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Note

Method for fluorescent marker swapping and its application in *Steinernema* nematode colonization studies

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ABSTRACT

An allelic exchange vector was constructed to replace *gfp* by *mCherry* in bacteria previously tagged with mini-Tn*5* derivatives. The method was successfully applied to a *gfp*-labeled *Yersinia pseudotuberculosis* strain and the re-engineered bacterium was used to study the colonization of *Steinernema* nematodes hosting their *Xenorhabdus* symbiont using dual-color confocal microscopy.

Fluorescent markers are largely used to quantify or to track microorganisms in various microbiological studies. The most common labeling system is based on the Green Fluorescent Protein (GFP) originally isolated from *Aequorea victoria* (Heim et al., 1994). Genetic tagging supposes that the *gfp* gene is brought inside the target organism either via a mobile episome (plasmid, transposon) or by site-specific recombination at a pre-identified genetic locus. When dealing with gram-negative bacteria with limited genetic engineering possibilities, mini-transposons are valuable options to consider for conducting strain tagging. Mini-transposons are modified versions of original elements bearing an antibiotic resistance marker, an optional marker typically conferring fluorescence, luminescence or an easily detectable enzymatic activity and devoid of transposase, the latter being brought transiently together with the mini-transposon delivery vector in the target strain. The mini-Tn-*gfp* family, initially developed in 1996 (Christensen et al., 1996) and further improved in several subsequent works (Eberl et al., 2006; Errampalli et al., 1999; Matthysse and Dandie, 1996) was successfully used in a variety of microbiological studies ranging from simple biofilm formation to sophisticated pathogenicity model experimentations (Cho and Kim, 1999; Gjermansen et al., 2005; Richter and Smalla, 2007; So et al., 2002; Wang et al., 2011).

When applied to complex biological systems, the success of GFP-labeling is however mitigated because of interferences frequently occurring between the fluorescent marker and components of the environmental matrix. Bleaching and quenching are the most common of such interferences (McVey and Crain, 2014) and the choice of the fluorescent label is therefore critical. In a similar way, co-localization studies requiring different markers rely on carefully selected fluorescent labels displaying non-interfering fluorescence characteristics. The choice of available fluorescent markers was limited in the early times of fluorescent organism tagging but rapidly improved following the discovery of new fluorescent proteins and the engineering of GFP variants displaying brighter fluorescence, shifted light excitation and/or emission spectra and improved robustness (Ai et al., 2007; Cubitt et al., 1995; Shaner et al., 2005). Nowadays, while the number of available fluorescence markers has exploded (Shaner et al., 2005), the palette of mini-transposons available for strain tagging is still limited. Considering the work required to obtain transposon-labeled bacterial strains having the desired genetic and physiological characteristics with the right fluorescence expression level, systems allowing fluorescent marker swapping in previously characterized mini-transposon insertion mutants would allow re-engineering of such mutants with new label characteristics.

The aim of the present work was to develop an allelic exchange vector able to replace *gfp* or variants thereof by *mCherry*, a gene coding for a red fluorescence protein with spectral characteristics optimized for double labeling with GFP (Shaner et al., 2005). A fluorescence exchange

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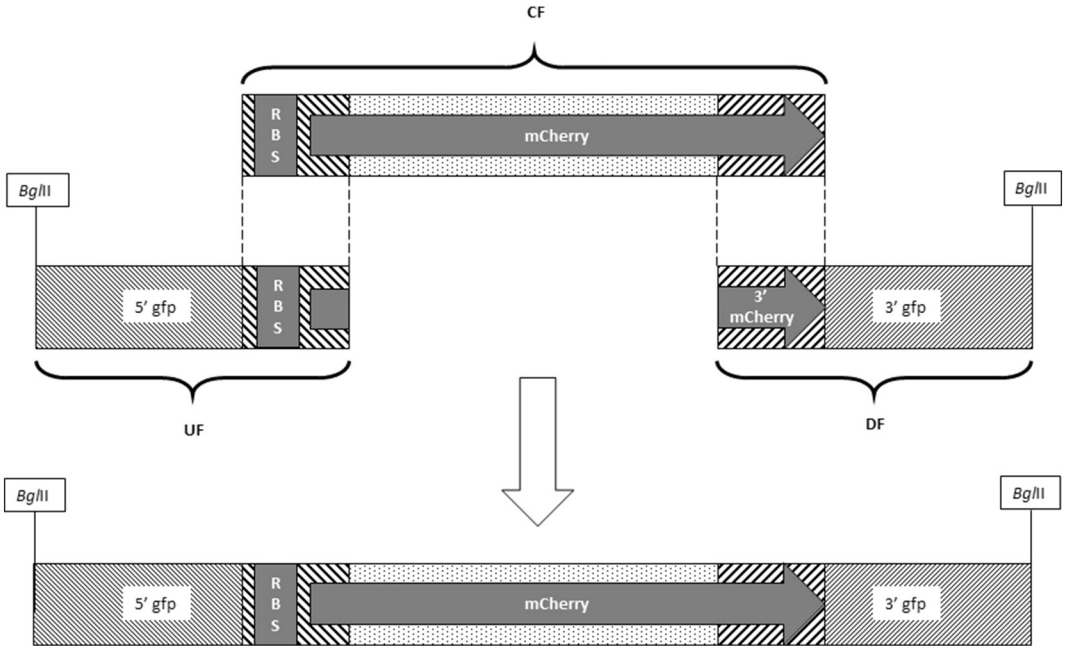

