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Construction of stable fluorescent laboratory control strains for several food safety relevant *Enterobacteriaceae*

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ABSTRACT

Using naturally-occurring bacterial strains as positive controls in testing protocols is typically feared due to the risk of cross-contaminating samples. We have developed a collection of strains which express Green Fluorescent Protein (GFP) at high-level, permitting rapid screening of the following species on selective or non-selective plates: *Escherichia coli* O157:H7, *Shigella sonnei*, *S. flexneri*, *Salmonella enterica* subsp. *Enterica* serovar Gaminera, *S.* Mbandaka, *S.* Tennesse, *S.* Minnesota, *S.* Senftenberg and *S.* Typhimurium. These new strains fluoresce when irradiated with UV light and maintain this phenotype in absence of antibiotic selection. Recombinants were phenotypically equivalent to the parent strain, except for *S.* Tennessee Sal66 that appeared Lac^- on Xylose Lysine Deoxycholate (XLD) agar plates and Lac^+ on Mac Conkey and Hektoen Enteric agar plates. Analysis of closed whole genome sequences revealed that Sal66 had lost one lactose operon; slower rates of lactose metabolism may affect lactose fermentation on XLD agar. These fluorescent enteric control strains were challenging to develop and should provide an easy and effective means of identifying cross-contamination.

1. Introduction

As of 2015, *Salmonella*, *Shigella* and Shiga-toxin producing *Escherichia coli* (STEC) remained, along with *Campylobacter*, the top bacterial pathogens causing foodborne illnesses in the United States (Huang et al., 2016). The U.S. Food and Drug Administration (FDA) and the U.S. Department of Agriculture (USDA) are together responsible for monitoring the safety of all domestic and imported food and feed sold in interstate commerce. These data can only be as good as the methods of detection used to identify contamination and the risks that could compromise food safety. Food/feed laboratories may submit analytical data in support of government food safety initiatives and routine enforcement and, to support the new prevention-based approach to food safety (Taylor, 2011), food facilities are now required to implement a written preventive control plans that will involve, among other things, evaluating the hazards that could affect the safety of their food. The FDA's preferred laboratory procedures for microbiological analyses of foods are collected in the Bacteriological Analytical Manual (BAM) but other standard methods are accepted by regulatory agencies, provided that these have been evaluated and validated.

Accurate interpretation of pathogen detection assays requires the inclusion of control samples that serve as a baseline against which to compare the sample of interest. The validity of these tests depends, in turn, on unequivocal results from the negative control, where no effect is expected, as well as from the positive control, known to reliably produce the expected effect. Proficiency testing conducted from 1999 to 2013 by the American Proficiency Institute (Snabes et al., 2013) in regard to analysis of foodborne pathogens showed that false positive rates for *Salmonella* varies between 2.1 and 6.9% in the tested laboratories and averaged 2.5% for *E. coli*. Although the causes of these false positive results have not been established, cross-contamination during analytical processing is known to be one such event that leads to these biological false-positives.

Among the panel of positive control strains used by FDA analysts for Quality Assurance and Quality Control of media and methods, only one strain, *S. flexneri* 2457M can be differentiated from natural strains, using a PCR assay that detects the insertion of a kanamycin-resistant cassette in place of a virulence gene (Binet et al., 2014;

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Deer and Lampel, 2010). However, considering that antibiotic resistance is becoming more prevalent among both clinical and food isolates, these marker genes are becoming less reliable methods for differentiating laboratory control strains from naturally-occurring isolates. An alternative, Green Fluorescent Protein (GFP) is a particularly attractive marker because simple irradiation by blue light or near-ultraviolet (UV) light will suffice to evoke the expression of fluorescence.

Nonetheless, an important consideration for generating control strains useful for food microbiologists is achieving a level of GFP expression high enough for the resulting fluorescence to be routinely monitored using hand-held instruments. Over the past 20 years, several GFP variants green fluorescent protein (GFP), with excitation and emission properties different from those of the *Aequorea* and *Renilla* wild-type proteins, have been developed. One such protein, GFP_{UV} (Clontech, Palo Alto, Calif.), emits bright green light (maximum at 509 nm) when exposed to standard UV light (360–400 nm) (Crameri et al., 1996). Variants GFP_{mut2} and GFP_{mut3} have emission maxima of 507 and 511 nm when excited by blue light (450–495 nm) (Cormack et al., 1996), however, unlike GFP_{UV}, the fluorescence from the GFP_{mut2} and GFP_{mut3} sources cannot be seen by the naked eye unless an amber filter is used on the emitted light (Seville, 2001).

Ideally, control strains integrating these forms of fluorescence would not require complex media or expensive equipment, allowing easy recognition of incidents where inadvertent cross contamination may have occurred. Our initial attempts to generate stable control strains involved a GFP/promoter cassette, carried by a transposon-like moiety into natural endogenous plasmids of *S. sonnei* and *E. coli*, which harbored the partitioning (*par*) genes for stability in the absence of antibiotic selection (Monday et al., 2003). Unfortunately, GFP fluorescence was weak in these low copy number plasmids, and in LacI-positive strains carrying the repressor for the promoter driving GFP expression, the fluorescence was almost imperceptible.

To construct a more effective positive control, we generated new GFP expressing cassettes driven by the T_7RNA polymerase or placed under the control of the synthetic *trc* promoter. We took advantage of the transposon Tn7 site-specific mode of insertion to engineer and construct laboratory control strains for two species of *Shigella*, seven different *S. enterica* isolates, and one O157:H7 STEC, each of which express GFP at levels allowing easy detection with handheld UV lamps and do not need antibiotic selection during the testing protocol, including differential selective agars. In the course of this work, we have also gained insights into the lactose fermenting ability of our *S.* Tennessee strain of interest and its related phenotypes, as displayed on selective agars used for the routine isolation of *Salmonella* from clinical samples and from food and used whole genome sequences analyses to reconcile phenotypes and genotypes of the *S.* Tennessee recombinants.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The *E. coli*, *Shigella*, and *Salmonella* strains used in this study are listed in Table 1.

One of two broths was used as a basic medium; either Luria-Bertani (LB) medium or Tryptic Soy Broth (TSB). To assess the stability of the fluorescent phenotype in all engineered strains, repeated inoculation in antibiotic-free media was carried out at 42 °C in a Whitley A35 anaerobic chamber (Microbiology International, USA), using *Shigella* broth, a low carbohydrate medium typically used in anaerobic enrichment protocols for the recovery of *Shigella* (Binet et al., 2014). When needed, culture media were supplemented with antibiotics at the following concentrations: ampicillin, 100 μg/ml (for *E. coli* O157:H7 strains) or 150 μg/ml (for *Salmonella* strains); chloramphenicol (Cm), 25 μg/ml for plasmid marker or 10 μg/ml for chromosomal marker; kanamycin 10 μg/ml for chromosomal marker. All the antibiotics were purchased from Sigma (St. Louis, MO).

In addition to LB agar or Tryptic Soy Agar (TSA), the following selective agars were used to confirm the phenotypes of interest of the *Salmonella* control strains: MacConkey (MAC), Xylose Lysine Desoxycholate (XLD), Hektoen Enteric (HE), Bismuth Sulfite (BS). Triple Sugar Iron (TSI) agar and Lysine Iron agar (LIA). Media were prepared as specified in FDA Bacteriological Analytical Manual (BAM) [http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm055778.htm].

2.2. Plasmid constructions

The plasmids used in this study are listed in Table 1. Basic sequence alignments were performed with Clone Manager 9 (SciEd Software, Denver, CO).

