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Codon-optimized Fluorescent mTFP and mCherry for Microscopic Visualization and Genetic Counterselection of Streptococci and Enterococci

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Abstract

Despite the powerful potential of fluorescent proteins for labeling bacteria, their use has been limited in multispecies oral biofilm models. Fermentative metabolism by streptococcal species that initiate biofilm colonization results in an acidic, reduced microenvironment that may limit the activities of some fluorescent proteins which are influenced by pH and oxygen availability. The need to reliably distinguish morphologically similar strains within biofilms was the impetus for this work. Teal fluorescent protein (mTFP1) and red fluorescent protein (mCherry) were chosen because their fluorescent properties made them promising candidates. Since tRNA availability has been implicated in efficient translation of sufficient quantities of protein for maximum fluorescence, a streptococcal codon optimization approach was used. DNA was synthesized to encode either protein using codons most frequently used in streptococci; each coding region was preceded by an engineered ribosomal binding site and restriction sites for cloning a promoter. Plasmids carrying this synthesized DNA under control of the *Streptococcus mutans* lactate dehydrogenase promoter conferred fluorescence to nine representative streptococcal and two *Enterococcus faecalis* strains. Further characterization in *Streptococcus gordonii* showed that mTFP1 and mCherry expression could be detected in cells grown planktonically, in biofilms, or in colonies on agar when expressed on an extrachromosomal plasmid or in single copy integrated into the chromosome. This latter property facilitated counterselection of chromosomal mutations demonstrating value for bacterial strain construction. Fluorescent and non-fluorescent bacteria were distinguishable at acidic pH. These codon-optimized versions of mTFP1 and mCherry have promising potential for use in multiple experimental applications.

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Keywords

biofilms; codon-optimized; fluorescent proteins; mCherry; mTFP; oral streptococci

1. INTRODUCTION

Fluorescent proteins expressed by bacteria are powerful tools for microbiology research that allow visual detection of cells and their components. Successive modifications of these proteins have improved their usefulness in a variety of chemical and biological systems (Shaner *et al.*, 2005, Day *et al.*, 2009). The goal of the present studies was to select and characterize fluorescent proteins that could be used to differentially label streptococcal strains in order to examine early-colonizing commensal or cariogenic species in oral biofilm models. Since the facultative streptococci and other species within dental plaque may be aciduric and/or acidogenic, the microenvironment within oral biofilms often has niches with low oxygen tensions and low pH (Kleinberg, 2002). Thus, for maximum usefulness fluorescent labels should have intrinsic properties that allow their stable expression under these conditions.

Most previous studies using fluorescent proteins in oral streptococci have used Green Fluorescent Protein (GFP) derivatives optimized for use in bacteria (Cormack *et al.*, 1996). Although valuable data and images were obtained, there were some limitations such as diminished brightness and unexplained variability or loss of fluorescence (Aspiras *et al.*, 2000). These difficulties may have been due to autofluorescence of the cells in the green wavelengths and sensitivity of GFP to acidic pH (Shaner *et al.*, 2005) or low oxygen tensions (Heim *et al.*, 1994, Hansen *et al.*, 2001). As there are now many additional fluorescent proteins available (Day *et al.*, 2009, Ai *et al.*, 2008, Shaner *et al.*, 2005), their properties were examined with a goal of choosing two fluorescent proteins to be used for distinguishing different streptococcal cell types under biologically relevant conditions. Brightness, which is influenced by speed of maturation and photostability of the fluorescent protein, pH stability, and distinct, nonoverlapping emission wavelengths were considered. Monomeric proteins were given preference since oxygen-dependent protein maturation, necessary for fluorescence detection after anaerobic growth, is faster in monomers than in multimeric proteins (Shaner *et al.*, 2005). Proteins that could be detected by optical properties of widely-available imaging systems were favored (Shaner *et al.*, 2005).

Two monomer proteins with distinct wavelengths were chosen as candidates. Red fluorescent protein mCherry is synthetically derived from successive modifications of the *Discosoma* sp. fluorescent protein DsRed (Shaner *et al.*, 2004). Teal Fluorescent Protein mTFP1 is a synthetic derivative from the Cyan protein cF_48 from *Clavularia* soft coral (Ai *et al.*, 2006). Commercially available genes encoding both proteins have been designed for expression in mammalian cells or *Escherichia coli* both of which have a higher G+C content than streptococcal DNA. Codon-optimization to ensure availability of sufficient tRNA to translate genes encoding fluorescent proteins has been shown to significantly improve cell fluorescence (Sastalla *et al.*, 2009). This approach has facilitated expression of fluorescent proteins in several microorganisms including *Botrytis cinerea* mold (Leroch *et al.*, 2011), as

well as *Bacillus anthracis* (Sastalla *et al.*, 2009) and *Clostridium difficile* (Ransom *et al.*, 2015). Accordingly, we synthesized streptococcal codon-optimized genes preceded by an appropriately-placed Shine Delgarno site to encode either mTFP1 or mCherry; these DNA fragments were cloned into convenient plasmids for streptococcal genetic experiments. Proof of concept results presented here demonstrate that these engineered genes express bright fluorescent proteins in representative strains of streptococci and closely-related enterococci and may be valuable new molecular tools to facilitate genetic and multi-strain biofilm studies.

