Appl Microbiol Biotechnol
DOI 10.1007/s00253-007-1253-9

APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

Fluorescent reference strains of bacteria by chromosomal integration of a modified green fluorescent protein gene

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Received: 3 October 2007 / Revised: 18 October 2007 / Accepted: 19 October 2007
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Abstract Fluorescent reference strains of bacteria carrying a stable chromosomally integrated single copy of the *gfp* gene have been developed. A modified version of the *gfp* gene has been generated by mutagenesis and expressed under the control of the bacteriophage lambda promoter P_L. A cassette comprising bacteriophage Mu transposon arms flanking the modified *gfp* gene and regulatory regions was irreversibly integrated as an in-vitro-assembled transposition complex into the genomes of *Escherichia coli* and *Salmonella* spp. The modified green fluorescent protein (GFP) protein retained the fluorescence excitation and emission wavelengths of wild-type GFP. However, it fluoresced more brightly in *E. coli* and S*almonella* compared to wild-type GFP, presumably due to improved protein maturation. Fluorescent *E. coli* and *Salmonella* strains carrying the *gfp* gene cassette were easily differentiated from their respective non-fluorescent parental strains on various growth media by visualization under UV light. The bacterial strains produced by this method remained viable and stably fluorescent when incorporated into a matrix for delivery of exact numbers of viable bacterial cells for use as quality control agents in microbiological procedures.

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Introduction

Microbiology testing laboratories maintain in-house bacterial culture collections for quality control (QC) purposes. These QC strains are used as reference standards and for quality control of the testing methods employed. A problem faced by testing laboratories is the inadvertent cross-contamination of samples with a QC strain. They use species of bacteria that are rarely detected in their samples as QC strains to help with identifying instances of cross-contamination. For example, in Australia, *Salmonella salford* is used as a QC strain because it is detected rarely in clinical, food, or environmental samples. When a laboratory detects *Salmonella*, tests are performed to check whether or not it is *S. salford*. While the use of rare species helps to identify cross-contamination problems, confirmation of the identity of the strain that has been detected takes considerable time, and lengthy delays can have serious implications. A further problem with the use of rare species as QC strains is that they may have biochemical or physiological properties that are different to those of commonly isolated organisms. A possible solution for this

problem and to speed up and improve the validation of results from microbiological testing methods would be inclusion of a stable and easily detectable molecular marker into QC bacterial strains.

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has been the marker system used most successfully to confer a visual phenotype for detection of bacteria (Errampalli et al. 1999). Bacteria marked with GFP can be detected simply under UV light illumination, without the requirement for exogenous substrates or complex media (Chalfie et al. 1994). A number of bacteria expressing GFP have been created by incorporating the *gfp* gene into plasmid vectors (Chalfie et al. 1994; Matthysse et al. 1996; Fratamico et al. 1997; Scott et al.1998; Prachayio et al. 2000). The main advantage of strains carrying plasmids is that several hundred gene copies can be present within a single cell, resulting in highly fluorescent bacterial cultures. However, plasmids normally carry an antibiotic resistance or other selective marker gene, and strains containing the plasmid only retain fluorescence when grown under selection, especially if they are cultured through many generations. The resulting effect of growth on non-selective media is the loss of the fluorescent phenotype due to selective enrichment of non-fluorescent plasmid-free cells. This is an undesirable characteristic for bacterial strains used in quantitative QC methods.

The *gfp* gene can be integrated into the genome of bacteria as an alternative to the use of plasmids for GFP expression. Transposon-based systems have been used widely for chromosomal integration of genes in bacteria. Often, these systems employ suicide vectors for random delivery of transposon constructions into the bacterial genome as single or multicopy genes (Tombolini et al. 1997; Tresse et al. 1998; Errampalli et al. 1998; Cho et al. 1999; Scott et al. 2000; Ahn et al. 2001). The most successful results for detection of bacterial strains expressing chromosomally integrated *gfp* were in cultures marked with mutant genes encoding GFP variants with enhanced brightness and better protein folding (Eberl et al. 1997; Errampalli et al. 1999; Koch et al. 2001; Hauterfort et al. 2003; Baldridge et al. 2005). Ideally, only a single copy of the *gfp* gene should be integrated into the bacterial chromosome for QC strain development purposes. This reduces the likelihood of genetic instability resulting in gene inactivation as a result of homologous recombination. However, the requirement for a single copy gene in the bacterial genome may not provide sufficient fluorescence for visual detection of cultures.

The work reported in this paper describes the generation of a modified *gfp* gene, construction of an expression cassette, and its use for chromosomal integration for development of fluorescent *Escherichia coli* and *Salmonella* reference strains.

Materials and methods

Bacterial strains, plasmids, and reagents

E. coli strain DH5α was used for all DNA manipulations. *E. coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028 and *S. abaetetuba*, ATCC 35640, supplied by BTF Pty (North Ryde, NSW, Australia), were used for chromosomal integration experiments. *E. coli* and *Salmonella* spp. were grown in either Luria–Bertani (LB) broth (Sambrook et al. 1989) or nutrient medium (Oxoid, Basingstoke, Hampshire, England) at 37°C. Chloramphenicol (Cm) was used at a concentration of 25 $\mu g\ ml^{-1}$ for plasmid selection. The following culture media were used in comparing the growth and differentiation of parental and fluorescent strains: Nutrient Broth (Oxoid, Thebarton SA Australia); Nutrient Agar (NA, Oxoid); mFC agar (Oxoid); Violet Red Bile agar (VRB, Oxoid); mEndo Chromocult (CCA), Plate Count Agar (PCA) Xylose Lysine Deoxycholate (XLD), Bismuth Sulfite (BS), Brilliant Green (BG), and Rambach agar (all from Merck, Kilsyth VIC Australia).