2.2.1. Blue light fluorescence: gfp_{mut3}

Most Gfp expressing cassettes used by bacteriological laboratories have been optimized for immunofluorescence microscopy. For example Gfp_{mut3} proteins reach maximal fluorescence when excited at 501 nm, corresponding to blue light (450_495 nm) (Cormack et al., 1996). We ligated a 1865 bp *Not*I fragment from pSM1606, that contained an unstable variant of gfp_{mut3} gene (Sternberg et al., 1999), into the mini-Tn7 donor plasmid vector pGRG25 (McKenzie and Craig, 2006), thereby creating pRBO1, before correcting gfp_{mut3} to its wild-type form. Site directed mutagenesis was performed by PCR with Platinum Pfx DNA polymerase (Invitrogen) using RB322 - RB325, and RB323 - RB324 primer sets, followed by RB326 and RB327 primers. That PCR product was then cut with *Pac*I and *Bst*1107I and subcloned into pRBO1, cut with those same restriction enzymes, to create pRBO8. Finally, a *Pac*I *cat* cassette generated from pBCSK + vector, intended to confer Cm resistance, was cut from pRAK146 and inserted into pRBO7 near the *rrnB*P_1-gfp_{mut3} expression cassette, at a unique *Pac*I site, thereby creating pRBO11.

2.2.2. UV fluorescence: gfp_{UV}

The gfp_{UV} gene, which codes for a GFP variant optimized for maximal fluorescence when excited by standard UV light (360–400 nm), was placed under the control of T7-*lac* promoter as a *Sph*I - *Not*I 882 bp insert, creating pSR2. The vector pTrc99, containing *rrnB* transcription terminators and an inducible $LacI^q$ repressor, was used to put expression of the T7 RNA polymerase gene from BL21(DE3), a *Eco*RI - *Bam*HI 2666 bp fragment, in control of the vector *trc* promoter. A 4345 bp *Pvu*II *Not*I fragment from pSR2 was then subcloned into pGRG36 (McKenzie and Craig, 2006) and linearized with those same enzymes to generate pSR3. However, this construct appeared toxic to the typical *E. coli* cloning strain DH5α; despite the *recA* background of DH5α, that is known to inhibit homologous recombinations (Bell and Kowalczykowski, 2016), numerous non-fluorescent colonies formed and plasmid rearrangements were observed when the plasmid was extracted from the cells (data not shown).

We were able to partially alleviate the burden of pSR3 by using *E. coli* K12 XL-1 Blue MRF' or XL10-Gold Kan^r strains that provide an episomal source of $LacI^q$. A *Pac*I pKD3 derivative of *frt*-flanked *cat* cassette (Datsenko and Wanner, 2000) from pRBO7 was then inserted into pSR3 near gfp_{uv} at a unique *Pac*I site, to generate pSR4. Nonetheless, colonies transformed with pSR3 and pSR4 were not uniformly fluorescent, despite the use of $LacI^q$ expressing cells.

Table 1
Bacterial strains and plasmids used in this study.

Strains and plasmids	Description	Source or reference
Strain		
E. coli		
DH5α	F⁻ϕ80Δ(*lacZY-argF*)U169 *deoR recA1 endA1 phoA hsdR17 supE44 λ⁻ thi-1 gyrA96 relA1 Δ(lacZ)M15*	(Hanahan, 1983)
BL21(DE3)	*E. coli* str. B F⁻ *ompT gal dcm lon* $hsdS_B(r_B m_B)$ λ(DE3 [*lacI lacUV5-T7p07 ind1 sam7 nin5*]) [$malB^+$]$_{K\text{-}12}(\lambda^S)$ contains the T7 RNA polymerase gene under the control of the lac_{UV5} promoter, integrated into the chromosome	Agilent Technologies
XL-1 Blue MRF′	Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)173 *endA1 supE44 thi-1 rec*A1 *gyrA96 relA1 lac Hte*[F *proAB lacI*^{q}ZΔM15*Tn*10(Tet^r)]	Agilent Technologies
XL10-Gold Kan^r	Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)173 *endA1 supE44 thi-1 rec*A1 *gyrA96 relA1 lac*[F' *proAB lacI*^{q}ZΔM15*Tn*10(Tet^r) Tn5 (Kan^r) Amy]	Agilent Technologies
EC27	ATCC43890 (serotype O157:H7; *stx1*$^+$ *stx2*$^-$); ESC00042[a]	(Monday et al., 2003)
EC32	Cm$^r_{10}$; EC27 *attTn*7:: *rrnB*P_1- gfp_{mut3}-T_o-T_1- *cat*	This study
EC39	Cm$^r_{10}$; EC27 *attTn*7:: P_{trc}-*T7Pol-rrnB*T_1T_2-P $_{T7\text{-}lac}$-gfp_{UV}-*cat*; 10% loss phenotype after 3 passages	This study
EC43	Cm$^r_{10}$; EC27*attTn*7:: P_{trc}-gfp_{UV}-*cat;* ESC1177[a]	This study
Shigella		
SF3	2457T; SHI00027[a]	
SF82	Cm$^r_{10}$; 2457T *attTn*7:: *rrnB*P_1- gfp_{mut3}-T_o-T_1- *cat*	This study
SF48	Km$^r_{10}$; *S. flexneri* 2457M; SHI00365[a]	(Deer and Lampel, 2010)
SF84	Km$^r_{10}$ Cm$^r_{10}$; SF48 *attTn*7:: P_{trc}-gfp_{UV}-*cat;* SHI0552[a]	This study
SS58	*S. sonnei* F2353; SHI00549[a]	(Hunter et al., 2005)
F2353-GFP	P_{tac} gfp_{UV} $aphA_3$ transposon into resident plasmid of SS58	(Monday et al., 2003)
SS61	Cm$^r_{10}$;SS58 *attTn*7:: P_{trc}-*T7Pol-rrnB*T_1T_2-P $_{T7\text{-}lac}$-gfp_{UV}-*cat;* SHI0551[a]	This study
S. enterica		
Sal32	*S.* Typhimurium LB5000 *r-m+;* H_2S^+; Lac$^-$	(Bullas and Ryu, 1983)
Sal42	Sal32 *attTn*7:: *rrnB*P_1- gfp_{mut3}-T_o-T_1- *cat*	This study
Sal46	Sal32 *attTn*7:: P_{trc}-gfp_{UV}-*cat;* SAL5693[a]	This study
Sal48	*S.* Typhimurium LT2; H_2S^+; Lac$^-$	Jie Zheng
Sal54	Sal48 *attTn*7:: P_{trc}-gfp_{UV}-*cat* obtained from P22 transduction on Sal46; SAL5694[a]; CFSAN070646[b]	This study
Sal49	*S.* Gaminara 24 N; typical H_2S^+; 1995 orange juice environmental isolate Lac$^-$; SAL2544[a]	T Hammack
Sal57, Sal63	Sal49 *attTn*7:: P_{trc}-gfp_{UV}-*cat*; SAL5695[a]; CFSAN070644[b]	This study
Sal50	*S.* Mbandaka 37 N; H_2S^+; 2001 halva candy Suc $^+$; SAL2634[a]	T Hammack
Sal58	Sal50 *attTn*7:: P_{trc}-gfp_{UV}-*cat;* SAL5696[a]	This study
Sal51	*S.* Senftenberg 2064H; H_2S^-; 1973 coconut isolate Lac$^-$; SAL2775[a]	T. Hammack
Sal59	Sal51 *attTn*7:: P_{trc}-gfp_{UV}-*cat;* SAL5697[a]	This study
Sal53	*S.* Minnesota 2938H; 1974 dry milk environmental isolate weak H_2S^+; Lac$^-$; SAL2643[a]	T. Hammack
Sal60	Sal53 *attTn*7:: P_{trc}-gfp_{UV}-*cat;* SAL5698[a]	This study
Sal52	*S.* Tennessee 4083H; H_2S^-; Lac$^+$,; 1976 dairy environmental isolate; SAL2820[a]; CFSAN024439[b]	T. Hammack
Sal61a	Sal52 *attTn*7:: P_{trc}-gfp_{UV}-*cat*;	This study
Sal62	Sal61a dull colony after 2 anaerobic passages at 42C; SAL10992[a] CFSAN070645[b]	This study
Sal66	Sal62 spontaneous variant exhibiting bright fluorescence; SAL5699[a]; CFSAN070643[b]	This study
Plasmid		
pBCSK$^+$	Ap^r; ColE1 Ori; source of *cat* for pRAK146	Agilent Technologies
pGEMT	Ap^r; ColE1 Ori; general cloning vector for PCR products;	Promega
pKD3	Ap^r Cm^r; Source of the *frt*-flanked *cat* cassette for pRBO7	(Datsenko and Wanner, 2000)
pTrc99a	Ap^r; pBR322 Ori; Bacterial expression vector with *rrnB* transcription terminators and inducible LacI repressor;	Pharmacia
pET-24a	Source of the T_7-*lac* promoter	Novagen
pSM1606	Km^r; pACYC177 Ori; source of *rrnB*P_1-$gfp_{mut3AAV}$-T_o-T_1 cassette	(Sternberg et al., 1999)
$pGFP_{UV}$	Source of the GFP variant optimized for maximal fluorescence when excited by standard UV light (360–400 nm)	Clontech
pGRG25	Ap^r pSC101 Ori *ts;* source for the site-specific recombination machinery of the bacterial transposon Tn7	(McKenzie and Craig, 2006)
pGRG36	Ap^r pSC101 Ori *ts;* source for the site-specific recombination machinery of the bacterial transposon Tn7	(McKenzie and Craig, 2006)
pRAK146	Ap^r Cm^r ColE1 Ori; pBCSK + *cat Pac*I PCR in pGEMT	This study
pRBO7	Ap^r Cm^r ColE1 Ori; pKD3 *cat Pac*I PCR in pGEMT	This study
pRBO1	Ap^r pSC101 Ori *ts*; delivery plasmid for the *rrnB*P_1- $gfp_{mut3AAV}$-T_o-T_1 Tn7 transposon	This study
pRBO8	Ap^r pSC101 Ori *ts*; delivery plasmid for the *rrnB*P_1- gfp_{mut3}-T_o-T_1 Tn7 transposon, generated by site directed mutagenesis on pRBO1	This study
pRB011	Ap^r Cm^r pSC101 Ori *ts*; delivery plasmid for the *rrnB*P_1-gfp_{mut3}-T_o-T_1-*cat* Tn7 transposon	This study
pSR2	Ap^r; Source of the P_{trc}-*T7Pol-rrnB*T_1T_2-P $_{T7\text{-}lac}$-gfp_{UV} cassette in pTRC99a background	This study
pSR3	Ap^r; pSC101 Ori *ts*; delivery plasmid for the P_{trc}-*T7Pol-rrnB*T_1T_2-P $_{T7\text{-}lac}$-gfp_{UV} Tn7 transposon ***unstable***	This study
pSR4	Ap^r; Cm^r; pSC101 Ori *ts*; delivery plasmid for the P_{trc}-*T7Pol-rrnB*T_1T_2-P $_{T7\text{-}lac}$-gfp_{UV} −*cat* transposon ***unstable***	This study
pSR7	Ap^r; Cm^r; pSC101 Ori *ts*; delivery plasmid for the P_{trc}-gfp_{UV}-*cat* Tn7 transposon	This study