2. METHODS

2.1. Bacterial strains and medium

All strains were stored in 50% glycerol at -80°C . *S. gordonii* strain Challis CH1 was used for streptococcal cloning and biofilm studies. Strains for determining the range and usefulness of plasmid-borne fluorescent genes included: *Streptococcus salivarius* 25975, *Streptococcus mitis* 49456, *Streptococcus anginosus* 33397, *Streptococcus sanguinis* 10556, and *Streptococcus gallolyticus* 43143, all obtained from American Type Culture Collection (ATCC; Manassas, VA USA), *Streptococcus mutans* strain 3209 (obtained from J. Ferretti, University of Oklahoma Health Sciences Center), *S. sanguinis* SK36 (obtained from M. Kilian, Aarhus University Denmark), *Streptococcus oralis* 34 (obtained from J. Cisar, National Institute of Dental and Craniofacial Research, Bethesda, MD), and *Enterococcus faecalis* strains JH2-2 and OG1X (obtained from D. Clewell, University of Michigan). Streptococcal and enterococcal strains were grown in Todd Hewitt (TH), or Brain Heart Infusion (BHI) (Becton, Dickinson and Co., Franklin Lakes, NJ USA) medium aerobically with 5% CO_2 or in defined FMC (Terleckyj *et al.*, 1975) medium anaerobically in a GasPak jar (Becton, Dickinson and Co.). *E. coli* DH5 α (Stratagene) or CopyCutter (EP1400; Epicentre; Madison, WI USA) strains used for cloning were grown in Luria-Bertani (LB) medium (Becton, Dickinson and Co., Franklin Lakes, NJ USA) with aeration. When necessary for selection or plasmid maintenance antibiotics were used in the following concentrations: erythromycin 5–10 $\mu\text{g/mL}$ and kanamycin 500 $\mu\text{g/mL}$ in Gram-positive species and erythromycin 250 $\mu\text{g/mL}$, kanamycin 50 $\mu\text{g/mL}$, and ampicillin 100 $\mu\text{g/mL}$ in *E. coli* strains.

2.2. DNA manipulations and transformation

Standard bacterial cloning methods were used (Ausubel *et al.*, 1987). Plasmid DNA was prepared using Qiagen kits according to the manufacturer's instructions. Modifications for Gram-positive strains included growing bacteria in medium supplemented with 0.5% glycine, and using mutanolysin and lysozyme to facilitate cell lysis. For transformation of *E. coli*, cells were made chemically competent with CaCl_2 . Naturally competent streptococcal species were transformed (Lawson and Gooder, 1970) using horse serum. For noncompetent Gram-positive species, cells were grown in 1% or 4% glycine for streptococcal or enterococcal strains, respectively, and electroporated with closed circular plasmid (Flannagan and Clewell, 1991). All genetic constructs were confirmed by nucleotide sequence analysis. Strains with chromosomal insertions were further verified by Southern

blot analysis using digoxigenin-labeled probes (Roche Diagnostics Corporation, Indianapolis, IN) according to the manufacturer's directions.

2.3. Synthesis and cloning of codon-optimized genes for Teal Fluorescent Protein and Cherry Fluorescent Protein

Genes for mTFP1 and mCherry were synthesized by GenScript Corporation (Piscataway, NJ) using the nucleotide sequence we provided. The coding sequence of each gene maintained the amino acid sequences found in GenBank (DQ676819.1 for mTFP1; AY678264 for mCherry). Codons were changed from those in GenBank to those used with the two highest frequencies in the oral cariogenic species *S. mutans* based on a codon usage table found at <http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=1309> (Nakamura *et al* 2000). A ribosome binding site was placed –12 to –8 basepairs (bp) upstream of the ATG start codon. A *KpnI* restriction enzyme site was engineered 40 nucleotides downstream of the stop codon. The synthesized genes were obtained from GenScript in plasmid pUC57 and maintained in an *E. coli* CopyCutter host.