The *gfp* gene was obtained from plasmid pGFP (Clontech, Mountain View, CA, USA) and regulatory regions for the *gfp* gene cassette from plasmid pJLA602 (Schauder et al. 1987). Plasmid pEntranceposon CmR (Finnzymes, Espoo, Finland) was used for chromosomal insertion of gene cassettes. Plasmid DNA was extracted from cells using QIAprep Systems (Qiagen) and DNA fragments purified from agarose gels using QIAquick gel extraction kits (Qiagen). Total genomic DNA was extracted from cells using a FastDNA® Kit and FastPrep® instrument (BIO 101, Carlsbad, CA, USA). Restriction endonucleases were obtained from MBI Fermentas (Burlington, ON, Canada), AmpliTaq Gold DNA polymerase was from Applied Biosystems (Applied Biosystems, Foster City, CA), and MuA transposase was obtained from Finnzymes and T4 DNA ligase from Roche (Roche Diagnostics, Basel, Switzerland). Oligonucleotides were obtained from Sigma-Genosys (Sigma-Aldrich, Castle Hill, NSW, Australia).

Electrocompetent cells

Electrocompetent *E. coli* and *Salmonella* spp. cells were prepared as described by Sambrook et al (1989).

PCR reactions

Standard polymerase chain reactions (PCR) were carried out in 50 μl reaction volumes using AmpliTaq-Gold Polymerase according to the manufacturer's recommendations in a Gene Amp 2400 PCR system (Applied Biosystems).

Overlap extension PCR

Mutagenesis and reassembly of the *gfp* gene and regulatory regions into a *gfp* gene cassette was achieved using overlap-extension PCR (Ho et al. 1989) using the oligonucleotide primers depicted in Fig. 1 and listed in Table 1. The PCR reagents used were identical to those used for standard PCR reactions except for the use of the *gfp* gene cassette segments (~10 ng) as template DNA in a 50 µl PCR reaction volume. PCR for assembly of the wild-type *gfp* gene and upstream λ regulatory regions into a single cassette was performed using an initial primer-less step-down overlap extension stage (annealing temperatures of 50 down to 35°C over 15 cycles with 1°C decrease per cycle (30 s), followed by addition of primers (GFPF0 and GFPR0) and a second standard PCR (95°C, 30 s; 55°C, 30 s; 72°C, 1 min) repeated for 25 cycles. A primer-less overlap extension stage (55 down to 30°C over 25 cycles) followed by the addition of primers GFPF1 and GFPR0 and standard PCR was performed to recombine the mutant *gfp* gene segments into a pool of mutated full-length *gfp* genes. After amplifying the assembled mutant *gfp* gene, another PCR reaction round was carried out using the same conditions used for the assembly of the wild-type *gfp* gene cassette.

Assembly of transpososomes in vitro

Transpososomes are stable protein DNA complexes formed by the binding of transposase protein to specific binding sites at each end of the transposon DNA (Goryshin et al. 2000; Lamberg et al. 2002). Transpososome formation reactions were optimized by titration of transposon DNA against a fixed amount of MuA transposase enzyme (Finnzymes). Transpososome assembly reaction mixtures (20 µl) comprised ~6 pmol MuA transposase, 50% (*v*/*v*) glycerol, 0.025% (*v*/*v*) Triton X-100, 150 mM of Tris–HCl (pH 6.0), 150 mM NaCl, 0.1 mM ethylenediaminetetra-acetic acid (EDTA), and 0.1 mM dithiothreitol. Reactions were performed by adding ~0.125 and ~1.0 pmol transposon DNA to the mixtures, followed by incubation at 30°C for 2–3 h. Transpososome formation was visualized by electrophoresis on 1.5% agarose-Tris-acetate-EDTA (TAE) gel containing 80 µg/ml of bovine serum albumin.

Transposon DNA carrying the *gfp* triple mutant (S72A, M153T, V163A) *gfp* gene cassette was constructed by cloning into the MCS site of the pEntranceposon vector. *Bgl*II digestion of the vector allowed excision of the transposon DNA containing the mutant *gfp* gene that was then used for transpososome assembly reactions. Titration of the amount of transposon DNA (~0.125 to ~1.0 pmol) against a fixed amount of MuA transposase (~6 pmol) resulted in successful transpososome complex formation as monitored by agarose gel electrophoresis. Selected complexes were used for the electrotransformation of *E. coli* and *Salmonella* strains.