[a] Designation in CFSAN strain collection.
[b] CFSAN internal strain accession number.

Plasmid preparations from individual fluorescent colonies frequently showed aberrant restriction profiles post-enrichment (data not shown). We then used a shorter, 2226 bp Gfp expression cassette carrying chloramphenicol resistance, obtained by PCR on Sal54 using primers RB320 and RB334 with Platinum Pfx DNA polymerase, cut with *Not*I and ligated into pGRG36 linearized with NotI and *Sma*I thus creating pSR7. Unlike pSR4, pSR7 was able to generate fluorescent recombinants in the six *Salmonella* strains of interest.

2.3. Genetic methods

2.3.1. Tn7 transposition

Tn7 based insertion of the GFP expression cassettes was performed using donor plasmid vectors, as previously described (McKenzie and Craig, 2006). Briefly, the DNA to be mobilized is placed between the *Tn7R* and *Tn7L* end sequences, which enables the transposition proteins to move that DNA segment. Replication of the donor plasmid is thermosensitive, so when cells are grown at 42 °C, the plasmid is lost. Under optimal conditions, 50–100% of colonies obtained at 42 °C should have carried the mobilized element inserted at *Tn7* insertion site; however, integration of gfp_{mut3} via pRBO11 and the first version of gfp_{uv} via pSR3 was inefficient until we added a *cat* chloramphenicol (Cm) resistance cassette to the mobile piece of DNA in our recipient strains. Thus, recipients of these chromosomal insertions could be selected on either TSA or LB agar plates which were supplemented with 10 μg/ml Cm and incubated at 42 °C to induce loss of the ampicillin-resistant donor plasmid.

We verified integration by PCR (Table 2) using the universal *glmS* primer RB357 paired the appropriate primer for annealing to *gfp:* either RB319, for the strains harboring the gfp_{mut3} gene, thereby amplifying a 1267 bp fragment, or RB327, for the strains harboring the gfp_{uv} gene, amplifying a 894 bp fragment in all strains, except for *S.* Tennessee isolates Sal62 and Sal66, that both showed a 2,934bp product.

2.3.2. P22 mediated transduction

A P22HFT*int* lysate prepared from propagation on Sal46 could transduce the GFP expression cassette into serotype Typhimurium strain LT2, thereby conferring resistance to Cm and fluorescence to all transductants, including Sal54. The other five serotypes of *Salmonella* tested (Gaminara, Mbandaka, Senftemberg, Minnesota, and Tennessee) were resistant to P22 phage infection, and therefore could not be transformed in this manner.

2.4. Whole genome sequencing

2.4.1. Short-read sequencing

This procedure has been described previously (Pettengill et al., 2015).

2.4.2. Long-read sequencing

The sequence resolution for the *S.* Tennessee isolates Sal52, Sal62 and Sal66 was not fine enough to infer the lactose genetic region; therefore we sequenced these, along with Sal63, on the Pacific Biosciences (PacBio) *RS* II Sequencer, as previously described (Hoffmann et al., 2013, 2014). Specifically, we prepared a library using 10 μg genomic DNA, that had been sheared to fragments of 10 kb by g-tubes (Covaris, Inc., Woburn, MA), according to the manufacturer's instructions. The SMRTbell 10-kb template library was then constructed using their DNA Template Prep Kit 1.0 with the 10-kb insert library protocol (Pacific Biosciences; Menlo Park, CA, USA). This library was sequenced using the P4/C2 chemistry on 4 single-molecule real-time (SMRT) cells using a 90-min collection protocol. The 10-kb continuous long read (CLR) data were *de novo* assembled using the PacBio hierarchical genome assembly process (HGAP)/Quiver software package, followed by Minimus2, and polished by Quiver (Chin et al., 2013).