To create a construct that could facilitate expression of the fluorescent proteins in Gram-positive cocci, a streptococcal promoter was subcloned upstream of the fluorescent protein gene. The synthesized coding region of mTFP1 or mCherry was released from the pUC57 vector with *BamHI* and *KpnI* and ligated into the similarly-digested shuttle vector pDL276 (LeBlanc *et al.*, 1991). The *ldh* gene had previously been determined by transcriptome microarray analysis of *S. mutans* UA159 to be among the most highly expressed constitutive genes in both planktonic and biofilm growth conditions (J. Banas, unpublished data). The *ldh* promoter region was amplified from strain UA159 (obtained from J. Ferretti) chromosomal DNA by PCR using primers containing engineered restriction sites (forward primer 5'TCTCTAGATTCTAGTCTAAACTCTTTTATATTATATCAC3'; reverse primer 5'TCGGATCCGTTCTAAACATCTCCTTATAATTTATTAAG3') and then cloned into *XbaI*-*BamHI* sites upstream of the fluorescent protein genes in pDL276 using an *E. coli* host.

2.4. Construction of fluorescent replicative plasmids

The *HindIII*-*SacI* fragment carrying the *ldh* promoter and the fluorescent gene was subcloned from pDL276 and ligated into compatible sites in pAMS749-12 which has only the broad host range Gram-positive pVA380-1 replicon (LeBlanc *et al.*, 1991) for expression in streptococcal and enterococcal species. All replicative plasmid construction was done in *S. gordonii* strain CH1 to create closed circular plasmid DNA for transformation or electroporation into other streptococcal or enterococcal strains. The characterized plasmids carrying genes encoding mCherry and mTFP1 were designated pVMCherry and pVMTeal, respectively (Fig. 1A).

2.5. Construction of fluorescent integrative plasmids

To integrate either fluorescence gene into the chromosome in single copy, the *HindIII*-*SacI* fragments carrying the *ldh* promoter and fluorescent gene from pDL276 were subcloned into compatible restriction sites in pVA8912 (Steiner and Malke, 1995) which has only an *E. coli* p15A replicon (Chang and Cohen, 1978) and cannot replicate in streptococci. All cloning

and construction involving fluorescent genes was done in *E. coli* CopyCutter. The resulting plasmids were designated p8912Cherry and p8912Teal (Fig. 1B).

2.6. Chromosomal integration and counterselection

To determine the fluorescence of a single chromosomal copy of each of the genes with minimal predicted effects on flanking genes, a region containing the apparent remnants of a temperate bacteriophage attachment *attB* site downstream of SGO_2004 (van der Ploeg, 2008) was cloned upstream of the *ldh* promoter in the integrative plasmids p8912Teal and p8912Cherry (Fig. 1B). The 210-bp *attB* region was amplified from strain CH1 chromosomal DNA template using forward primer 5'TA AAG CTT AAC CTG ATT TAT CAG GAA GCA TC3' and reverse primer 5'TA GCA TGC AAG GCA TTT GTC TTT AAT TCT ACT G3' with engineered *Hind*III and *Sph*I sites (underlined), respectively, to facilitate cloning. As a non-fluorescent control, the *attB* amplicon was cloned into the *Hind*III and *Sph*I sites of pVA8912 (Steiner and Malke, 1995) to create p8912att. Strains with the chromosomally-integrated fluorescent plasmid DNA were designated CH1Tealatt and CH1Cherryatt; the nonfluorescent control strain with integrated p8912att was designated CH1att. Cells were passed in broth successively from late log cultures without antibiotic for 5 days and then plated on antibiotic free agar and colonies were examined for fluorescence to ascertain stability.

To examine potential for use of integrative plasmids encoding fluorescent proteins for genetic counterselection methods, a 1.6-kb fragment from pAMS24 encoding an internal fragment of *gtfG* encoding Glucosyltransferase (GtfG), (Vickerman *et al.*, 1997), was cloned into the *Hind*III site of p8912Teal; the resulting plasmid was transformed into strain CH1. Putative transformants and revertants with and without the chromosomally integrated plasmid, respectively, were screened on TH agar with 3% (w/v) sucrose for a hard or soft colony phenotype indicating the presence or absence of glucans synthesized by a functional GtfG enzyme (Vickerman *et al.*, 1996).

2.7. Colony fluorescence on agar plates

Strains carrying fluorescent genes, and nonfluorescent controls either as single or mixed cultures, were diluted and plated on agar plates and incubated overnight at 36°C, aerobically with 5% CO₂. Colonies were visualized using a ChemiDoc MP Imaging System (Bio-Rad, California, USA) and the Image Lab software (version 4.1). Teal fluorescence of colonies was detected using the 530/28 nm emission filter and Cherry fluorescence of colonies was detected using the 605/50 nm emission filter. To visualize both mTFP1 and mCherry colonies on the same agar plate a multichannel image software option was used to overlay the two emission filter images.

