent experiments with >100 cells scored per experiment) (Fig. 4).

Next, we sought to determine whether mCherryOpt could be used to verify the identity of a predicted *C. difficile* cell division protein. ZapA is a widely conserved FtsZ-binding protein that has been studied primarily in *E. coli* and *Bacillus subtilis*, where it localizes sharply to the septal ring (25–30). ZapA proteins have two domains, a globular head that binds FtsZ and a C-terminal coiled-coil that mediates tetramerization (31, 32). In *C. difficile* 630, locus CD0701 is annotated as a “putative cell division protein” based on its similarity to ZapA (13, 33), and we will refer to it henceforth as ZapA_{Cd}. However, whether ZapA_{Cd} is in fact a ZapA ortholog is not completely clear based on bioinformatic approaches alone; ZapA_{Cd} is ~100 amino acids longer than ZapA_{Ec} or ZapA_{Bs}, and amino acid identity in the region of overlap

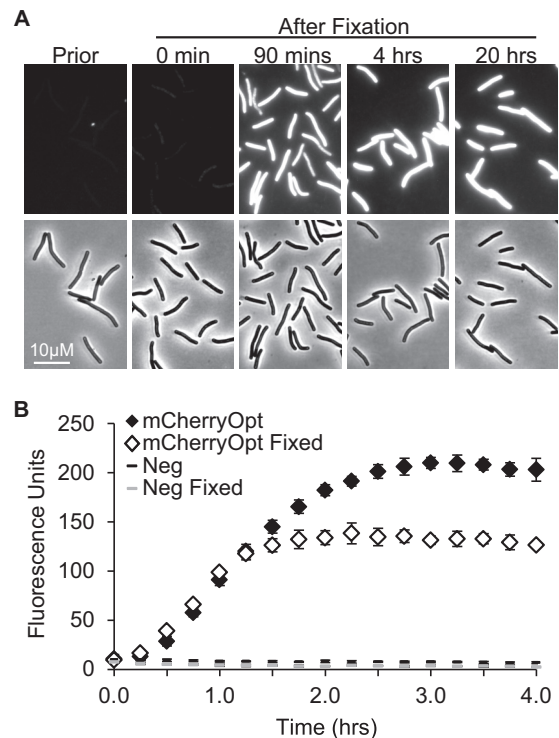


FIG 3 Time course of fluorescence development. (A) *C. difficile* exconjugants producing mCherryOpt from pDSW1728 were induced with 400 ng of aTet per ml, fixed, removed from the anaerobic chamber, and photographed at the times indicated. “Prior” refers to an unfixed sample photographed immediately after removal from the anaerobic chamber. (B) *C. difficile*/pDSW1728 (mCherryOpt) or *C. difficile*/pRPF185 (Neg) were grown and induced as described above. Cells were fixed or not as indicated, transferred to PBS, and removed from the anaerobic chamber. Fluorescence (red filter) was recorded using a plate reader at 15-min intervals and normalized to the OD₆₀₀ at time zero. The data points represent the means and standard deviations of three independent cultures per group, all grown and processed on the same day. These results are representative of three experiments.

is modest, e.g., 14% identity to ZapA_{Ec} and 33% identity to ZapA_{Bs} (see Fig. S2 in the supplemental material) (19). Nevertheless, we found that an mCherryOpt-ZapA_{Cd} fusion protein localized near the midcell in 38% ± 3% of *C. difficile* cells (*n* = three independent experiments with >100 cells scored per experiment) (Fig. 4). Because not all mCherryOpt-ZapA_{Cd} bands were precisely at the midcell, we sought independent evidence that they correspond to division sites. Measurements of the distance from the cell poles to the bands revealed most are at or near the midcell, and their spatial distribution mimics division septa as determined by staining with the membrane dye FM4-64 (see Fig. S3 in the supplemental material). These findings are consistent with the prediction that CD0701 is a ZapA ortholog. The anomaly of ZapA_{Cd} being ~100 amino acids longer than ZapA_{Ec} or ZapA_{Bs} can be attributed to a much longer predicted coiled-coil domain (Pfam version 27.0 [34]).