2.4.3. Sequence accession numbers

All sequences for generated for the recombinant strains in this study are available at the National Center for Biotechnology Information (NCBI) under BioProject PRJNA414243 with accession numbers PEHQ00000000 (CFSAN070646; Sal54); CP024165, CP024166 and CP024167 (CFSAN070644; Sal63); CP024164 (CFSAN070645; Sal62); CP024168 (CFSAN070643; Sal66).

3. Results

3.1. glmS genetic region in E. coli O157:H7, S. sonnei, S. flexneri, and S. enterica

Most bacterial strains, including *E. coli*, harbor a unique chromosomal attachment Tn7 site, referred to as *att*Tn7, downstream of the essential *glmS* gene, which encodes the glucosamine-6-phosphate synthetase involved in cell wall biosynthesis. The primers designed by McKenzie and Craig to flank *att*Tn7 site in *E. coli* K12 (McKenzie and Craig, 2006) (Table 2) gave no PCR product when used in a PCR reaction on our strains of interest, suggesting differences in the *glmS* regions (data not shown). When we aligned the *glmS* DNA regions of our three strains (*E. coli* O157:H7 str EDL933 [AE005174], *S. flexneri* serotype 2a [AE014073] and *S. sonnei* [NC_007384]) against the reference strain *E. coli* K12 str. MG1655 [NC_000913] we identified one single nucleotide polymorphism (SNP) in O157:H7 with the last 36bp of the *glmS* ORF, corresponding to the TnsD transposase binding site (Kuduvalli et al., 2001) while the same site was perfectly conserved in the two species of *Shigella.* However, in those three strains, *pstS*, the gene that immediately followed *glmS* in K12, was separated from about 7 kb in O157:H7 str EDL933 to 23 kb in the *S. flexneri* 2a isolate and more than 85 kb in *S. sonnei*. Importantly, *S. sonnei* appeared to harbor an intact Tn7 transposon downstream to *glmS*.

In *S.* Typhimurium [NC_003197] *pstS* was more than 4 kb downstream to *glmS*. Alignment of the *glmS* DNA region, which comprised *glmS* and about 1 kb downstream, using *E. coli* K12 as the reference [NC_000913] against 13 different *Salmonella* serovars (Typhimurium [NC_003197], Abaetetuba [CP007532], Agona [NC_022991], Anatum [CP007531], Bovismorbificans [HF969015], Choleraesuis [AE017220], Cubana [NC_021818], Dublin [CP001144], Heidelberg [NC_011083], Montevideo [CP007530], Newport [CP007530], Tennessee [CP007505] and Thompson [CP006717]) revealed, in the last 36bp of the *glmS* ORF, two SNPs in five serovars, including Tennessee, and three SNPs in the remaining 8 serovars analyzed, including Typhimurium, in which Tn7 transposition had been previously documented (Crepin et al., 2012; Shivak et al., 2016). Primers generated from these various alignments were verified on our panel of strains (Table 2).

3.2. Construction of fluorescent laboratory control strains for E. coli O157:H7, S. sonnei, S. flexneri, and six different serotypes of S. enterica

The mini-Tn7 transposon is a great tool for single copy tagging of bacteria in a site-specific manner at a unique and neutral site without any deleterious effects (Choi and Kim, 2009). The pSR4 mini-T7 donor carrying the large T7 RNA polymerase GFP expression cassette generated fluorescent Cm^R *gfp-cat* colonies in three of the eight strains transformed. Several of the initial fluorescent integrants in *E. coli,* including EC39, and *S.* Typhimurium Sal32,

including Sal46, showed sensitivity to ampicillin suggesting that the pGRG36 plasmid construct was lost when selected for at the non-permissive temperature, but only one colony displayed this sought-after phenotype for *S. sonnei* (SS61). While evaluating the stability of the fluorescence in the absence of antibiotic selective pressure to ensure their suitability as laboratory control strains, we observed that all colonies issued from SS61 and Sal46 were fluorescent after three passages at 42 °C in the absence of oxygen while numerous *E. coli* colonies remained non-fluorescent under the UV light. PCR analyses with RB357 and RB327 primers (Table 2) confirmed integration of the expression cassette downstream of *glmS* and presence of *gfp* in all candidates including the non-fluorescent *E. coli* integrants. PCR analyses with primers pucF and RB334 amplified a 4106 bp fragment corresponding to the T7 RNA

Table 2
Primers used for polymerase chain reaction amplification, site-directed mutagenesis and sequencing.

Primer target and designation	Position	Sequence (5' → 3')
Plac [a]		
puc-F[b]	749,910	AGCGGATAACAATTTCACACAGGAAAC
T7 RNA polymerase [a]		
3′ T7 pol.8	750,504 C	GTAATGGTCAGCCAGAGTGTTG
3′ T7 pol.7	750,895 C	CTTCAAGGTCACGGATACG
3′ T7 pol.6	751,280 C	CAATAGCCACCACCAGTAATG
3′ T7 pol.1	752,976 C	CTGGTCAGAGAACTGGTCGTAG
5′ T7 pol.1	750,542	ATGAGTCTTACGAGATGGGTGAA
5′ T7 pol.3	751,152	CGAACTCGCACCTGAATACG
5′ T7 pol.4	751,522	GACATGAATCCTGAGGCTCTC
5′ T7 pol.5	751,906	CCACTGGAGAACACTTGGTG
5′ T7 pol.6	752,291	GTGTTACTCGCAGTGTGACTAAG
5′ T7 pol.7	752,716	GATAGCGAGATTGATGCACAC
cat [c]		
RB320	30	AGATTAATTAATGTGTAGGCTGGAGCTGCTTC
RB321	1038c	AGATTAATTAATATCCTCCTTAGTTCCTATTCC
gfp [d]		
RB318	179	TCCATGGCCAACACTTGTC
RB319	698 C	CCCAGCAGCTGTTACAAACTC
RB323	322	ACGGGAACTACAAGACaC
RB324 mut1F	746 C	CGTCGTTTGCTGCAGGTCA-TTTGTAtAGtTCATC
RB325 mut1R	714	GATGAaCTaTACAAATGA-CCTGCAGCAAACGACG
RB329	639 C	TCGAAAGGGCAGATTGTG
RB327	332	CAAGACACGTGCTGAAGTCAAG
RB333	550 C	GCTAGTTGAACGctTCCATC
3′T7 GFP.3	63 C	CAACAAGAATTGGGACAACTC
stx1 [e]		
RB345	2,996,868 C	CGCTGAATGTCATTCGCTCTG
RB346	2,996,152	CAGATGCCATTCTGGCAACTC
S. sonnei [f]		
RB222	3,532,891 C	ACGTTACCTCGCCTATTTCC
RB223	3,532,501	AACACCGGTTTCCACTACCC
Tn7 delivery plasmids [g]		
RB334	1491; 12,377 C	TGCCCGTCGTATTAAAGAGG
RB358	1,499 C; 12,369;	CGACGGGCAATTTGCACTTC
RB322	12,126	GATGGGAACTGGGTGTAG
RB326	12,256	AACACCGGTTTCCACTACCC
glmS region		
E. coli K12 [h]		
glmS1 [i]	3,910,450 C	GATGCTGGTGGCGAAGCTGT
glmS2 [i]	3,909,772	GATGACGGTTTGTCACATGGA
RB355	3,910,043 C	AGGATGCGGGTTTTGTAAGTAG
RB357	3,910,057 C	TGTCTTCGCCGATCAGGATG
E. coli O157:H7 [e]		
glmS1 [i]	4,774,853 C	GATGCTGGTGGCGAAGCTGT
RB355	4,774,446 C	AGGATGCGGGTTTTGTAAGTAG
RB356	4,774,149	TCAACGGTGCCCCAAATAATAG
RB357	4,774,460 C	TGTCTTCGCCGATCAGGATG
S. sonnei [f]		
glmS1 [i]	4,099,962	GATGCTGGTGGCGAAGCTGT
RB357	4,100,355	TGTCTTCGCCGATCAGGATG
RB334	4,100,747	TGCCCGTCGTATTAAAGAGG
RB322	4,114,560 C	GATGGGAACTGGGTGTAG
RB355	4,100,369	AGGATGCGGGTTTTGTAAGTAG
S. flexneri [j]		
glmS1 [i]	3,853,597	GATGCTGGTGGCGAAGCTGT
RB355	3,854,004	AGGATGCGGGTTTTGTAAGTAG
RB357	3,853,990	TGTCTTCGCCGATCAGGATG
RB	C	