Chromosomal integration of the *gfp* gene cassette into *E. coli* cells

The *gfp* gene was amplified directly from chloramphenicol-resistant transformed cells by colony PCR using the GFPF1

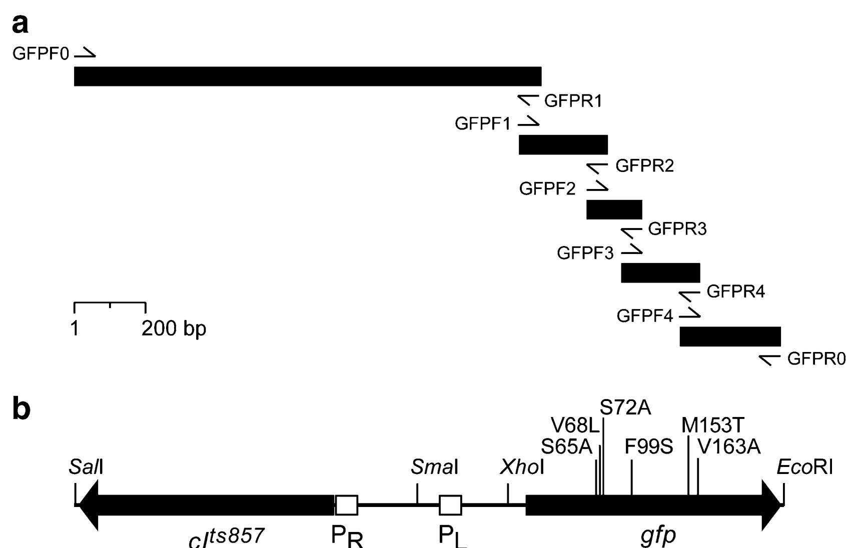

Fig. 1 Strategy used for *gfp* gene cassette construction and PCR mutagenesis. **a** Graphical representation of the binding positions of overlapping degenerate and non-degenerate oligonucleotide primers and the five PCR products used for the overlap-extension PCR assembly of the *gfp* gene cassette depicted in **b**. Oligonucleotide sequences are listed in Table 1. **b** Overview of the structure of the temperature inducible *gfp* cassette, comprising the bacteriophage lambda promoters P_R and P_L, the gene encoding the lambda thermolabile cI_{ts857} repressor protein, and the *gfp* gene. Highlighted along the *gfp* gene are the six amino acid modifications randomly introduced during the overlap-extension PCR

Table 1 Oligonucleotide primers used for the *gfp* gene cassette construction and mutagenesis and for genomic walking to determine cassette integration points

Primer	Sequence	GFP Codon Alteration[a]
GFPF0	5′-TTTTTTGAATTCTTATTTGTATAGTTCATC	
GFPR1	5′-CTTTACTCATGGCAGTCTCCAGTTTGT	
GFPF1	5′-GAGACTGCCATGAGTAAAGGAGAAGA	
GFPF2	5′-ATGGTSTTCAATGCTTTKCRAGATACCCAGATCATA	S65A,S65G,V68L,S72A
GFPF3	5′-AACTATATYTTTCAAAGATGACGGGA	F100S
GFPF4	5′-CAAACAAAAGAATGGAATCAAAGYTAACTTCAAAATTAGA	M153T
GFPR0	5′-TTTTTTGAATTCTTATTTGTATAGTTCATC	
GFPR2	5′-AAAGCATTGAASACCATAMSMGAAAGTAGTGACAAGT	S65A,S6G,V68L
GFPR3	5′-CTTTGAAARATATAGTTCTTTCCTGTA	F100S
GFPR4	5′-TTCCATTCTTTTGTTTGTCTGCCRTGATGTATACATTGTGT	M153T
PENTGWF	5′-TGATCTTCCGTCACAGGT	
PENTGWR	5′-GTAACAGCTGCTGGGATT	

[a] Relative binding position of each oligonucleotide is shown in Fig. 1.

and GFPR0 primers. Successful amplification indicated the presence of an integrated copy of the *gfp* gene cassette into the bacterium genome. Genomic DNA from four selected putative integrant recombinants representing both mutant and wild-type *gfp* integrants were used for PCR amplification of the *gfp* gene cassette followed by DNA sequencing to confirm the presence of an intact error-free *gfp* gene cassette (regulatory region and *gfp* open reading frame).

GWPCR for the identification of the insertion points of the *gfp* gene cassette

Genomic-walking PCR (GWPCR; Morris et al. 1998) was employed for the identification of the insertion point of the *gfp* gene cassette in *E. coli* and *Salmonella* spp. recombinants. The specific primers, PENTGWF and PENTGSWR, were designed for GWPCR from each end of the pEntrancesposon MuA cassette (see Table 1). Both primers were used in combination with primers complementary to the generic GWPCR reaction linker. For construction of linker libraries, restriction fragments were generated by overnight digestion at 37°C of ~1.5 µg genomic DNA with 30 U of *Nco*I, *Hin*dIII, *Eco*RI, *Xba*I, *Bam*HI, *Sal*I, *Pst*I, *Sac*I, *Kpn*I, or *Aat*II. GWPCR templates were prepared by ligating ~0.5 µg digested genomic DNA to a ~10-fold molar excess of linker DNA. All GWPCR were performed using 0.2 µl of linker library ligation mixture as template. PCR products ranging from 300 to 800 bp were sequenced and results used to search bacterial genome sequences for identification of the region flanking the *gfp* gene cassette insertion point.

Southern blotting

Southern blots were performed using the DIG Easy-Hyb system according to the manufacturer's recommendations (Roche). The *gfp* gene cassette DNA probes were prepared using a PCR DIG Probe Synthesis kit (Roche). Positive bands were detected using the DIG system wash and Block Buffer Set and CPD-Star detection reagent according to manufacturer's protocols (Roche) and exposed to CL-XPosure ™ X-Ray Film (Pierce Biotechnology, IL, USA).

DNA sequencing

DNA sequencing was performed using dye terminator chemistry (Applied Biosystems). Sequencing results were analyzed using the GCG Wisconsin software package version 8 (Devereux et al. 1984).