Fig. 1. Schematic view of the fluorescence exchange cassette. UF and DF consist of 244 and 223 bp from the *gfp* coding sequence, respectively. CF consists of the *mCherry* coding sequence with a RBS added upstream. Overlapping sequences are shown in spaced hatched bars. The whole cassette is generated by a short triple fusion PCR (Shevchuk et al., 2004). The cassette is flanked by *Bgl*II restriction sites.

cassette was generated using an overlap PCR protocol adapted for short fragments as described by Shevchuck et al. (Shevchuk et al., 2004). Three PCR fragments were amplified, a central fragment (CF) and two flanking fragments located upstream and downstream (UF and DF, respectively) as described in Fig. 1. CF contains *mCherry* nucleotide sequence with a ribosome binding site added upstream to ensure optimal translation. UF and DF are used for homologous recombination and target the *gfp* gene already present in the mini-transposon. The primers used are listed in Table 1. UF, DF and CF were separately amplified using 30 PCR cycles each consisting in 30 s at 95 °C, 30 s at 60 °C and 1 min at 72 °C followed by a final elongation at 72 °C for 10 min. The full-length exchange cassette was generated by triple fusion PCR using primers BG883 and BG886 as described (Shevchuk et al., 2004). The final PCR product was restricted by *Bgl*II and ligated into the unique *Bam*HI site of the mobilizable suicide vector pKNG101, which confers resistance to streptomycin and carries the counter-selectable marker *sacBR* (Kaniga et al., 1991). After *Bam*HI re-digestion of the ligation product, the resulting plasmid termed pSGCG (= LMBP 9561) was electroporated in *Escherichia coli* SM10 λPir resulting in *E. coli* 10WP . To assess the functionality of the developed vector, pSGCG was conjugated into *Yersinia pseudotuberculosis* 4N1G, a nalidixic-acid resistant field isolate labeled with a mini-Tn*5*-*gfp* transposon (Gengler et al., 2015). Allelic exchange was conducted in two steps. Initial integration of pSGCG was first selected on specific agar plates containing nalidixic-acid (35 μg ml^{-1}) and streptomycin (100 μg ml^{-1}). After purification of the recombinant strain, a second recombination event was selected on agar plates containing nalidixic-acid and sucrose (100 μg ml^{-1}). Recombinant *Y. pseudotuberculosis* 4N1C colonies expressing mCherry but not GFP were validated by both epifluorescence microscopy and PCR. To evaluate the suitability of combined mCherry and GFP fluorescent proteins in dual-labeling experiments, *Steinernema* entomopathogenic nematodes (EPNs) hosting a GFP-tagged derivative of their natural symbiont *Xenorhabdus* sp. TZ03 (Gengler et al., 2015) were allowed to graze on an agar plate covered with a lawn of the mCherry-tagged *Y. pseudotuberculosis* 4N1C described above. After one week, EPNs harboring the two fluorescent bacteria were stored for 48 h at 4 °C in physiological water, a treatment that kills the nematodes while allowing *Xenorhabdus* and *Yersinia* to survive and slowly multiply. Confocal imaging was then conducted using a Zeiss LSM710 confocal microscope equipped with a spectral detector and a water-immersion

Table 1
List of primers used in this study.

Name	Sequence (5′ to 3′)	Description	Amplicon[1]
BG883	CCGAGATCTGC*CTGGAGTTGTCCCAATTCTTGTTGA*	*gfp* forward primer with *Bgl*II restriction site (underlined) and nucleotide sequence matching bases 27 to 50 of *gfp*[2] (italics)	UF
BG884	*TTGCTCAACAT*TCCGATTTCCTCCTTGGTCACTAGTTTCGGGCATGGCACTCTT	*gfp* reverse primer with nucleotide sequence matching bases 270 to 253 of *gfp*[2] (underlined) and bases 11 to 1 of *mCherry*[3] (italics)	
BG885	*GCTGTACAAGTAA*GCAGGCGCAAAATTAGACACAACATTGAAGATGGAAGC	*gfp* forward primer with nucleotide sequence matching base 495 to 525 of *gfp*[2] (underlined) and bases 699 to 711 of *mCherry*[3] (italics)	DF
BG886	GGCAGATCTCGGTTA*TTTGTATAGTTCATCCATGCCATGTGTAATC*	*gfp* reverse primer with *Bgl*II restriction site (underlined) and nucleotide sequence matching bases 714 to 684 of *gfp*[2] (italics)	
BG887	*GTGACCAAGGAGGAAATCGGAATGTTGAGCAAGGGCGAGGAGG*	*mCherry* forward primer with ribosome binding site and start codon (underlined), overlap sequence with UF (italics)	CF
BG888	*CAATGTTGTGTCTAATTTTGCGCCTGCTTACTTGTACAGC*TCGTCCATGCC	*mCherry* reverse primer with stop codon (underlined), overlap sequence with DF (italics)	

[1] As specified in Fig. 1. UF, CF and DF: Upstream, Central and Downstream PCR Fragments.
[2] GenBank accession number KC551806.
[3] GenBank accession number AY678264.1.