2.8. Fluorescence of broth cultures and buffer suspensions

Mid- to late-log cells were measured either directly in medium to assess the extent of autofluorescence or in buffer. Sodium acetate or sodium phosphate buffers (0.1 M) over a pH range of 4.6 to 7.2 in 0.2 increments were used. Cells were pelleted, washed once with buffer, and pelleted again. Pellets were resuspended to the same OD₅₂₀ of 1.0 in buffer and 200 µl aliquoted per well of a Corning 96-well black plate with clear, flat bottoms.

Fluorescence of cell suspensions or media was read on a FlexStation 3 (Molecular Devices, California, USA) plate reader using the SoftMax Pro software (version 5.4.5). Teal fluorescence was read using an excitation wavelength of 485 nm and emission at 515 nm. Cherry fluorescence was measured using an excitation wavelength of 544 nm and emission at 600 nm. Default settings were used and each well was scanned to give an endpoint read out in Relative Fluorescent Units (RFU). Absorbance of the resuspended cells was measured at an optical density (OD) at wavelength 520 nm to approximate cell number per well. A minimum of two independent biological replicates were done for each experiment. Readings from a minimum of four wells per strain or test condition were averaged and differences compared for statistical significance by a twotailed Student *t* test with unequal variances using Excel software.

2.9. Confocal Laser Scanning Microscopy (CLSM)

S. gordonii strains expressing mTFP1, mCherry, either on an extrachromosomal plasmid or integrated within the chromosome, and empty vector strains (plasmid alone or plasmid integrated with no fluorescent gene), were grown overnight in BHI broth at 37°C in an aerobic incubator with 5% CO₂. Broth cultures were washed twice and cells were resuspended in phosphate buffered saline (PBS) to remove fluorescent elements of the culture medium. Cell suspensions were normalized to an OD₆₀₀ of 0.1. Equivalent volumes of selected strains were placed on a microscope slide, covered with a glass coverslip and edges sealed with nail polish to avoid desiccation. These slides were used to microscopically distinguish cells carrying the two fluorophores from each other and nonfluorescent cells.

For biofilms, *S. gordonii* strains were grown under the same conditions as for fluorescent microscopy except that the BHI contained 1% (w/v) sucrose and biofilms were allowed to form on circular glass coverslips (12-mm diameter) in Costar 24-well polystyrene flat-bottom culture dishes (Corning Inc., Corning, NY) and incubated statically for 18 hours. The biofilms were washed by gentle immersion and agitation in PBS. The coverslip containing the biofilm was then placed on a microscope slide, wetted with PBS for optimum hydration, covered with a rectangular glass coverslip and edges sealed with nail polish for visualization.

Single plane and stacked images at 1-μm intervals were collected on a Zeiss LSM 510 laser scanning confocal microscope using excitation/emission optima of 458/488 for mTFP1 and 543/610 for mCherry. Images were initially saved as 'lsm' files with the internal microscope software. The channels were merged and formatted in Image J and saved as tiff files.

2.10. Nucleotide accession numbers

The nucleotide sequences of the streptococcal codon-optimized mCherry and mTFP1 genes are deposited in GenBank with Accession numbers KR608257 and KR608258, respectively.

3. RESULTS AND DISCUSSION

3.1. Broad host range of the replicative plasmids

Due to an observed spontaneous loss of fluorescence in the shuttle plasmid pDL276 in *E. coli* (data not shown) and previous reports that different cloning vectors can influence the stability and expression of other fluorescent proteins, such as the green fluorescent protein (Aspiras *et al.*, 2000), in oral streptococci, we chose to clone the fluorescent genes into a plasmid, pAMS749-12 (Flannagan *et al.*, 2008), that has only one replicon that functions in streptococci and enterococci. The *ldh* promoter-driven codon-optimized fluorescent genes were expressed in a range of representative species (Table 1). The pVMTeal and pVMCherry conferred significant fluorescence to a variety of oral streptococcal species, the representative non-oral species *S. gallolyticus*, and two strains of *E. faecalis*. The basis for the observed differences in fluorescence among strains is not known and may be due to a plasmid copy number effect, or the relative strength of the *S. mutans* *ldh* promoter and/or the efficiency of the *S. mutans* codons in the heterologous host cells. Modification of the plasmids, for example with species-specific promoters, may be necessary to optimize fluorescence in different hosts. Although the G+C content of genome sequences of representatives of the species tested ranged from ca. 37% to 41% (National Center for Biotechnology Information Genome database; <http://www.ncbi.nlm.nih.gov/genome/>) these percentages did not correlate directly with the relative fluorescence of mTFP1 or mCherry in the strains tested (data not shown). Fluorescence was strongest in *E. faecalis* strains. The lowest fluorescence was seen in *S. mitis* for both mTFP1 and mCherry, yet both were statistically different than plasmid-free cells. The replicative plasmids carry unique *HindIII*, *SphI*, *XbaI* *KpnI* and *SacI* sites on either side of the promoter and fluorescence genes making these plasmids convenient for cloning experiments (Fig. 1A). Although originally designed to facilitate studies of strains in mixed oral biofilms, the fluorescence detected among a range of species suggests that use of these plasmids may also be widely applicable for studies of non-oral streptococcal species.