Application of mCherryOpt as a reporter of gene expression. In *C. difficile* the *pdaV* promoter (P_{pdaV}) directs transcription of a seven-gene operon necessary for resistance to lysozyme (17, 35). One of the genes in the operon is *csfV*, which codes for an extracytoplasmic function (ECF) sigma factor required for transcription from P_{pdaV} (35). To test the suitability of mCherryOpt as a

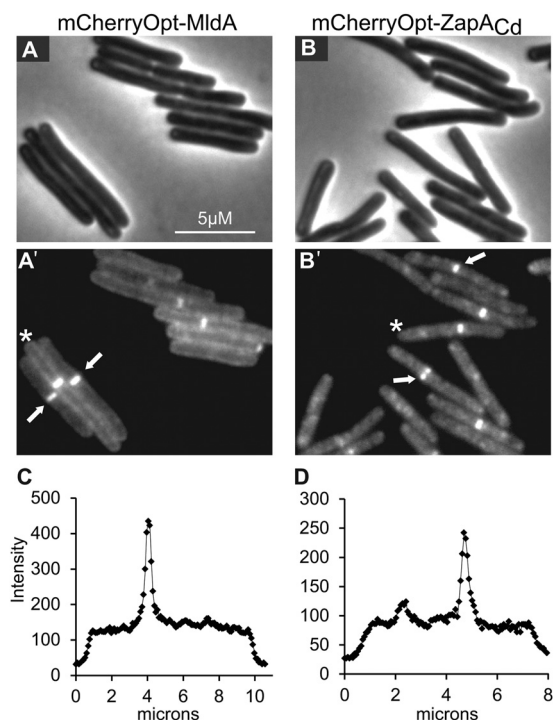


FIG 4 Septal localization of mCherryOpt fusions to the division proteins MldA and ZapA. *C. difficile* cells harboring pRAN535 (mCherryOpt-MldA) or pRAN534 (mCherryOpt-ZapACd) were fixed, removed from the anaerobic chamber, and photographed the next morning. (A and B) Phase-contrast images. (A' and B') Fluorescence images. Arrows indicate examples of septal localization. (C and D) Quantitation of fluorescence intensity along transects through the cells marked with an asterisk in panels A' and B'. The transects begin $\sim 1 \mu\text{m}$ before each cell (i.e., the end with the asterisk) and extend $\sim 1 \mu\text{m}$ beyond each cell. As noted elsewhere (6) and as documented in Fig. S3 in the supplemental material, *C. difficile* does not always divide precisely at the midcell.

gene expression reporter in *C. difficile*, we replaced the P_{tet} promoter-regulatory region in pDSW1728 with an ~ 300 -bp DNA fragment carrying the promoter-regulatory region for the *pdaV* operon. The resulting $P_{\text{pdaV}}::\text{mCherryOpt}$ reporter plasmid is designated pRAN738.

C. difficile exconjugants harboring pRAN738 were grown to mid-log phase in TY medium, exposed to lysozyme for 1 h, and then fixed and exposed to air overnight to allow for chromophore maturation. Fluorescence microscopy revealed the expected lysozyme-dependent increase in fluorescence intensity, which was completely dependent upon the ECF sigma CsfV (Fig. 5A and B). Fluorescence was relatively uniform from cell to cell, indicating the plasmid is well maintained and that lysozyme reaches all cells in the population. The fact that the wild-type reporter strain exhibited faint fluorescence even in the absence of lysozyme, whereas the *csfV* mutant background did not, confirms basal transcription at P_{pdaV} requires σ^{V} (17, 35). Quantifying fluorescence with a plate reader revealed that there was a 9.4-fold induction of $P_{\text{pdaV}}::\text{mCherryOpt}$ (Fig. 5C; 0 versus 20 μg of lysozyme/ml).

Fluorescent proteins require considerable time to fold and mature, so mCherryOpt might under-report induction if a significant fraction of the protein fails to become fluorescent. We explored this possibility by using both qRT-PCR and fluorescence to measure gene expression after cells had been exposed to lysozyme for