(*continued on next page*)

Table 2 (*continued*)

Primer target and designation	Position	Sequence (5' → 3')
S. Typhimurium [k]		
RB357	4,069,229 C	TGTCTTCGCCGATCAGGATG
RB359	4,069,243 C	GCGGTCAGTTGTACGTCTTC
RB360	4,068,920	AGGCGTAGCACCTCTTAGTC
RB378	4,069,641 C	CCAGTTAACCGTGCTGTTGATG
RB379	4,068,537	TATGATGACTGGGCGAATGG
RB380	4,069,415 C	GCGCTGAAGCTGAAAGAG

[a] Position in GenBank entry NC_012971 [BL21(DE3)] for the first base of the primer. C: complementary strand.
[b] (Binet and Maurelli, 2007).
[c] Position in GenBank entry AY048742; pKD3, for the first base of the primer. C: complementary strand. The added *Pac*I site is underlined.
[d] Position in GenBank entry KU248761; gfp_{mut3} for the first base of the primer. C: complementary strand. Nucleotides in lower case correspond to mismatch in gfp_{UV} [GenBank entry U62636]. The TAG codon that was introduced by site directed mutagenesis is underline.
[e] Position in GenBank entry NC_002655; *E. coli* O157:H7 EDL933 complete genome, for the first base of the primer. C: complementary strand.
[f] Position in GenBank entry NC_007384; *S. sonnei* Ss046 complete genome, for the first base of the primer. C: complementary strand.
[g] Position in GenBank entry DQ460223; pGRG36. C: complementary strand.
[h] Position in GenBank entry NC_000913; *E. coli*. K-12 MG1655, complete genome, for the first base of the primer. C: complementary strand.
[i] (McKenzie and Craig, 2006).
[j] Position in GenBank entry AE014073; *S. flexneri* 2457T complete genome, for the first base of the primer. C: complementary strand.
[k] Position in GenBank entry NC_003197; *S.* Typhimurium str. LT2, complete genome, for the first base of the primer. C: complementary strand.

polymerase gene and its promoter region and P_{lacUV5}-*gfp* in pSR4, SS61 and EC39. The DNA fragment amplified from Sal46 appeared about 3 kb smaller than expected. Sequence analysis verified a molecular rearrangement had occurred in that new shorter cassette, such as the *trc* promoter was now controlling GFP expression, instead of the T7 RNA polymerase, whose gene had been deleted. When Sal46 was used as donor for a bacteriophage P22 transduction experiment with the wild-type *S.* Typhimurium LT2 strain, the resulting LT2 Cm^R transductants, including Sal54, harbored the shorter P*trc*-gfp_{uv} cassette at the *glmS att*Tn7 site and were also stably fluorescent. P22 phages prepared using Sal46 and Sal54 failed to infect S. Gaminara 24 N, S. Mbandaka 37 N, S. Senftenberg 2064H, S. Minnesota 2938H and S. Tennessee 4083H, making transduction an unsuitable means for constructing the desired *Salmonella* control strains. When the pSR7 mini-T7 donor plasmid harboring the shorter P*trc*-gfp_{uv}-*cat* cassette was prepared from *E. coli* K12 and transformed in *E. coli* O157:H7 and *S. flexneri*, stable *gfp-cat* integrants, including EC43 and SF84, respectively, were readily obtained. Plasmid pSR7 prepared in the R-M + *S.* Typhimurium produced integrants in four of the five *Salmonella* serovars, with varying degrees of transformation efficiency. It was not possible to successfully transform the *S.* Gaminera serotype until plasmids derived from the atypical isolates were used. PCR analysis of the integrants using RB357 and RB327 confirmed insertion immediately downstream *glmS* in all recombinants, except for *S.* Tennessee, in which the insertion appeared 2040 bp further, past a conserved ORF following *glmS*, in an unrelated *att*Tn7 site.

3.3. Verifying the biochemical identity of the Salmonella strains

To differentiate *Salmonella* from other *Enterobacteriaceae*, bacteriologists use lack of lactose and sucrose fermentation and hydrogen sulfide (H_2S) production as biochemical screens (Ewing, 1986). The panel of *Salmonella* strains used by the FDA field laboratories for Quality Assurance and Quality Control of Media and Methods (Andrews et al., 2016) includes typical Lac^- H_2S^+ *Salmonella*, such as *S.* Gaminera 24 N, as well as atypical strains, including *S.* Minnesota 2938H, that only produces a small amount of H_2S, the H_2S^- *S.* Senftenberg 2064H (Yi et al., 2014), the Lac + *S.* Tennessee 4083H, and the Suc + *S.* Mbandaka 37 N. While all of our fluorescent recombinant strains were biochemically identical to the parent strains, as seen by the color of the colonies formed the differential selective agar plates typically used for *Salmonella* (HE agar, XLD agar, TSI agar, BS agar, LIA agar) (Table 3), we observed intriguing differences in the lactose fermenting phenotype among *S.* Tennessee isolates (Fig. 1). During passages at 42 °C in the absence of oxygen, we observed white sectors on several fuchsia colonies formed by the parent strain (Sal52) on MAC, indicating instability of Lac^+. While *gfp-cat* integrants obtained on Sal52 (including Sal62) were fuchsia on MAC and exhibited low level fluorescence, a few Lac^+ bright colonies (including Sal66) could be seen after two anaerobic passages (Fig. 1). Stability testing showed no loss of either phenotype when monitored on MAC agar plates with handheld UV lamps. However, although Sal66 colonies were fuchsia on MAC and orange on HE agar, indicating their lactose-fermenting phenotype, these colonies were surprisingly red instead of yellow on XLD agar which contains 7.5 g/l lactose compared to 10 and 12 g/l for MAC and HE agars, respectively (Fig. 1).