Further characterization of the fluorescent genes was carried out in *S. gordonii* strain CH1. A representative of commensal oral species, this strain is well characterized genetically and genomically (Vickerman *et al.*, 2007), and expressed intermediate fluorescence levels of both mTFP1 and mCherry among the strains tested (Table 1).

3.2. The pH sensitivity of the plasmid-encoded mCherry and mTFP1 in *S. gordonii*

A valuable application of these codon-optimized fluorescent genes would be to examine individual cell types in oral biofilm models. Early dental plaque models involve the initial colonization of oral surfaces by streptococcal species which create a drop to acidic pH as facultative metabolism occurs (Kleinberg, 2002, Deng *et al.*, 2004). A potential limitation of some fluorescent proteins is limited usefulness at low pH due to acid sensitivity (Shaner *et al.* 2005). To examine the pH-dependent fluorescence of mTFP1 and mCherry in *S. gordonii*, cells were washed and resuspended in buffers of pH 4.6 to pH 7.2. After 30 minutes incubation at room temperature to allow equilibration, the cells were then excited at appropriate wavelengths and the resulting fluorescence detected (Figure 2). Fluorescence readings for plasmid-carrying cells were significantly different from those of plasmid-free

control cells at all pH levels ($p < 0.000001$, Student t test). At lower pH the difference between control cells and fluorescent cells was greater for CH1/pVMCherry than for CH1/pVMTeal suggesting that the former marker might be more useful at acidic pH ranges found in oral biofilm studies. Although the emission levels were pH-dependent, fluorescence due to both pVMCherry and pVMTeal was readily detectable over biologically relevant pH ranges.

3.3. Chromosomal integration of genes encoding mTFP1 and mCherry

Although pVA749 is relatively stable in *S. gordonii* (Macrina *et al.*, 1981), one potential limitation of plasmid-carrying strains as markers of individual cell types would be plasmid loss during long-term experiments if cells were grown without antibiotic pressure to ensure plasmid maintenance. Accordingly, we designed a plasmid for chromosomal integration of the fluorescence genes. A derivative of pVA891 was chosen since the replicon of this *E. coli* integrative plasmid has been widely used with streptococcal and enterococcal DNA (Macrina *et al.*, 1983) and the copy number is significantly lower (Chang and Cohen, 1978) than those of pUC based *E. coli* plasmids such as pDL276 (LeBlanc *et al.*, 1991). Although we did not do a controlled evaluation of fluorescence stability of mTFP1 and mCherry in different cloning vectors, we did not observe spontaneous loss of fluorescence in the the replicative or integrative plasmids shown in Figure 1.

A previous study (Van der Ploeg, 2008) identified a putative bacterial attachment site, *attB*, for an unknown temperate bacteriophage in several streptococcal species. Plasmid DNA integrated into this chromosomal site via a Campbell-type insertion duplication mechanism was relatively stable over 100 generations without antibiotic pressure (Van der Ploeg, 2008). In *S. gordonii*, the putative *attB* region is located between convergent open reading frames, suggesting that disruption and separation of this region would have minimal effects on flanking gene expression and bacterial growth. A BLAST analysis (Altschul *et al.*, 1990) of the *S. gordonii* genome sequence (Vickerman *et al.*, 2007) verified that the 210-bp putative *attB* region was unique in this strain. Therefore, the putative *attB* region was cloned into pVA8912 with or without *ldh* promoter-driven fluorescent genes (Fig. 1B) and integrated into the strain CH1 chromosome by a single cross-over recombination. Loss of the integrated plasmid would require homologous recombination of the duplicated 210-bp flanking regions; theoretically, the recombination potential diminishes as the size of the regions of homology decreases.

Stability of the integrated plasmid DNA was measured by successive passages of cells in broth cultures without antibiotic selection. Cells were then plated on antibiotic-free agar and colonies were picked and tested for erythromycin resistance and/or examined directly on a ChemiDoc Imager for fluorescence indicating the presence of the integrated plasmid. After ca. 120 generations, ca. 1800 colonies were examined and all were fluorescent and/or erythromycin resistant, indicating the presence of integrated plasmid DNA. These results support the usefulness of these strains for experiments of at least this duration even in the absence of antibiotics. Growth curves of strains with integrated plasmid DNA and parental strain CH1 in antibiotic-free TH medium under 5% CO₂ aerobic and anaerobic conditions were determined spectrophotometrically and compared. Doubling times and final cell

numbers attained were similar for all strains (data not shown) indicating that the fluorescent proteins were not toxic to the cells and that the integrated plasmid DNA did not affect growth under these conditions.