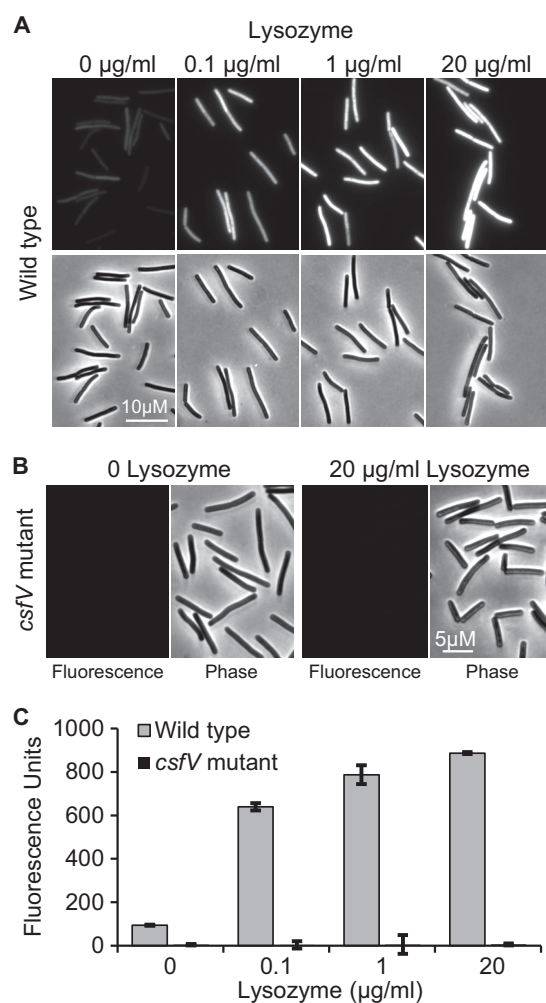


FIG 5 Use of mCherryOpt to monitor induction of the *pdaV* promoter by lysozyme. Wild-type (A) or *csfV* mutant (B) strains of *C. difficile* containing pRAN738 ($P_{\text{pdaV}}::\text{mCherryOpt}$) were grown to an OD_{600} of 0.3, exposed to lysozyme for 1 h, fixed, and removed from the anaerobic chamber. Fluorescence and phase contrast micrographs were obtained 16 h later. (C) Same as in panels A and B, except that fluorescence was quantified using a plate reader and normalized to the OD_{600} .

1 or 4 h. At the 1-h time point, $P_{\text{pdaV}}::\text{mCherryOpt}$ was induced 5-fold as measured by fluorescence but 28-fold as measured by qRT-PCR (see Fig. S4 in the supplemental material). This is a ~ 6 -fold difference. At the 4-h time point, the two methods gave more similar results: 45-fold induction by qRT-PCR and 30-fold induction by fluorescence (see Fig. S4 in the supplemental material). Collectively, these data indicate mCherryOpt tends to under-report induction, particularly if the protein has not had several hours to mature prior to fixation. Thus, like all proxies for gene expression, the results obtained using mCherryOpt must be interpreted with caution.

Plasmid stability and copy number. Our mCherryOpt vectors contain a pCD6 replicon. Although pCD6-based plasmids are widely used in *C. difficile*, the published estimates of their segregational stability vary widely (22, 36, 37), and we could not find any published estimates of copy number. To assess plasmid stability, we passaged JIR8094 exconjugates containing pRPF185,

TABLE 4 Plasmid stability

Plasmid	Mean % \pm SD ^a	
	Thi ^r	Stability
pRAN738	80.9 \pm 1.6	99.5 \pm 0.0
pDSW1728	78.9 \pm 2.9	99.5 \pm 0.1
pRPF185	66.8 \pm 20.5	99.1 \pm 0.7

^a Thi^r, the fraction of cells resistant to thiamphenicol; stability, the fraction of daughter cells that retain the plasmid after each cycle of cell division, as calculated based on Thi^r data (see Materials and Methods). The means for three experiments are shown.

pDSW1728, and pRAN738 in TY media without antibiotic selection. After 46 generations, ~70% of the cells in the culture could form a colony on TY plates containing Thi at 10 μ g/ml (Table 4). This corresponds to a rate of plasmid loss of <1% per generation, assuming progeny grew at equal rates with or without the plasmids.

To determine the plasmid copy number, *C. difficile* cells containing either pRPF185 or pRAN416 (a pRPF185 derivative with *mldA*) were grown in TY medium with thiamphenicol to an OD₆₀₀ of ~0.6. Total plasmid and chromosomal DNA were extracted using phenol-chloroform, and the relative amounts of various plasmid-borne and chromosomal genes were determined by qPCR. Three primer sets were used to target three genes: *rpoB* encodes a subunit of RNA polymerase and resides on the chromosome, *cat* confers resistance to thiamphenicol and resides on the plasmids, and *mldA* codes for a *C. difficile* cell division protein and is found on the chromosome and one of the plasmids, pRAN416. The ratio of *cat* to *rpoB* ranged from 4 to 7, while the ratio of *mldA* to *rpoB* was closer to 10 (Table 5). Thus, we estimate that the plasmids are present at approximately 4 to 10 copies per chromosome during exponential growth in TY.