3.4. Further exploration of S.Tennessee Sal52 and its derivatives by whole genome sequencing

A recent study by Leonard et al. (2015) showed the *lac* operon could be on a plasmid or in the chromosome in *Salmonella,* depending on the strains tested, and identified two chromosomal *lac* operons in two of their four *S.* Tennessee sequenced. Complete and closed genomes of Sal52 and Sal62 revealed two parallel but inverted lactose operons in the chromosome, 43 kb apart, containing duplicate intact *lacI*, *lacZ*, and *lacY* genes, and a truncated *lacA* ORF followed by two IS_1 elements each, sharing 98% identity over 6792 bp, most of the differences residing in *lacZ*. Nevertheless both *lacZ* sequences were closer together (97% identity) than to *E. coli* K12 *lacZ* (96% and 95% identity). Genome comparisons of Sal52 with *S.* Tennessee Lac^- strain TXSC_TXSC08-19 (Timme et al., 2013) showed a 117,325 bp insertion upstream of two phage-like integrases in the 4,700,000 region of *S.* Tennessee Lac^- strain, using NCBI accession number # CP007505 as the reference; it was flanked by a 50-bp direct repeat sequence (i.e. *GGGATTGAAAATCCCCGTGTCCTTGGTTCGATTCCGAGTCCGGGCACCAC)* at the joint point/that demarcates the insertion site. That inserted element contains ORFs for multiple transposases and integrases, including the tyrosine recombinase XerD integration element (Midonet and Barre, 2014), a DNA_BRE_C domain-containing tyrosine recombinase with no significant sequence similarity to

Table 3
Phenotypes relevant to the fluorescent bacterial strains developed in this study.

Strain	Parent Strain	Fluorescence level	*gfp* promoter	phenotype of interest	MAC	XLD	HE	BS	TSI	LIA
EC43	*E. coli* serotype O157:H7	bright (UV)	*trc*	*stx1*$^+$ *stx2*$^-$; *cm*^{R}; Lac$^+$	fuchsia	yellow	orange	NT	NT	NT
EC32	*E. coli* serotype O157:H7	Fluorescence microscopy only (blue light)	*rrnBP*$_1$	*stx1*$^+$ *stx2*$^-$; *cm*^{R};Lac$^+$	fuchsia	yellow	orange	NT	NT	NT
SF82	*S. flexneri* 2457T	Fluorescence microscopy only	*rrnBP*$_1$	*cm*^{R};Lac$^-$	white	red	green	NT	NT	NT
SF84	*S. flexneri* 2457M	bright (UV)	*trc*	*cm*^{R};*Km*^{R}, Lac$^-$	white	red	green	NT	NT	NT
SS61	*S. sonnei* F2353	bright (UV)	T_7	*cm*^{R}; Lac$^-$	white	red	green	NT	NT	NT
Sal42	*S.* Typhimurium R$^-$M$^+$	Fluorescence microscopy only	*rrnBP*$_1$	*cm*^{R}, Lac$^-$, H_2S^+	white	black	black	black metallic	Purple top, black bottom	Purple; black stabs
Sal46	*S.* Typhimurium R$^-$M$^+$	bright (UV)	*trc*	*cm*^{R}, Lac$^-$, H_2S^+	white	black	black	black metallic	Purple top, black bottom	Purple; black stabs
Sal54	*S.* Typhimurium	bright (UV)	*trc*	*cm*^{R}; Lac$^-$, H_2S^+	white	black	black	black metallic	Purple top, black bottom	Purple; black stabs
Sal57	*S.* Gaminara	bright (UV)	*trc*	*cm*^{R}; Lac$^-$, H_2S^+	white	black	black	black metallic	Purple top, black bottom	Purple; black stabs
Sal58	*S.* Mbandaka	bright (UV)	*trc*	*cm*^{R}; Suc$^+$, H_2S^+	white	yellow	Orange with black center	black metallic	Yellow top and bottom	Black stabs [H_2S+]
Sal59	*S.* Senftenberg	bright (UV)	*trc*	*cm*^{R}; Lac$^-$, H_2S^-	white	red	green	black metallic	Red top, yellow bottom	Purple stabs [H_2S-]
Sal60	*S.* Minnesota	bright (UV)	*trc*	*cm*^{R}; Lac$^-$, weak H_2S^+	white	black center on some	black center on some	Brown green	Red top, yellow bottom with some black area	Black stabs [light H_2S producer]
Sal62	*S.* Tennessee	dull (UV)	*trc*	*cm*^{R}; Lac$^+$, H_2S^-	fuchsia	yellow	orange	black metallic	Yellow top and bottom	Purple
Sal66	*S.* Tennessee	bright (UV)	*trc*	*cm*^{R}; weak Lac$^+$, H_2S^-	fuchsia	red	orange	black metallic	top is yellow to red, yellow bottom	Purple

other integrases of integrons (Dillon et al., 2005), and various conjugative elements suggesting an integrative conjugative element (ICE) (Johnson and Grossman, 2015). WU-Blast2 search against the ICEs in the online tool ICEberg v1.0 identified *E. coli* strain BEN374 AGI-5 integrative and conjugative element as a close homolog, with an E value = 0; however, the Sal52 ICE homolog does not seem to contain all the conjugative elements identified in other ICEs and is likely not mobile anymore (Bi et al., 2012). This suggests that both lactose operons had been acquired simultaneously by Sal52.

Interestingly, *lac1* contains genes for a ferric citrate uptake system (Fec) that is flanked by 2 IS elements and typically is missing in *Salmonella*. Much like the *E. coli fec* locus, the Sal52 *fec* locus consists of two operons carrying the regulatory genes, *fecI* and *fecR*, and the downstream structural genes, *fecABCDE* encoding the citrate-dependent iron (III) transport system, that allows bacteria to utilize otherwise insoluble ferric hydroxide (Fe^{3+}) (Braun and Killmann, 1999). Sal52 *fec* genes shared 96% identity with *E. coli fec* locus but are identical to the *fec* locus identified on various *Klebsiella pneumoniae* plasmids or in the chromosome of *Cronobacter sakazakii* strain ATCC29544 and various *Enterobacter* sp. A 15,599 bp region containing multiples ORFs sharing homologies with metal transporters [copper, lead, cobalt] follows *fec* in the element. Those particular transporters can be also found in the 320,000 bp sector of the Lac$^-$ strain TXSC08-19 chromosome (NCBI accession number CP007505). The presence of two copies of *lacI* in Sal52 was responsible for the low level fluorescence observed for Sal62 recombinants.

The complete and closed genome of Sal66 revealed a 49,046 bp deletion in that strain harboring a single *lac* operon. Sequence alignments showed that *lacAYZ* were identical to Sal52 *lac* element 1 while *lacI* was identical to Sal52 *lac* element 2, indicating that Sal66 arose from Sal62 from recombination between the two inverted lactose operons yielding to the deletion of the *fec* system and of the metal transporters DNA regions that were acquired by that isolate of *S.* Tennessee. The presence of a single operon of lactose is likely to affect the rate of metabolism of lactose by the strain, which appears to be too low for XLD to reveal, unlike MAC and HE agars (Fig. 1).