3.4. Detection of colony fluorescence on agar plates and counterselection

Colonies of cells with plasmid-borne fluorescent genes were readily distinguished from each other on agar plates, and colonies arising from mixed cultures could be readily differentiated using appropriate filters to determine the proportion of cells in mixed growth experiments (Fig. 3 panels A–C). Neither CH1/pVMCherry nor CH1/pVMTeal had an advantage when co-cultured in TH from an equal starting inoculum and showed similar numbers of cells after dilution and plating of a mid-log culture. The chromosomally integrated single copies of genes encoding both mTFP1 (Fig. 3D) and mCherry (Fig. 3E) were also fluorescent on agar plates compared with nonfluorescent CH1att cells.

Because the mTFP1 colonies were subjectively brighter to our eyes in comparison to the nonfluorescent colonies, the p8912Teal plasmid was chosen to demonstrate the usefulness of this plasmid for insertion duplication mutagenesis and counterselection for genetic strain construction. Detection of double crossovers for allelic exchange during bacterial mutant construction has previously been shown to be facilitated by the commercially-available mCherry in *Actinomyces oris* (Wu and Ton-That, 2010); thus, a similar approach should be feasible using genes and vectors optimized for use in streptococci. Integrating plasmids with only an *E. coli* replicon for streptococcal chromosomal gene disruption and then allowing plasmid DNA loss by homologous recombination of duplicated flanking regions (one of parental and one of the cloned desired genotype), is a routine genetic method used to construct stable chromosomal mutations. Since the potential for homologous recombination increases with the size of the duplicated flanking chromosomal regions, a larger DNA fragment is generally cloned into an integrative plasmid when this method is used. To demonstrate the feasibility of using fluorescent-labeled plasmids to facilitate counterselection, the *gtfG* gene was chosen for disruption because of its readily-observable phenotype. The parental GtfG enzyme synthesizes insoluble glucans resulting in hard, adherent colonies on sucrose agar plates. Disruption of *gtfG* by plasmid integration results in a soft colony phenotype due to lack of enzyme activity (Vickerman *et al.*, 1996). A transformant of strain CH1 with pVA8912Teal integrated in *gtfG* was erythromycin resistant, soft on sucrose agar, and fluorescent at wavelengths for TFP1. After this strain was passaged for approximately 100 generations in antibiotic-free medium to encourage loss of the integrated plasmid, cells were diluted and plated on sucrose agar. A total of 200 colonies were examined independently for reversion to parental hard colony phenotype and loss of fluorescence; all non-fluorescent colonies were hard indicating a reversion rate of ca. 4% and 100% correlation of the loss of fluorescence with the loss of plasmid (Fig. 3F). Screening agar plates for nonfluorescent colonies is considerably more efficient than traditional colony picking methods. For counterselection of mutations that do not have a readily observable colony phenotype and require screening only by their antibiotic sensitivity, use of integrative plasmids expressing fluorescent proteins will result in significant time savings.

3.5. Sensitivity of detection of cells carrying fluorescent genes in multiple plasmid-borne and single chromosomal copies

The usefulness of fluorescent cell markers is influenced by potential autofluorescence of medium and cell components in the wavelengths being measured (Shaner *et al.*, 2005). When *S. gordonii* cells were resuspended in PBS and excited, they emitted fluorescence proportional to cell number for strains carrying either pVMTeal or pVMCherry (Fig. 4); plasmid-carrying cells could be distinguished from plasmid-free CH1 cells at an OD₅₂₀ = 0.15 ($p < 0.01$). As expected, cells carrying single chromosomal copies of fluorescent genes were less fluorescent than those carrying the genes on multi-copy plasmids (17.7 vs. 56.4 RFU for CH1Tealatt vs. CH1/pVMTeal and 2.5 vs. 12.2 RFU for CH1Cherryatt vs. CH1/pVMCherry when resuspended to an OD₅₂₀ of 1.13 ± 0.05). Nevertheless, cells carrying chromosomal fluorescent genes were more fluorescent than the nonfluorescent control cells (Fig. 4), and could be distinguished at cell concentrations as dilute as OD₅₂₀ of 0.74 ($p < 0.008$ for CH1Tealatt vs. CH1att) or OD₅₂₀ of 0.25 ($p < 0.03$ for CH1Cherryatt vs. CH1att) indicating a high degree of sensitivity for detecting the fluorescence of both mTFP1 and mCherry. Similarly, cells carrying plasmid-borne (Fig. 5A) or chromosomally-integrated (Fig. 5B) fluorescent genes could be distinguished from each other and from non-fluorescent cells when resuspended in PBS and examined by CLSM.