Growth and differentiation of parental strains and GFP integrants on standard medium

Pure cultures of each recombinant GFP mutant were streaked onto various growth media used in standard analyses of food and/or water for the respective organism (s). The same procedure was followed for each parental strain. Mixed cultures of each GFP variant and its parent were streaked onto the same medium. Media and incubations for *E. coli* were mFC (44.5°C/24 h), VRB agar (30°C/24 h) mEndo, and CCA and PCA (37°C/24 h). VRB medium inoculated with *E. coli* was also used in a pour plate format to evaluate the appearance of subsurface colonies. Media for *S. typhimurium* were XLD, BS, BG, Rambach agar (Merck), and PCA with all incubations at 37°C for 48 h. All test bacteria were cultured aerobically. All plates were examined under UV light (366 nm) illumination in a dark room after 24 and 48 h incubation. Fluorescence was scored qualitatively as: 0 (no fluorescence), 1 (weak fluorescence), or 2 (strong fluorescence).

Production of BioBalls™ using *gfp* integrant strains

BioBall™ freeze-dried pellets, containing a precise number of viable bacterial cells, were prepared as described previously (Morgan et al. 2004). In summary, cells were grown in a proprietary medium and dispensed in precise numbers into droplets of a proprietary lyoprotectant mixture using a specially designed flow cytometer. Droplets were ejected into liquid nitrogen and freeze-dried in sterile glass vials under vacuum.

Results

Construction and mutagenesis of the temperature-inducible *gfp* gene cassette

A gene cassette was constructed comprising the *gfp* gene under the control of the strong bacteriophage lambda promoters P_R and P_L and the gene encoding the lambda thermolabile cI_{ts857} repressor protein to obtain visibly detectable levels of GFP expressed from a single copy *gfp* gene in the bacterial chromosome. A modified *gfp* gene was created by PCR mutagenesis using overlapping sets of non-degenerate and degenerate oligonucleotide primers to reconstruct the full-length *gfp* gene with six modified codon positions (Table 1). The degenerate primers were designed to introduce randomly either the wild-type codon or a codon for an amino acid known to alter the fluorescence or maturation characteristics of GFP. The sites and the selected residue alterations were chosen for their proven ability to improve the maturation and fluorescence of GFP in bacteria (Crameri et al. 1996; Heim and Tsien 1996; Cormack et al. 1996; Youvan and Michel-Beyerly 1996; Siemering et al. 1996). It was expected that using the degenerate oligonucleotide primers would result in the creation of a library of *gfp* genes with an average three of the six altered codon positions present. The binding positions of all oligonucleotide primers with respect to the *gfp* cassette are shown in Fig. 1a. The wild-type *gfp* gene was also incorporated into the cassette in the same manner for use as a control. PCR reactions using non-degenerate and degenerate oligonucleotide primers resulted in products corresponding to the regulatory regions and four different segments of the mutant *gfp* gene. The locations of the individual mutations in the *gfp* gene are shown in Fig. 1b. The initial plasmid construction included both the λ cI_{ts857} gene and the λ_{P_r} regions (Fig. 1b), but these sequences were deleted following initial assays of the level of fluorescence observed (see "Construction and chromosomal integration of the unrepressed *gfp* gene cassette into *E. coli* and *Salmonella*").

Screening of mutant *gfp* gene cassette recombinants

The *gfp* gene cassette was designed to include two unique restriction enzyme sites, *Sal*I and *Eco*RI (Fig. 1b) that were used for directional ligation of the mutated *gfp* gene cassette into the multiple cloning site of the plasmid vector pEntranceposon CmR (Fig. 2a). The vector is a high copy number plasmid constructed by replacing the multiple cloning site of plasmid pUC19 with the bacterial phage Mu transposon and the chloramphenicol resistance gene. PCR products comprising the complete mutant *gfp* gene cassette and the plasmid vector pEntranceposon were digested with *Sal*I and *Eco*RI, ligated, and used to