4. Discussion

4.1. Making stable fluorescent strains

Conventional labeling of bacterial strains is impractical, as it relies on transformation with plasmids carrying the GFP open reading frame (ORF) under promoters driving protein expression and, therefore, requires continuous antibiotic selection to be maintained within the bacteria (Ma et al., 2011). Using endogenous plasmids may offer some stability in the absence of selection (Monday et al., 2003); however, when F2353-GFP was grown at 42 °C in *Shigella* broth supplemented or not with 0.5 μg/ml novobiocin in the absence of oxygen, at each repeated passage, 0.6% of the population lost the ability to fluoresce under UV (data not shown). PCR analyses of those non-fluorescent colonies using the primer pairs RB222 - RB223 (391bp) and RB318 - RB319 (520 bp) (Table 2) confirmed that these were *S. sonnei* colonies that had lost *gfp*. Colonies of F2353-GFP given four repeated passages at 44 °C in *Shigella* broth supplemented with 0.5 μg/ml novobiocin in aerobic environmental conditions did stably retain fluorescence (Monday et al., 2003), suggesting that lack of oxygen is unfavorable to the maintenance of the endogenous plasmid carrying the Gfp expression cassette. This instability of the fluorescent phenotype behavior made this strain unreliable as a laboratory control strain. We believed inserting *gfp* into the bacterial chromosome would provide the stability necessary for laboratory control strains and used

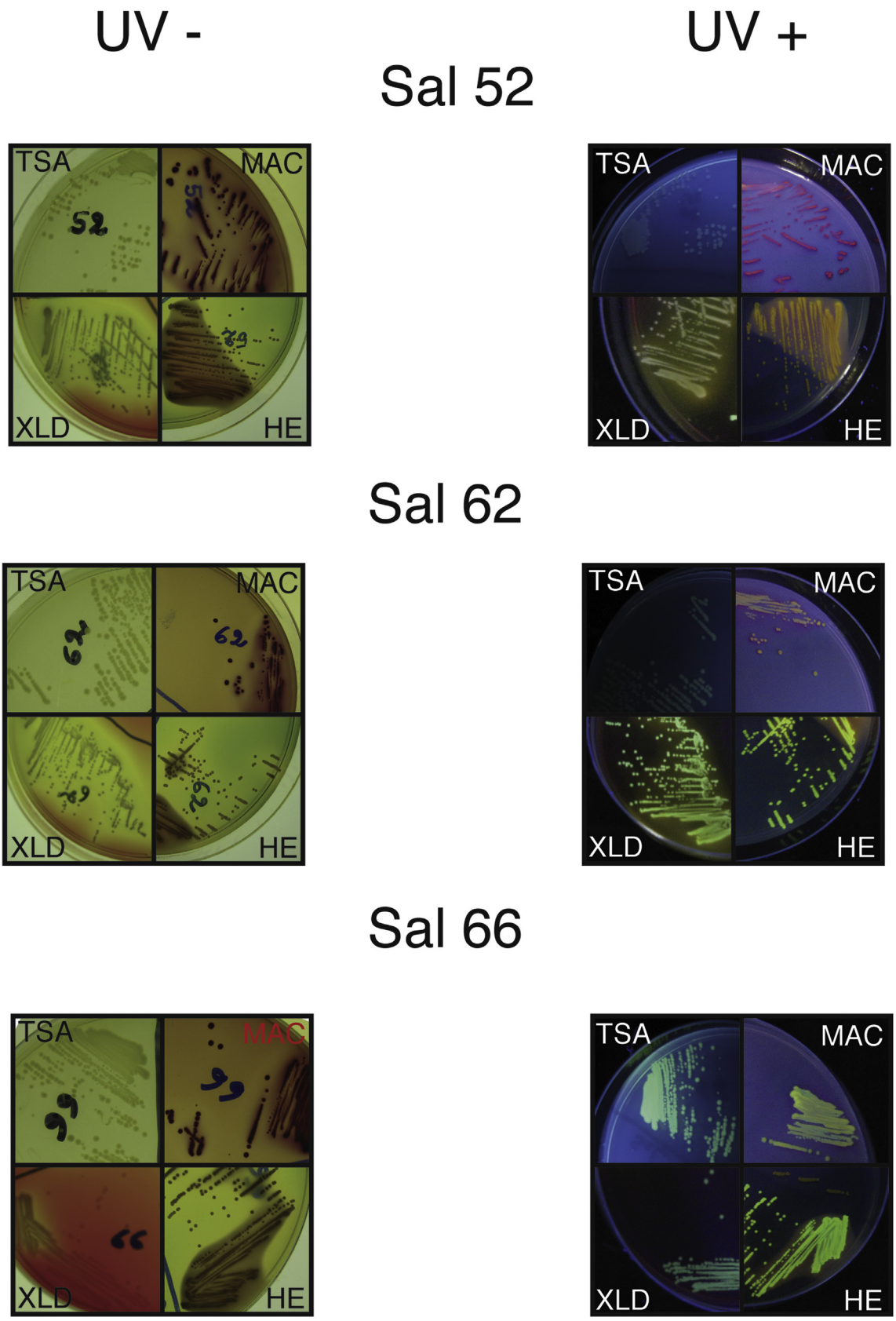

Fig. 1. *S.* Tennessee growing on TSA, MAC, XLD, HEA. Sal52 is the parent strain, Sal62 is the *gfp* recombinant with two lactose operons and Sal66 is the spontaneous mutant carrying only one lactose operon. Plates were illuminated with white light (UV−) or handheld UV lamp (UV +) and photographed.

serial passages under anaerobic environmental conditions in absence of antibiotic to assess the stability of the fluorescent strains.

Mini-Tn7 transposons are frequently used for single-copy integration of DNA fragments into chromosomes of Gram-negative bacteria at the unique *att*Tn*7* position immediately downstream of *glmS*. This position would prevent the disruptions of genes that might have discernible fitness costs to the host (McKenzie and Craig, 2006). Integrating this transposable element requires the proteins encoded by Tn*7* genes (*tnsABCD/E*) (Choi and Kim, 2009). Although the Tn7 system was recently shown to be efficient for site-specific-integration in pathogenic *E. coli*, *S.* Typhimurium and *S.* Typhi (Crepin et al., 2012; Shivak et al., 2016), no data were available for *S. sonnei* or *S. flexneri,* or the remaining five *Salmonella* serovars (Gaminara, Mbandaka, Senftemberg, Minnesota and Tennessee) at the time of this project. Despite a few suspected SNPs in the DNA motif corresponding to the transposase binding site in some strains, we were able to obtain chromosomal insertion immediately downstream *glmS* in all recombinants, except for *S.* Tennessee, in which the insertion appeared 2 kb further in an unrelated *att*Tn7 site. Secondary *glmS*-unrelated *att*Tn7 sites have also been identified in *Proteus mirabilis* (Choi and Kim, 2009).

4.2. Making high-level of fluorescence

Ribosomal promoters are among the strongest described in *E. coli* and the activity of *rrnB*P1, in particular, nicely mimics bacterial growth rate, increasing to a high point during the exponential growth phase in *E. coli* (Dennis et al., 2004); it will also function in phylogenetically-unrelated species, including *Pseudomonas putida* (Sternberg et al., 1999). Unfortunately, integration of a *rrnB*P1-gfp_{mut3} cassette derived from pSM1606 (Sternberg et al., 1999) via pRBO11 (Table 1) into *E. coli* O157:H7 (EC32), *S. flexneri* (SF82), and *S.* Typhimurium (Sal42) only allowed detection of fluorescence by microscopy, that fluorescence was too weak to be seen with the Dark Reader Transilluminator (Clare Chemical Research, USA.) (Table 3). This is a well-known limitation of GFP expression levels derived from single copy ORFs.

