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The pUltra plasmid series: A robust and flexible tool for fluorescent labeling of Enterobacteria

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ABSTRACT

Fluorescent labeling has been an invaluable tool for the study of living organisms and bacterial species are no exception to this. Here we present and characterize the pUltra plasmids which express constitutively a fluorescent protein gene (GFP, RFP, YFP or CFP) from a strong synthetic promoter and are suitable for the fluorescent labeling of a broad range of Enterobacteria. The amount of expressed fluorophore from these genetic constructs is such, that the contours of the cells can be delineated on the basis of the fluorescent signal only. In addition, labeling through the pUltra plasmids can be used successfully for fluorescence and confocal microscopy while unambiguous distinction of cells labeled with different colors can be carried out efficiently by microscopy or flow cytometry. We compare the labeling provided by the pUltra plasmids with that of another plasmid series encoding fluorescent proteins and we show that the pUltra constructs are vastly superior in signal intensity and discrimination power without having any detectable growth rate effects for the bacterial population. We also use the pUltra plasmids to produce mixtures of differentially labeled pathogenic *Escherichia*, *Shigella* and *Salmonella* species which we test during infection of mammalian cells. We find that even inside the host cell, different strains can be distinguished effortlessly based on their fluorescence. We, therefore, conclude that the pUltra plasmids are a powerful labeling tool especially useful for complex biological experiments such as the visualization of ecosystems of different bacterial species or of enteric pathogens in contact with their hosts.

1. Introduction

The discovery of the green fluorescent protein (GFP) in 1961 (Shimomura et al., 1962), its crystallization twelve years later (Morise et al., 1974) and the first production of fluorescence *in vivo* from a genetically engineered construct ten years after that (Chalfie et al., 1994) marked the beginning of a new era for cell biology, biochemistry and even ecology. Since its discovery, GFP has been modified to produce a plethora of differently colored variants (Day and Davidson, 2009), fusion proteins (Chalfie et al., 1994) and biosensors (Miyawaki et al., 1997) which have been used in a wide spectrum of applications. Microbiology is one of many fields that has reaped the benefits of these new tools. Fluorescent tagging of proteins has allowed the investigation of the function and assembly of the most impressive molecular machines in bacteria (for instance protein translocation systems (Alcock et al., 2013), cellular appendages (Baker et al., 2016; Delalez et al., 2010; Turner et al., 2000) and toxin secretion structures (Basler et al., 2013; Basler and Mekalanos, 2012; Diepold et al., 2010)) while fluorescent labeling of the entire bacterial cell has led to impressive results in the study of naturally evolving bacterial populations (Kim et al., 2014; Mitri et al., 2015) and of pathogenic bacteria in interaction with their hosts (Helaine et al., 2014; Young et al., 2014).

The small size and high division rate of bacteria enforce the use of fluorescent proteins that are particularly bright and mature rapidly following expression (Shaner et al., 2005). For this reason, numerous application-specific fluorescent tools have been developed (Chalfie et al., 1994; Aymanns et al., 2011; Singer et al., 2010). These are often specialized constructs that have been optimized for certain bacterial species and microscopy setups, and have been engineered using only one of two types of fluorescent protein. As a result, these constructs are likely to have limited utility in the study of bacterial communities or pathogenic bacteria. In parallel, series of mini-Tn*7* vectors expressing an array of fluorescent proteins which can be used in a broad range of bacterial species have also been developed (Kim et al., 2014; Mitri et al., 2015; Choi et al., 2006; Choi and Schweizer, 2006). The integration of these constructs at a conserved site in the chromosome (specifically at the 3′ end of the *glmS* gene) has the advantage that the fluorescent

Abbreviations: GFP, green fluorescent protein; RFP, red fluorescent protein; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; OD600, optical density A_{600}; DMEM, Dulbecco's Modified Eagle Media; MOI, multiplicity of infection; EHEC, enterohaemorrhagic *Escherichia coli*; PFA, para-formaldehyde.