As expected, measuring the fluorescence of bacterial cultures was confounded by autofluorescence of the medium. Both TH and BHI alone were as fluorescent as late-log cultures of plasmid-bearing *S. gordonii* cells at the excitation and emission wavelengths for both mTFP1 and mCherry. Although FMC was less autofluorescent than these complex media, its fluorescence at the excitation and emission wavelengths for mTFP1 precluded spectrophotometrically distinguishing late-log cultures from medium alone (data not shown). However, FMC was significantly less autofluorescent than complex medium in the wavelength for detection of mCherry (0.34 RFU for FMC vs. 34.0 RFU for TH). Strain CH1Cherryatt cells were distinguishable from strain CH1att control cells at a dilution in FMC as low as OD₅₂₀ = 0.3 ($p < 0.001$) similar to the levels seen for cells resuspended in buffer (Fig. 4), indicating that the mCherry gene carried in single or multiple copies could be measured directly in early-log FMC cultures. FMC cultures were grown anaerobically in order to obtain higher final cell numbers (OD₅₂₀ ~ 1.3 anaerobically vs. OD₅₂₀ 0.6 aerobically in 5% CO₂). Although fluorescent proteins require oxygen to fluoresce (Heim, *et al.*, 1994, Ransom *et al.*, 2015), FMC culture aliquots that were allowed to equilibrate for 30 minutes in air at room temperature before RFU measurements were made showed stable, reproducible fluorescence indicating that anaerobic growth did not preclude subsequent fluorescence detection.

Although complex medium components did interfere with quantifying fluorescence spectrophotometrically, the mTFP1 and mCherry fluorescent proteins were detected and individual cells could be readily distinguished from each other microscopically in 18-hour mixed cell biofilm cultures. The fluorescence of both mTFP1 and mCherry was readily distinguishable in cells carrying the genes encoding these proteins in either plasmid (Fig. 6A) or chromosomal (Fig. 6B) copies. Although there are limitations of these constructs which may become apparent in future studies of mature biofilms with less oxygenation or

lower pH depending upon the complexity of the microbial community, the fluorescent proteins effectively distinguished cell types under the current test conditions. The differentiation of morphologically similar cell types such as parent versus mutant cells, or commensal versus pathogenic streptococcal or enterococcal species, will facilitate experiments designed to understand bacterial colonization and biofilm development.

3.6. Conclusion

Codon-optimized genes for fluorescent proteins that are expressed in oral streptococci and enterococci under the control of a strong streptococcal promoter have a variety of potential uses for bacterial cell detection and visualization; these tools will be especially valuable in mixed strain or multi-species biofilm studies. The plasmids carrying these genes were constructed to be useful in a variety of cloning applications with restriction digest sites flanking the fluorescent protein genes for convenience of cloning promoters or other DNA of choice. Furthermore, both encoded fluorescent proteins were readily detectable in a range of species, even in single chromosomal copy in *S. gordonii*. The integrative plasmids presented here will facilitate the laborious counterselection process of picking putative revertant colonies in streptococcal and enterococcal genetic studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

1. Codon-optimized *mTFPI* and *mCherry* genes expressed in streptococci and enterococci
2. Plasmids for distinguishing different streptococci or enterococci in biofilms
3. Fluorescence-based oral streptococci detection maintained at cariogenic pH
4. Fluorescence-based differentiation of colonies on agar plates
5. Counterselection of single-copy integrated plasmid facilitates genetic techniques

Fig. 1. Plasmids

Streptococcal replicative and integrative plasmids carrying an *XbaI-KpnI*-flanked ca. 180-bp *S. mutans ldh* promoter and 797-bp codon-optimized structural gene for mTFP1 or mCherry to express fluorescence in streptococcal and enterococcal species. Maps are not drawn to scale.

A. Replicative. pAMS749-12 (Flannagan *et al.*, 2008) is a ca. 5-kb replicative plasmid derived from pVA749 (Macrina *et al.*, 1981) with a broad streptococcal host range 380-1 replicon (rep), *ermAM* to encode erythromycin resistance, and a multiple cloning site. Plasmids carrying the cloned fluorescence genes were designated pVMTeal or pVMCherry.

B. Integrative. pVA8912 (Steiner and Malke, 1995) is a ca. 2.8-kb plasmid derivative of pVA891 (Macrina *et al.*, 1983) with an *E. coli* p15A replicon (Chang and Cohen, 1978) (rep). The *ermAM* gene is expressed in both streptococci and *E. coli* (5 ug/ml selection in streptococci and 250 ug/ml selection in *E. coli* CopyCutter). The plasmids carrying the cloned fluorescence genes were designated p8912Teal and p8912Cherry.

