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The *Escherichia coli motA* Flagellar Gene as a Potential Integration Site for Large Synthetic DNA

(Gen Flagelum *Escherichia coli motA* sebagai Tapak Integrasi yang Berpotensi untuk DNA Sintetik Besar)

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ABSTRACT

Escherichia coli *is used as a chassis for many synthetic biology applications. However, the limitations of maintaining recombinant plasmids extra-chromosomally include increased metabolic burden to the host, constant selective pressure, variable plasmid copy number and plasmid instability that leads to curing. Hence, to overcome these limitations, DNA constructs are integrated into the bacterial chromosome to allow stable control of copy number and to reduce the metabolic burden towards the surrogate host. Non-essential* E. coli *flagellar genes have been proposed as potential chromosomal insertion target sites. In this study, we validated and compared the efficiency of two loci, namely* motA *and* flgG, *as target sites for synthetic biology applications. To enable this comparison, a dual reporter strain (DRS) that utilises two reporter proteins, EforRED and Venus, was developed as a test case. Initially, a yellow reporter plasmid k14.1_*Venus *was constructed and subsequently used as the plasmid backbone for the generation of two other plasmids, k14.1_*eforRED *and* pcat_Venus, *required to build the dual reporter strain. In the DRS, the* eforRED *gene was inserted into* flgG *whereas motA was disrupted by* Venus. *This mutant strain was defective in motility (*$p<0.001$*) but growth rate was unaffected. The fluorescence emitted by Venus was higher (*$p<0.05$*) compared to EforRED, suggesting that motA is the better chromosomal target locus compared to* flgG. *Hence, this study proposes the use of* E. coli motA *as the site for chromosomal insertion for future synthetic biology applications.*

Keywords: Chromosomal integration; protein expression; reporter system; synthetic biology

ABSTRAK

Bakteria Escherichia coli *digunakan sebagai kes dalam banyak aplikasi biologi sintetik. Walau bagaimanapun, cabaran untuk mengekalkan plasmid rekombinan di luar kromosom termasuk peningkatan beban metabolik kepada perumah, tekanan memilih yang berterusan, pelbagai bilangan salinan plasmid dan ketidakstabilan plasmid membawa kepada penyingkiran plasmid daripada bakteria. Untuk mengatasi batasan tersebut, binaan DNA diintegrasikan ke dalam kromosom bakteria untuk membenarkan bilangan salinan gen yang terkawal dan mengurangkan beban metabolik kepada perumah pengganti. Gen flagelum yang tidak perlu telah dicadangkan sebagai tapak sasaran penyisipan kromosom yang berpotensi. Dalam kajian ini, kami mengesah dan membandingkan kecekapan dua lokus, iaitu* motA *dan* flgG, *sebagai tapak sasaran untuk aplikasi biologi sintetik. Untuk membenarkan perbandingan ini, strain dwipelapor (DRS) yang menggunakan dua protein pelapor, EforRED dan Venus, telah dibangunkan sebagai kes ujian. Pada mulanya, plasmid pelapor kuning, k14.1_Venus dibina dan kemudiannya digunakan sebagai tulang belakang plasmid untuk menjana dua plasmid lain, k14.1_*eforRED *dan pcat_*Venus, *yang diperlukan untuk membina DRS. Dalam DRS, gen* eforRED *diselitkan ke dalam* flgG *manakala* motA *disisip dengan* Venus. *Kemortilan strain mutan ini dimansuhkan (*$p<0.001$*) tetapi kadar pertumbuhannya tidak terjejas. Pendarfluor yang dipancarkan oleh Venus lebih tinggi (*$p<0.05$*) berbanding dengan* EforRED, *menunjukkan bahawa* motA *merupakan lokus sasaran kromosom yang lebih baik berbanding dengan* flgG. *Oleh itu, kajian ini mencadangkan penggunaan* E. coli motA *sebagai tapak untuk penyisipan kromosom dalam aplikasi biologi sintetik pada masa depan.*

Kata kunci: Biologi sintetik; integrasi kromosom; pengungkapan protein; sistem pelapor

INTRODUCTION

Synthetic biology is a new way of doing biological sciences by enabling high-level synthesis of products from DNA constructs or modified pathways. It holds great potential in engineering complex biological systems for the synthesis of many useful products such as drugs, polymers, food, biomass and fuels (Haseloff & Ajioka 2009). Examples of products derived using the synthetic biology platform include the antimalarial artemisinin (Keasling 2008), terpenoids (Chang et al. 2007) and green biofuels (Atsumi et al. 2008). Synthetic DNA constructs or pathways are inserted into *Escherichia coli* which is routinely used as an intermediate or final chassis (Ajikumar et al. 2010; Juhas 2016; Juhas et al. 2013).