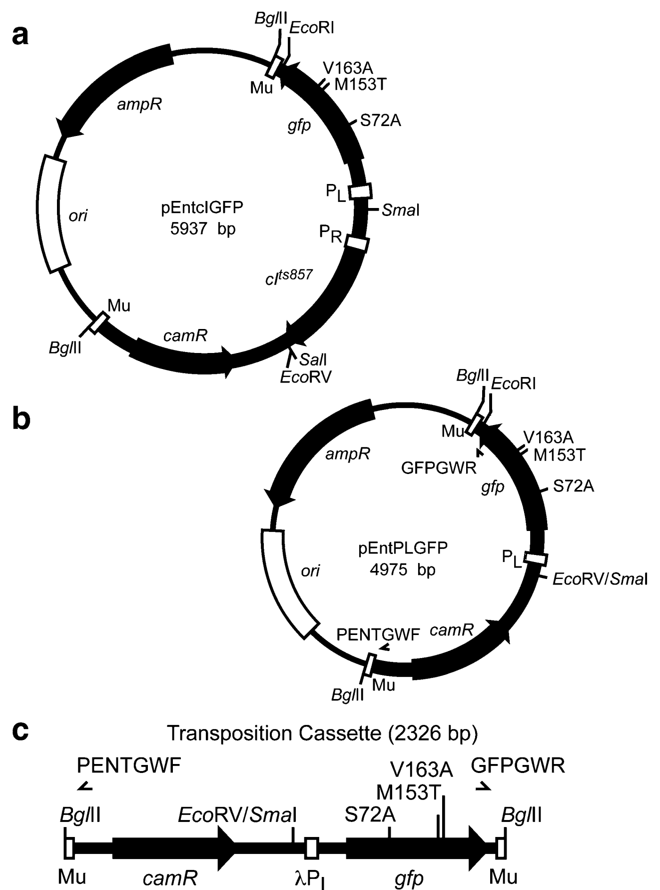

Fig. 2 Plasmid and transposon constructions. **a** A diagram of the plasmid pEntcI*gfp* generated by ligation, via *Eco*RI and *Sal*I sites, of the *gfp* gene cassette depicted in Fig. 1b into the plasmid pEntransposon-CamR. The relative positions of the three most frequently occurring combination of amino acid alterations observed to result in the brightest fluorescence intensity of recombinant colonies (S72A, M153T, and V163A) are highlighted along the *gfp* gene. The transposon arms are labeled Mu and the pBR322 origin of replication is marked *ori*. **b** Diagram of the plasmid pEntPL*gfp* generated by an *Eco*RV/*Sma*I deletion of the cI_{ts857} gene and the lambda P_R promoter. **c** Structure of the unrepressed *gfp* transposition cassette and binding positions of primers PENTGWF and GFPGWR used for identification of insertion points in the genome of the fluorescent strains

transform competent *E. coli* DH5α to generate a library of recombinants containing the *gfp* gene mutated randomly at the six selected positions. Similarly, the wild-type *gfp* gene cassette was ligated into the digested pEntranceposon vector and transformed into competent *E. coli* DH5α. Transformed cells were plated onto LB plates containing Cm and incubated at 28°C and, after 48 h incubation, shifted to 42°C for 2–3 h for induction of GFP expression. Colonies expressing GFP were screened by visualization of fluorescence using a handheld UV lamp (365 nm). Colonies originating from the mutant *gfp* gene library showed variation in the green fluorescence intensity emitted. Recombinant colonies with a range of fluorescence intensities were isolated for further characterization.

Identification of the amino acid changes introduced in mutant GFP recombinants

Sequence alignment results revealed that mutant *gfp* genes with a combination of S72A, M153T, and V163A mutations resulted in colonies with the brightest fluorescence intensity, and they were considerably brighter than colonies bearing the wild-type *gfp* gene cassette. The mutant *gfp* gene cassette containing the combined S72A, M153T, and V163A changes and the wild-type *gfp* gene construction were selected for chromosomal integration experiments.

Chromosomal integration of *gfp* gene cassette into *E. coli* cells

Integration of the *gfp* gene cassette into the *E. coli* DH5α genome was performed by electroporation of bacteriophage Mu DNA transposition complexes into electrocompetent cells. Once inside the cell, the transposition complex is activated by Mg^{2+} ions in the intracellular environment, resulting in single copy integration of the transposon construction into the bacterial genome (Lamberg et al. 2002). However, after thermal induction at 42°C of these recombinants containing the λcI_{ts857} gene, no fluorescence was observed when colonies were illuminated with UV light. Therefore, we examined the removal of the cI_{ts857} gene to allow unrepressed, constitutive, high-level expression of the GFP protein from the λ P_L promoter.

Construction and chromosomal integration of the unrepressed *gfp* gene cassette into *E. coli* and *Salmonella*

Unique *Sma*I and *Eco*RV sites in pEntcIGFP (see Fig. 2a) were used to excise most of the cI_{ts857} gene and lambda P_R from the cassette to allow high-level constitutive transcription of *gfp* from the P_L promoter. The DNA was recircularized by blunt-end ligation and used to transform *E. coli* DH5α that were then plated onto LB+Cm plates and grown overnight at 37°C. The bacteriophage Mu DNA transposition complex derived from this plasmid construction (termed pEntPLGFP, Fig. 2b) was used for chromosomal integration of the unrepressed *gfp* gene cassette (after *Bgl*II digestion, Fig. 2c) into strains of *E. coli*, *S. typhimurium*, and *S. abaetetuba* as described in “Materials and methods.” Excision of the cI_{ts857} gene from the *gfp* gene cassette was confirmed by restriction digestion analysis of plasmids extracted from transformants. After 12–16 h growth at 37°C, colonies of *E. coli* and *Salmonella* expressing the GFP protein could be visualized by illumination of plates with the hand-held UV light (Fig. 3a).

PCR products of the expected size confirmed the presence of the unrepressed *gfp* gene cassette in all colonies tested and genomic DNA prepared from representative cultures resulted in PCR amplification of the entire unrepressed *gfp* gene cassette. Nucleotide sequencing of products indicated the presence of intact unrepressed *gfp* gene cassettes in the genomic DNA of both *E. coli* and *Salmonella* transformants.