Fig. 2. Fluorescence in different pH buffers

Representative experiment showing the fluorescence of late-log *S. gordonii* cells carrying pVMTeal (panel A) or pVMCherry (panel B) compared with plasmid-free strain CH1 controls. Cells were resuspended in buffers with a pH ranging from 4.6 to 7.2. Bars show the average RFU of eight wells per plate. Error bars indicate standard deviations.

Fig. 3. Agar plates viewed with a 530/28 nm emission filter and a 605/50 nm emission filter to visualize *S. gordonii* colonies expressing mTFP1 and mCherry, respectively

A. Mixture of CH1/pVMTeal and CH1/pVMCherry colonies viewed with the filter for mTFP1.

B. The same plate viewed with the filter for mCherry.

C. A multichannel overlay of the images in panels A and B confirms the specificity of the filters for each colony type.

D. Isolated nonfluorescent strain CH1att colonies (left) are readily distinguishable from those of CH1Tealatt (right).

E. Isolated nonfluorescent CH1att colonies (left) are distinguishable from CH1Cherryatt colonies (right).

F. Counterselection of revertant colonies that have lost the integrated p8912Teal that disrupted the *gtfG* structural gene encoding the GtfG enzyme. Non-fluorescent revertants (arrows) have the parental glucan-associated hard colony phenotype on sucrose agar plates.

Fig. 4. Spectrophotometric detection

Doubling dilutions of mid- to late-log *S. gordonii* cells resuspended in PBS, pH 7.2. Fluorescence was measured at the appropriate excitation and emission wavelengths for mTFP1 (panel A) and mCherry (panel B). The RFU value marked for each strain at each dilution represents the average readings in two biologically independent experiments.

Fig. 5. CLSM of *S. gordonii* cell suspensions in PBS

Views of nonfluorescent strain CH1 cells with plasmid-borne (panel A) or chromosomally-integrated (panel B) genes encoding mTFP1 and mCherry. Representative cell types are labeled.

Fig. 6. CLSM images of 18-hour biofilms

Composite and orthogonal horizontal and vertical images of *S. gordonii* cells with plasmid-borne (panel A) or chromosomally-integrated (panel B) genes encoding mTFP1 and mCherry.

Table 1Range of hosts carrying plasmid-borne genes encoding mTFP1 or mCherry^a.

Strain	RFU (Ex 485/Em 515)		RFU (Ex 544/Em 600)	
	Plasmid-free	pVMTeal	Plasmid-free	pVMCherry
<i>S. gordonii</i> CH1	14.02 ± 0.33	44.69 ± 0.73	1.31 ± 0.13	23.26 ± 0.40
<i>S. mutans</i> 3209	17.49 ± 0.42	68.27 ± 1.18	1.58 ± 0.07	39.54 ± 0.73
<i>S. sanguinis</i> ATCC 10556	13.60 ± 0.23	37.67 ± 0.60	1.20 ± 0.07	25.29 ± 0.78
<i>S. sanguinis</i> SK36	18.34 ± 0.32	76.61 ± 1.03	1.68 ± 0.05	10.02 ± 1.43
<i>S. salivarius</i> ATCC 25975	14.84 ± 0.56	36.66 ± 0.51	1.60 ± 0.21	28.81 ± 1.10
<i>S. mitis</i> ATCC 49456	14.10 ± 0.27	24.00 ± 0.54	1.46 ± 0.13	3.21 ± 0.14
<i>S. oralis</i> 34	16.59 ± 0.47	26.96 ± 0.46	1.28 ± 0.15	6.37 ± 0.20
<i>S. anginosus</i> ATCC 33397	13.80 ± 0.24	70.95 ± 0.79	1.50 ± 0.06	32.01 ± 1.13
<i>S. gallolyticus</i> ATCC 43143	15.50 ± 0.27	49.42 ± 0.54	1.28 ± 0.15	22.67 ± 0.30
<i>E. faecalis</i> OG1X	22.76 ± 1.01	73.20 ± 3.23	1.00 ± 0.05	32.60 ± 1.14
<i>E. faecalis</i> JH2-2	20.01 ± 1.04	256.48 ± 3.49	1.23 ± 0.41	169.60 ± 5.70

^aRelative Fluorescence Units (RFU) at the appropriate excitation/emission wavelengths for each fluorescent protein detected in bacterial strains that are either plasmid-free or carrying replicative plasmids pVMTeal or pVMCherry. Values are the average (± Standard Deviation) of 16 wells in two biologically independent experiments. All strains carrying plasmids differ from the corresponding plasmid-free controls (p < 0.000001 Student *t* test).