DISCUSSION

GFP and related fluorescent proteins are powerful tools for studying gene expression and protein localization in a wide variety of organisms, but the fact that these reporters require O₂ for chromophore maturation has hampered their use in strict anaerobes. Nevertheless, we recently used cyan fluorescent protein (CFP) to demonstrate septal localization of three new cell division proteins in *C. difficile* (6). This application was made possible by our discovery that CFP produced during anaerobic growth can acquire fluorescence even after cells are fixed to preserve their architecture. We show here that a codon-optimized mCherry red fluorescent protein is superior to CFP because it matures quickly and there is less interference from the intrinsic fluorescence of *C. difficile*.

We also constructed and characterized two plasmids that we think will prove generally useful to the *C. difficile* community (Fig. 1). pDSW1728 is designed to be used as a reporter of gene expression because unique restriction sites for NheI and SacI facilitate placing mCherryOpt under the control of essentially any promoter-regulatory region of interest. pRAN473 is designed for protein localization studies and has an in-frame MCS that can be used for fusing mCherryOpt to the N terminus of target proteins. The copy number of these plasmids is in the range of 4 to 10 per genome, and they are stably maintained even in the absence of antibiotic selection.

For studies of promoter activity, mCherryOpt has a couple

TABLE 5 Plasmid copy number

Target genes	Mean ratio \pm SD ^a	
	RAN433	RAN429
<i>catR</i> and <i>rpoB</i>	4.5 \pm 0.3	7.3 \pm 0.9
<i>mldA</i> and <i>rpoB</i>	1.4 \pm 0.9	9.6 \pm 0.9
<i>catR</i> and <i>mldA</i>	4.0 \pm 2.3	0.76 \pm 0.1

^a The means for three biological replicates are shown, each assayed with three technical replicates. RAN433 harbors pRPF185: *cat* is on the plasmid, and both *rpoB* and *mldA* are chromosomal. RAN429 harbors pRAN416: *cat* is on the plasmid, *rpoB* is on the chromosome, and *mldA* is on both the plasmid and the chromosome.

of advantages that should make it a useful complement to alternatives such as the *gus* reporter and RT-PCR. First, mCherryOpt is convenient because no manipulations are necessary beyond a 2-h (or less) exposure of the culture to air. The fact that fixation can be omitted for mCherryOpt should facilitate some experiments, such as high-throughput screening of small-molecule libraries to identify potential new drugs. However, to observe native patterns of protein localization fixation is essential because *C. difficile* dies and lyses after exposure to air. Second, when mCherryOpt is visualized by microscopy it can be used to ask whether gene expression is uniform across all cells in a population. Although this is often the case, there are well-documented exceptions that would be difficult to study using *gus* or RT-PCR, such as bistable regulatory switches and the spatial variation of gene expression in biofilms (38–41).

Despite the clear utility of mCherryOpt, investigators need to be mindful of its limitations. We found that mCherryOpt underestimated P_{pdav} transcription, particularly when cells were analyzed after 1 h of induction. Fluorescent proteins are generally not well suited for monitoring the kinetics of changes in gene expression because they fold slowly and have long half-lives (e.g., >24 h for wild-type GFP and 40 to 190 min for “unstable” GFP variants developed to facilitate studies of transient gene expression in bacteria [42]). When using fluorescent proteins to study protein localization, it should be remembered that heterologous tags can perturb protein function, sometimes in ways that are difficult to recognize (43). In addition, the requirement for O₂ precludes use of mCherryOpt for studies of dynamic protein localization in live *C. difficile* cells, although this should not be a problem in anaerobes that tolerate transient exposure to air (44). Some alternative fluorescent labels currently under development are smaller and do not require O₂, so they may overcome these limitations (45, 46). The limitations of mCherryOpt notwithstanding, our findings suggest it will prove to be a useful addition to the *C. difficile* molecular biology toolbox.

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