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protein is expressed as a single-copy gene, ensuring that the entire population is labeled uniformly (Choi et al., 2006; Choi and Schweizer, 2006). However, the expression levels of the fluorescent proteins are relatively low and often lead to insufficient signal, especially for red fluorescent proteins (Choi and Schweizer, 2006), while disruptive insertions of Tn*7* transposons at ectopic sites are also known to occur (Choi et al., 2006; McKenzie and Craig, 2006).

During this era of antibiotic resistance and superbug prevalence, the majority of research conducted on pathogenic Enterobacteria is related to their interactions with their host organisms (for example (Helaine et al., 2014; Young et al., 2014)). These studies require labeling of bacteria with bright and stable fluorescent proteins, expressed in large amounts by an easily transferable genetic construct (Schultz et al., 2005; Zhao et al., 2001). Ideally, one should be able to unambiguously distinguish between differently colored bacteria in the presence or absence of host cells, often for a length of time. Here we present and characterize the pUltra plasmids, a series of plasmids suitable for most Enterobacteria, where the fluorescent protein gene is expressed under the control of a strong synthetic promoter guaranteeing the constitutive production of the fluorescent protein in large amounts in the entire bacterial population. We have constructed pUltra plasmids that can express four different fluorescent proteins and we show that they can be used successfully in microscopy and flow cytometry experiments for the study of enteric bacteria, including pathogenic species.

2. Materials and methods

2.1. Construction of plasmids

Plasmids and oligonucleotides used in this study are listed in Tables S1 and S2, respectively. KOD Hot Start DNA polymerase (Novagen, Madison, Wisconsin, United States) was used for PCRs according to manufacturer's instructions, oligonucleotides were synthesized by IDT (Leuven, Belgium) and all constructs were sequenced and confirmed to be correct before use.

For the generation of pUltraYFP-KM and pUltraCFP-KM the *yfp* and *cfp* genes were amplified from pTn*7*YFP and pTn*7*CFP (Choi and Schweizer, 2006), respectively using primers P1 and P2. The strong synthetic Biofab promoter sequence (a modified version of the P2 (apFAB45) promoter, described as [PT7A1 (−35 region) + NM (linker) + PT525 (−10 region)] in (Mutalik et al., 2013)) of pUltraRFP-KM was included in the P1 primer. The *yfp* and *cfp* inserts were subsequently cloned into the *Eco*RI-*Bam*HI sites of pUltraRFP-KM. For the generation of pUltraGFP-GM, pUltraRFP-GM, pUltraYFP-GM, and pUltraCFP-GM, the gentamicin cassette of pTn*7*YFP was amplified using primers P3 and P4 and cloned into the *Nru*I site of pUltraGFP-KM, pUltraRFP-KM, pUltraYFP-KM, and pUltraCFP-KM. Since the original kanamycin cassette was disrupted in the construction process, the pUltra-GM plasmids only confer resistance to gentamicin.

2.2. Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table S3. Plasmids were transformed into all strains by electroporation and were maintained during growth by addition of 30 μg ml^{-1} gentamicin sulfate or 50 μg ml^{-1} kanamycin sulfate in the media. 5 ml of LB medium (10 g l^{-1} peptone, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl) in 50 ml polypropylene tubes were used for all liquid cultures, which were incubated for 12 h at 37 °C with shaking at 250 rpm before use. For the generation of mixed colonies, overnight cultures of the relevant strains were mixed in equal ratios and 2 μl of this mixture were spotted on 1.5% w/v LB agar medium and incubated for 12 h at 37 °C. For experiments involving colony counts as the final readout, cell suspensions prepared from overnight liquid cultures were diluted appropriately, platted onto 1.5% w/v LB agar medium and incubated for 12 h at 37 °C.