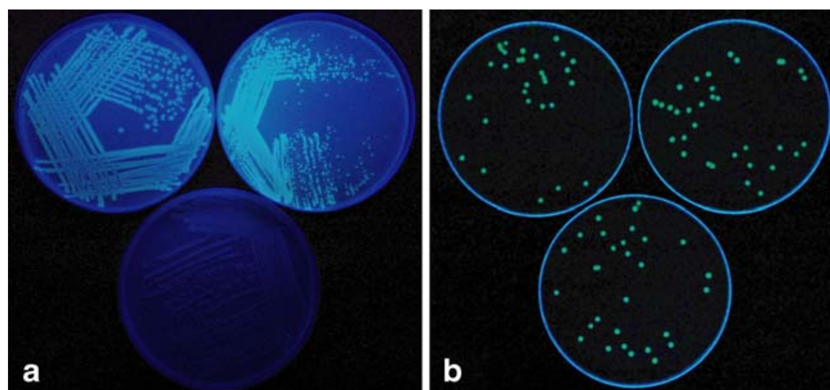

Fig. 3 Fluorescent strains of *E. coli* and *Salmonella* under U.V. light. **a** *Top left*, *E. coli gfp* chromosomal integrant strain; *top right*, *E. coli* carrying plasmids expressing GFP variant S72A, M153T, V163A; *lower plate*, untransformed *E. coli.* **b** Samples from the 30 CFU BioBall™stability control experiments using the fluorescent *S. abaetetuba* integrant strain

Table 2 Fluorescence intensity of parental and GFP variant colonies

E. coli[a]					*S. typhimurium*[a]				
Medium	Parent, 24 h	GFP, 24 h	Parent, 48 h	GFP, 48 h	Medium	Parent, 24 h	GFP, 24 h	Parent, 48 h	GFP, 48 h
PCA	0	1	0	2	PCA	0	1	0	2
mEndo	0	0	0	2	XLD	0	2	0	2
mFC	0	1	0	2	BG	0	1	0	2
VRB	0	1	0	2	BS	0	2	0	2
CCA	0	2	0	2	Rambach	0	2	0	2

[a] Pure cultures of *E. coli* and *S. typhimurium* and each respective GFP variant were streaked onto various solid growth media. All plates were incubated at 37°C, except VRB, which was incubated at 30°C. Plates were examined under UV light (366 nm) after 24 and 48 h incubation. Fluorescence of colonies was qualitatively scored as: 0 (not visibly fluorescent), 1 (weakly fluorescent), or 2 (strongly fluorescent).

Chromosomal DNAs from integration recombinants were extracted and digested with *Hin*dIII, *Bam*HI, *Nco*I, and *Rsa*I and were analyzed by Southern blot hybridization using a DIG-labeled *gfp* gene cassette probe. Detection of a single band within the *Hin*dIII- and *Bam*HI-digested chromosomal DNA confirmed the presence of a single copy of the *gfp* gene cassette in the integrants. Concurrent Southern blot analysis of *Nco*I- and *Rsa*I-digested chromosomal DNA (which both cleave the *gfp* gene cassette sequence once), showed two distinct bands, further indicating that a single copy of the *gfp* gene cassette was integrated into the chromosome of each recombinant (data not shown).

The appearance of colonies of the *E. coli* and *Salmonella abaetetuba* strains are shown in Fig. 3. The fluorescent *E. coli* strain with the chromosomally integrated *gfp* gene (Fig. 3a, left) is comparable to the plasmid-borne copy of the gene (Fig. 3b, right) and both can be distinguished clearly from the host strain that does not carry the *gfp* gene (Fig. 3a, bottom).

Chromosomal insertion points of the *gfp* cassettes

The insertion points of the *gfp* gene cassette into the genomes of *E. coli* and *Salmonella* spp. integrants were identified based on published genome sequence data for *S. typhimurium* and *E. coli* K12. For *E. coli* ATCC 25922, the *gfp* gene cassette was determined to be inserted into the gene encoding a zinc-binding periplasmic protein (ZnaP). The *gfp* gene cassette of the fluorescent *S. abaetetuba* ATCC 35640 isolate was inserted into a sequence encoding a *S. typhimurium* ATP-dependent helicase protein (*hrp*A), and for *S. typhimurium* ATCC 14028, it was inserted into a sequence encoding a common antigen found in the outer membrane of *Salmonella* and other enterobacteria. The integration of the *gfp* gene cassettes did not affect the growth rates or ability to express GFP by *E. coli* and *Salmonella* spp. strains (data not shown).

Growth and differentiation of parent strains and GFP integrants on standard media

Under white light illumination, colonies of all *gfp* integrants displayed identical colonial morphologies and equivalent differential media reactions compared to their respective parental strains. Fluorescence of all *gfp* integrants could be discerned under UV light on all media tested after 24 h incubation, and differentiation from each respective non-fluorescent parent strain could be established clearly (Table 2). The only exception was recombinant *E. coli* on mEndo that showed weak fluorescence in the primary inoculum (confluent growth) but not from isolated colonies after 24 h incubation on this medium. Fluorescence of *E. coli* on mEndo was clearly discernable, and fluorescence of all other *gfp* integrants had increased in intensity after 48 h incubation.