For this reason, we turned to one of the strongest and most specific expression systems known in bacteria, the T7 RNA polymerase/promoter combination. Its highly selective preference for transcriptional initiation at its own promoter sequence allows researcher to limit expression exclusively to those target proteins under the regulation of the T7 promoter (Tabor, 2001). To this end, we constructed a genetic cassette that containing the gene encoding the T7 bacteriophage RNA polymerase, under the control of the synthetic *trc* promoter, a hybrid of *trp* and *lac* promoters (Tegel et al., 2011), originating from the pTRC series of vectors (Pharmacia), and the GFP ORF, under the control of the T7 promoter from the pET vector series (Novagen). The two ORFs were separated by the *rrnB* T_1T_2 transcriptional terminator sequence to prohibit RNA:RNA interference. However these P_{T7} *gfp* fluorescent *att::*Tn7 integrants were difficult to find. The pGRG36 plasmid derivative carrying the T7-GFP expression cassette proved to be very prone to molecular rearrangements that favored the loss or reduction of GFP expression. Although this effect could be partially overcome in $lacI^q$-positive strains, which lowered GFP expression, the rare fluorescent integrants could only be obtained when a chloramphenicol resistance gene was engineered into the *gfp* cassette, creating pSR4, which allowed improved selection (Table 1). Once established, fluorescence could be monitored with a handheld UV lamp (Table 3). Although insertion of the large T7-GFP expression cassette was stable in *S. sonnei* and *E. coli* O157:H7, the expression of the fluorescent phenotype was stable in *S. sonnei* only. Failures of the bacteriophage T7 polymerase/promoter system in *E. coli* have been frequently reported and may be due to mutations affecting production of functional T7 RNA polymerase (Vethanayagam and Flower, 2005). However, it is not clear why the expression system appeared stable in our *S. sonnei* fluorescent recombinants.

Restriction-modification (R-M) systems, comprised of restriction enzymes and cognate methyltransferases, are a well-known barrier for horizontal gene transfer between bacteria by serving as an " immune defense" system for uptake of foreign DNA (Monk et al., 2012) (Binet and Maurelli, 2009). The restriction enzyme recognizes and digests foreign incoming DNA, whereas the methyltransferase performs methylation of the bacterium's own DNA to protect itself from degradation by the cognate restriction enzyme. Transformation of pSR4 into *S. sonnei* only gave one fluorescent integrant yet previous studies on the transformation of *Shigella* suggest the absence of a strong R-M in *S. sonnei* that would pose a genetic barrier with DNA originating from *E. coli* K12 (Ranallo et al., 2006; Shireen et al., 1990). The extremely low transposition efficiency in that strain is likely due to the presence of an internal Tn7 transposon, that is expected to exert immunity to transposition and therefore markedly reduce the likelihood of subsequent insertion of another copy of that transposon into the same target chromosome (Arciszewska et al., 1989). For *S. enterica* on the other hand, various R-M systems have been characterized in numerous serovars (Pirone-Davies et al., 2015; Roer et al., 2016). We used the data generated by Roer et al. (2016) who screened for the presence of R-M systems in 221 *S. enterica* genomes covering 97 different serovars of *S. enterica* subsp. *enterica* to generate a table listing the various R-M systems identified in the *Salmonella* strains used in this study [Supplemental Table 1]. The seven isolates of *S.* Typhimurium harbored 18 different R-M systems, 14 were identified in two *S.* Gaminara, 18 in three *S.* Mbandaka, 42 in seven *S.* Senftenberg, 10 in two *S.* Minnesota and 12 in three S. Tennessee. Of the 98 different R-M systems identified in these six *Salmonella* strains, only eight were shared by at least two strains. *S.* Typhimurium remains the strain of choice for classical genetic manipulation of *Salmonella*. The restriction profiles of pSR4 plasmid with *Eco*RV and *Nco*I after passage into Sal32, a restriction-minus, modification-positive (R^-M^+) strain of *S.* Typhimurium, showed molecular rearrangements, likely due to the lack of LacI repressors in that strain; it was, therefore, useless for our purposes. That same plasmid, prepared in the $lacI^q$ *E. coli* strain was only able to transform the R-M+ strain of *S.* Typhimurium, confirming a restriction barrier in the other strains. However the *gfp-cat* cassette in the fluorescent recombinants on Sal32 (Sal46) was about 3 kb shorter than the wild-type cassette on pSR4. Until this point, we did not know that the T7 promoter controlling Gfp expression in pSR4 derivatives was actually a hybrid T7-*lac* promoter (Dubendorff and Studier, 1991). Because both *trc* and T7-*lac* promoters share a 23 bp *lac* operator sequence which binds LacI repressors to inhibit transcription, passage of this promoter into *S.* Typhimurium likely promoted homologous recombination and excision of the DNA region between the two *lacO* boxes, and this event likely occurred before integration (Darmon and Leach, 2014). Apparently, such homologous recombination was less efficient in *S. sonnei* or *E. coli* O157:H7, since our tests showed that the cassette from pSR4 was full length in the integrants. Importantly, that single copy of the P*trc-gfp* recombined cassette allowed high-level expression of fluorescence, demonstrating that the activity of the synthetic P*trc* was higher than the *E. coli rrnB*P1 promoter.

4.3. Generating a representative panel of Salmonella control strains

Food microbiologists have traditionally used a panel of *Salmonell*a strains to represent the diversity of metabolic phenotypes observed for this pathogen (Andrews et al., 2016). Most differential plating media used today for the isolation of *Salmonella* contain

lactose and sucrose, HE agar (12 g/l), XLD agar (7.5 g/l), TSI agar (10 g/l) while MAC agar that only contains lactose (10 g/l) has been considered more appropriate for the isolation of *Shigella*. The presence of pH indicators, allows media acidified by the fermentation of the sugar(s) by the colony to develop distinctive colors: fuchsia on MAC, yellow on XLD and yellow – orange on HE for example (Table 3). Although still considered atypical, Lac + or Suc + variants of *Salmonella* have been reported (Gonzalez, 1966; McDonough et al., 2000). With few exceptions, typical *Salmonella* strains produce H_2S abundantly from sulfur-containing substrates, such as sodium thiosulfate added in combination to iron salts to various differential media, including BS, XLD, HE, TSI, and LIA agars causing blackened colonies. However, occasional strains of almost any *Salmonella* serotype may fail to produce H_2S in the above-mentioned media (Ewing, 1986). To work around that erratic behavior, the panel of *Salmonella* strains used by the FDA field laboratories for Quality Assurance and Quality Control of Media and Methods (Andrews et al., 2016) includes typical Lac^- H_2S^+ *Salmonella*, such as *S.* Gaminera 24 N, as well as atypical strains, including *S.* Minnesota 2938H, that only produces a small amount of H_2S, the H_2S^- *S.* Senftenberg 2064H (Yi et al., 2014), the Lac + *S.* Tennessee 4083H, and the Suc + *S.* Mbandaka 37 N. Caution is warranted when using the *S.* Tennessee recombinants Sal62 and Sal66. The first one exhibits the same phenotype as the parent on the selective agars used for the routine isolation of *Salmonella* from clinical samples and from food, yet fluorescence, although visible under UV irradiation, is not as intense as for the other *Salmonella* control strains harboring the same cassette. Fluorescence emitted by Sal66 is bright but that strain lost one of its two chromosomal lactose operons and appears Lac^+ on HE and Lac^- on XLD (Table 3). To the best of our knowledge, this dichotomy in expression of Lac by the same strain has yet to be observed with natural atypical strains of *Salmonella*.

5. Conclusions

Successful construction and dissemination of these control strains to testing facilities for use in their testing procedures, as well as to curated culture collections for distribution, provides an easy and effective means by which to ascertain that positive results are due to actual contamination of the matrices tested and not due to accidental laboratory cross-contamination.

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The Gfp_{UV} control strains developed in this study have been licensed to Microbiologics (St. Cloud, Minnesota) for distribution.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fm.2017.10.014.

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