For testing the frequency of plasmid loss in the absence of antibiotics, overnight liquid cultures of *Escherichia coli* MG1655 cells harboring the pUltraGFP-GM plasmid were diluted into 200 μl of LB medium, with and without 30 μg ml^{-1} gentamicin sulfate, in 96-well plates at a starting optical density A_{600} (OD600) of 0.001. Cells were grown for 24 h at 37 °C with shaking at 250 rpm for approximately 15 generations. Subsequently they were analyzed by flow cytometry as described in Section 2.5 of the Materials and methods. Seven biological replicates were analyzed for each tested growth condition (*i.e.* growth with or without antibiotics).

2.3. Growth rate measurements

Overnight liquid cultures were diluted into 200 μl of LB medium in 96-well plates at a starting OD600 of 0.001. Cells were grown with agitation at 37 °C using a plate reader (Tecan, Infinite M200 Pro) and their OD600 was measured automatically every 900 s. Growth rates were calculated for individual wells as the slope of the linear regression of the natural logarithm of OD600 over time, for OD600 > 0.1 and OD600 < 0.3. For each strain, four biological replicates were analyzed.

2.4. Mammalian cell infection

HeLa cells (ATCC) were maintained in Dulbecco's Modified Eagle Media (DMEM) (1 g l^{-1} glucose with addition of 2 mM glutamax and 10% w/v FCS) and routinely passaged every two or three days. For infections, cells were seeded at 7.5×10^4 cells per well into a 24-well plate while a glass coverslip was also inserted in each well. Each bacterial strain harboring either pUtraGFP-GM or pUtraRFP-GM was grown independently and strains were mixed in equal ratios immediately prior to each infection.

Stationary phase cultures of *Shigella sonnei* strain 53G (Formal et al., 1966) were diluted 1:200 into Tryptone Soy Broth (Oxoid) and incubated for 2 h at 37 °C with shaking at 200 rpm. Bacteria were pelleted and resuspended in DMEM before being added to HeLa cells at a multiplicity of infection (MOI) of approximately 100:1. Infected cells were centrifuged for 10 min at 750*g* and then incubated for 30 min at 37 °C in the presence of 5% v/v CO_2. Stationary phase cultures of *Salmonella typhimurium* strain SL1344 (Hoiseth and Stocker, 1981) were diluted 1:33 into LB medium and incubated for 3 h at 37 °C with shaking at 200 rpm. Bacteria were added to HeLa cells to give an MOI of approximately 300:1. Infected cells were incubated for 15 min at 37 °C in the presence of 5% v/v CO_2 and then they were washed three times with 1 ml of PBS per well (Dulbecco, pH 7.1–7.5), and further incubated for 60 min. Stationary phase liquid cultures of enterohaemorrhagic *E. coli* (EHEC) strain EDL933 (stx-) (Riley et al., 1983) were diluted 1:1000 into DMEM and incubated for 16–18 h at 37 °C in the presence of 5% v/v CO_2, without shaking. Bacteria were added to HeLa cells to give a MOI of approximately 200:1. Infected cells were centrifuged for 5 min at 500*g* and then incubated for 2.5 h at 37 °C in the presence of 5% v/v CO_2.

2.5. Flow cytometry

1–4 μl of overnight liquid cultures of the relevant strains were diluted into 200 μl of filter-sterilized M9 medium without a carbon source (12.8 g l^{-1} $Na_2HPO_4 \cdot 7H_2O$, 3 g l^{-1} KH_2PO_4, 0.5 g l^{-1} NaCl, 1 g l^{-1} NH_4Cl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$). The cell suspensions were analyzed on a BD Accuri C6 Flow Cytometer using 10,000 and 8000 as thresholds for the FSC and SSC parameters, respectively. 20,000 events per sample were quantified.

2.6. Imaging

For imaging of bacterial cultures at single-cell level, 5 μl of overnight liquid culture were placed onto a square piece of 1.5% w/v LB agar medium and covered with a 1.5 mm-thick cover slip. Epifluorescence images were acquired using a Zeiss Axio Observer inverted microscope with a Zeiss Plan-Apochromat 63× oil immersion objective (NA =

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