Fluorescence of GFP was not obscured by differential media reactions (Table 3). Irrespective of medium color or differential reactions, both *E. coli* and *Salmonella* fluorescent strains were clearly discernable from non-fluorescent colonies on both selective and non-selective media, and in some cases, fluorescence was easier to distinguish on selective media. For example, the *E. coli gfp* integrant exhibited stronger fluorescence on CCA than PCA at 24 h, and the *S. typhimurium gfp* integrant exhibited stronger fluorescence on XLD than PCA at 24 h (Table 3). Colony

Table 3 Observed appearance with UV illumination of *S. typhimurium* parental and *gfp* variant colonies on different growth media

Growth medium	*gfp* integrant	Untransformed parent
XLD	Green with black periphery	Black
BS	Green with black periphery	Black
BG	Light green	Lilac
Rambach	Yellow gold	Pink-red
PCA	Bright green	Colorless

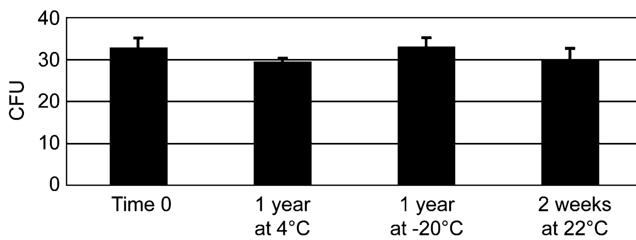

Fig. 4 Stability of fluorescent strains after incorporation into BioBall™ matrix. Results obtained for 30 CFU BioBall™stability control experiment using the fluorescent *S. abaetetuba* strain

fluorescence was generally easier to discern for media where the background (medium) color contrasted with the green fluorescent colonies under UV illumination.

Stability of *gfp* integrant strains

Batches of 300 BioBalls™ carrying a *gfp* integrant strain were produced and 50 BioBalls™ from each batch plated on nutrient agar to determine mean dose and standard deviation (n=50) of viable bacterial cell number. Stability experiments were conducted by storing BioBalls™ for 2 weeks at 22°C, 1 year at 4°C, and 1 year at −20°C. After incubation, BioBalls™ carrying *gfp* integrant strains were plated onto nutrient agar and incubated overnight at 37°C (Fig. 4) and counts of fluorescent bacterial colonies formation verified. Results for all three incubation conditions indicated that the *gfp* integrant strains remain viable and stably fluorescent after incorporation into the BioBall™ production process. Figure 3b shows the fluorescence of individual colonies of *S. aetetuba* carrying the integrated *gfp* gene after growth from the reconstitution of a single BioBall™.

The stability of the integrant strains during culturing was verified by maintenance of fluorescence after ten sequential subcultures of all strains in LB broth, performed by inoculating 50 µl of broth cultures into 50 ml of LB media free of antibiotics and grown in shake flasks overnight at 37°C. The appearance of any non-fluorescent cells was monitored by analyzing samples of each culture using a flow cytometer and also by streaking each subculture to single colonies on agar plates and examining colony fluorescence under UV light. After ten such subculture steps, no non-fluorescent cells could be detected for all integrant strains using both methods.

Discussion

Fluorescent strains of *E. coli* and *Salmonella* spp. carrying a stable single copy of the *gfp* gene have been developed for use as markers for the validation of quality control determinations. We have resolved the problems of fluorescent marker loss due to plasmid instability and segregation, by incorporating a single copy of the *gfp* gene into the chromosome of the host.

Initial experiments using *gfp* under the control of a heat-inducible repressor were unsuccessful. The cI^{ts857} repressor protein is inactivated by incubation at 42°C, thereby allowing transcription/translation from the lambda P_L. Normally, this repression/expression system is plasmid-based and in high copy number, and maximum levels of protein expression are usually achieved 3–5 h post-induction. Indeed, we observed GFP fluorescence when inducing the cI^{ts857}-controlled *gfp* cassette as a pEntrans-poson-based plasmid. However, once integrated into the genome as a single copy, we found that GFP fluorescence was not detectable after 3–5 h. Theoretically, longer incubation times at 42°C would allow the production of visible levels of GFP from a single copy gene. However, we believe that the metabolic stress caused by prolonged incubation of *E. coli* at 42°C most likely prevented production of visible levels of GFP.

Accordingly, we re-engineered the *gfp* cassette so that expression was constitutive and did not require heat induction. A modified version of *gfp*, with increased fluorescence compared to the wild-type gene, was generated by PCR mutagenesis and placed under the control of a strong bacteriophage λ promoter to obtain visibly detectable levels of GFP expression from the single copy gene. MuA transposition complexes carrying an integrative gene cassette with the modified *gfp* gene and regulatory regions were assembled in vitro and used for irreversible insertion of the *gfp* gene into the genomes of *E. coli* and *Salmonella* spp. strains. These fluorescent strains produced by expression of modified GFP from the λ_{PL} promoter could be differentiated from equivalent non-fluorescent parental strains on various growth media typically used for quality control. As a further demonstration of their utility in QC procedures, we showed that they remained viable and stably fluorescent when incorporated into the BTF Pty BioBall™ format for delivery of exact numbers of viable bacterial cells to liquid or solid media. The construction of these QC strains is significant because of their use for the quality control of media and testing processes reduces the risk of false positive results due to contamination with the QC strain. Because the fluorescence of the strains developed in this study is stable in the absence of antibiotics, they can be used for QC purposes with the knowledge that they will remain fluorescent. The fluorescent strains generated in this work have the potential to be used as rapidly and easily identified QC positive controls, thus saving laboratories time and money compared to molecular serotyping. They also have potential for use as internal standards for other microbiological analytical procedures.

Acknowledgements This research was supported by an Australian Research Council Linkage Grant in collaboration with BTF Pty Ltd, Australia and the National Measurement Institute of Australia.

References

Ahn YB, Beudette H, Trevors JT (2001) Survival of a GFP-labeled polychlorinated biphenyl degrading psychrotolerant *Pseudomonas* spp. in 4 and 22°C soil microcosms. Microbial Ecol 42:614–623

Baldridge GD, Burkhardt N, Herron MJ, Kurtti TJ, Munderloh UG (2005) Analysis of fluorescent protein expression in transformants of *Rickettsia monacensis*, an obligate intracellular tick symbiont. Appl Environ Microbiol 71:2095–2105

Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC (1994) Green fluorescent protein as a marker for gene expression. Science 263:802–805

Cho JC, Kim SJ (1999) Green fluorescent protein-based direct viable count to verify a viable but non-culturable state of *Salmonella typhi* in environmental samples. J Microbiol Methods 36:227–235

Cormack BP, Valdivia RH, Falkow S (1996) FACS-optimized mutants of the green fluorescent protein (GFP). Gene 173:33–38

Crameri A, Whitehorn EA, Tate E, Stemmer WP (1996) Improved green fluorescent protein by molecular evolution using DNA shuffling. Nat Biotechnol 14:315–319

Devereux J, Haeberli P, Smithies O (1984) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res 12:387–395

Eberl LSR, Ammendola A, Geisenberger O, Earhart R, Molin S, Amann R (1997) Use of green fluorescent protein as marker for ecological studies of activated sludge communities. FEMS Microbiol Lett 149:77–83

Errampalli D, Leung K, Cassidy MB, Kostrzynska M, Blears M, Lee H, Trevors JT (1999) Applications of the green fluorescent protein as a molecular marker in environmental microorganisms. J Microbiol Methods 35:187–199

Errampalli D, Okamura H, Lee H, Trevors JT, van Elsas JD (1998) Green fluorescent protein as a marker to monitor survival of phenanthrene-mineralizing Pseudomonas sp. UG14Gr in creosote-contaminated soil. FEMS Microbiology Ecol 26:181–191

Fratamico PM, Den Y, Strobaugh TP, Palumbo SA (1997) Construction and characterization of E. coli 0157:H7 strains expressing firefly luciferase and green fluorescent protein and their use in survival studies. J. Food Prot 60:1167–1173

Goryshin IY, Jendrisak J, Hoffman LM, Meis R, Reznikoff WS (2000) Insertional transposon mutagenesis by electroporation of released Tn5 transposition complexes. Nat Biotechnol 18:97–100

Hautefort I, Proenca MJ, Hinton JC (2003) Single-copy green fluorescent protein gene fusions allow accurate measurement of *Salmonella* gene expression in vitro and during infection of mammalian cells. Appl Environ Microbiol 69:7480–7491

Heim R, Tsien RY (1996) Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. Curr Biol 6:178–182

Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 77:51–59

Koch B, Jensen LE, Nybroe O (2001) A panel of Tn7-based vectors for insertion of the *GFP* marker gene or for delivery of cloned DNA into Gram-negative bacteria at a neutral chromosomal site. J Microbiol Methods 45:187–195

Lamberg A, Nieminen S, Qiao M, Savilahti H (2002) Efficient insertion mutagenesis strategy for bacterial genomes involving electroporation of in vitro-assembled DNA transposition complexes of bacteriophage mu. Appl Environ Microbiol 68:705–712

Matthysse AG, Stretton S, Dandie C, McClure NC, Goodman AE (1996) Construction of GFP vectors for use in Gram-negative bacteria other than *Escherichia coli*. FEMS Microbiol Lett 145:87–94

Morgan CA, Bigeni P, Herman N, Gauci M, White PA, Vesey G (2004) Production of precise microbiology standards using flow cytometry and freeze drying. Cytometry A 62:162–168

Morris DD, Gibbs MD, Chin CW, Koh MH, Wong KK, Allison RW, Nelson PJ, Bergquist PL (1998) Cloning of the *xynB* gene from *Dictyoglomus thermophilum* Rt46B.1 and action of the gene product on kraft pulp. Appl Environ Microbiol 64:1759–1765

Prachaiyo P, McLandsborough LA (2000) A microscopic method to visualize *Escherichia coli* interaction with beef muscle. J Food Prot 63:427–433

Sambrook J, Fritsch EJ, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbour Laboratory Press, New York

Schauder B, Blocker H, Frank R, McCarthy JEG (1987) Inducible expression vectors incorporating the *Escherichia coli atpE* translational initiation region. Gene 52:279–283

Scott KP, Mercer DK, Richardson AJ, Melville CM, Glover LA, Flint HJ (2000) Chromosomal integration of the green fluorescent protein gene in lactic acid bacteria and the survival of marked strains in human gut simulations. FEMS Microbiol Lett 182:23–27

Scott KP, Mercer DK, Glover LA, Flint HJ (1998) The green fluorescent protein as visible marker for lactic acid bacteria in complex ecosystems. FEMS Microbiol Ecol 26:219–230

Siemering KR, Golbik R, Sever R, Haseloff J (1996) Mutations that suppress the thermosensitivity of green fluorescent protein. Curr Biol 6:1653–1663

Tombolini R, Unge A, Davy ME, de Bruijn FJ, Jansson J (1997) Flow cytometric and microscopic analysis of GFP-tagged *Pseudomonas fluorescens* bacteria. FEMS Microbiol Ecol 22:17–28

Tresse O, Errampalli D, Kostrzynska M, Leung KT, Lee H, Trevors J, van Elsas JD (1998) Green fluorescent protein as a visual marker in a *p*-nitrophenol degrading *Moraxella* sp. FEMS Microbiol Lett 164:187–193

Youvan DC, Michel-Beyerle ME (1996) Structure and fluorescence mechanism of GFP. Nat Biotechnol 14:1219–1220