However, the introduction of extrachromosomal recombinant plasmids into *E. coli* for the synthesis of

desired products usually imposes a high metabolic burden onto the host as well as constant selective pressure to avoid plasmid curing as a result of plasmid instability (Cunningham et al. 2009). The quality and stability of extrachromosomal plasmids are dependent on active cell machinery for its synthesis while plasmid maintenance can elicit stress responses from the surrogate host that will lead to plasmid instability (Silva et al. 2012). In a previous study, a medium-copy recombinant plasmid was cured after the 35th generation and the host lost the ability to synthesise polyhydroxybutyrate (PHB) even in the presence of antibiotics, however, the marker-free host with the gene of interest inserted into the bacterial chromosome was able to produce PHB stably for an extended period of time (Tyo et al. 2009). Furthermore, a high copy number plasmid does not necessarily guarantee increased protein expression. A strain with six integrated gene copies could synthesise more L-serine compared to a strain harbouring a medium-copy (15-20 copies) recombinant plasmid. This is due to saturation of extrachromosomal plasmids in the cell, leading to host metabolic burden and a decrease in protein synthesis (Gu et al. 2015).

To overcome these drawbacks, synthetic DNA constructs are preferably integrated into the host chromosome to generate marker-free strains to lower host metabolic burden and enable controlled copy number and stable DNA propagation. Previously, flagellar genes in regions 1 (Juhas & Ajioka 2015a), 2, 3b (Juhas & Ajioka 2015b) and 3a (Juhas et al. 2014) of the *E. coli* chromosome were identified as potential sites for DNA integration due to high level of RNA polymerase binding to these regions (Juhas et al. 2014). The flagellar regions were identified as loci harbouring non-essential flagellar genes of *E. coli* that have high expression under laboratory conditions (Juhas et al. 2014). Integration of simple genetic circuits into the flagellar genes did not have a negative impact on the growth of the host (Juhas & Ajioka 2015a, 2015b; Juhas et al. 2014). Of all the tested genes, *flgG* of region 1 (Juhas & Ajioka 2015a) and *motA* of region 2 (Juhas & Ajioka 2015b) were deemed the most suitable integration target loci for chromosomal insertion.

Hence, in this study, we set out to determine whether *flgG* or *motA* was the better integration site for large DNA constructs. To achieve this, a dual reporter system (DRS) that utilises the red (*eforRED*) and yellow (*Venus*) reporter genes was used as proof-of-concept. Prior to the system development, a yellow reporter plasmid, k14.1_*Venus* was first constructed and used as the plasmid backbone for the development of two other reporter plasmids, k14.1_*eforRED* and p*cat*_*Venus*. These two plasmids were then used as the templates for the generation of recombineering cassettes for Red recombinase-based chromosomal integration to build the DRS. Bacterial growth and fluorescence emitted of the individual strains were evaluated to determine the better integration target site.

Materials and Methods

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Luria Bertani (LB) agar supplemented

TABLE 1. Bacterial strains and plasmids used in this study

	Characteristics	Source
Bacterial strains		
E. cloni® 10G	F $^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) *endA1 recA1* ϕ80d*lac*Z ΔM15 Δ*lac*X74 *ara*D139 Δ(*ara,leu*)7697 *gal*U *gal*K *rpsL* (Str^R) *nup*G λ*-ton*A	Lucigen, USA
One Shot® BL21 Star™ (DE3)	F$^-$ *ompT hsdSB* (rB$^-$mB$^-$) *gal dcm* (DE3) pLysS (CamR)	Invitrogen, USA
Plasmids		
pJS209_*eforRED*	β-lactamase FRT cassette with *eforRED* in pSB1K3	Department of Pathology, University of Cambridge (DPUC)
pLysS	chloramphenicol acetyltransferase cassette with T7 lysozyme gene	Invitrogen, USA
k14.1	kanamycin FRT cassette in pSB1K3	DPUC (Juhas et al. 2014)
k14.1_pJS209	kanamycin FRT and β-lactamase cassette with *Venus* in pSB1K3	DPUC
k14.1_*Venus*	kanamycin FRT cassette with *Venus* in pSB1K3	This study
k14.1_*eforRED*	kanamycin FRT cassette with *eforRED* in pSB1K3	This study
p*cat*_*Venus*	chloramphenicol acetyltransferase FRT cassette with *Venus* in pSB1K3	This study
pKM208	Red recombinase controlled by *lacZ*	DPUC (Murphy & Campellone 2003)
pCP20	FLP recombinase helper plasmid	DPUC (Datsenko & Wanner 2000)

with 100 μg/mL carbenicillin, 50 μg/mL kanamycin or 35 μg/mL chloramphenicol when required, was used to cultivate the *E. coli* strains. The plate cultures were grown aerobically for 16 h at either 30°C or 37°C. The *E. coli* liquid cultures were grown in LB and incubated at 250 r.p.m. for 16 h at either 30°C or 37°C.

PRIMERS

The primers used in this study were designed based on the DNA sequence of the templates. The melting temperature and secondary structure formation of each primer was evaluated using OligoAnalyzer 3.1 (https://sg.idtdna.com/calc/analyzer). These primers were synthesised by Integrated DNA Technology (USA).

PCR AMPLIFICATION AND DNA MODIFICATIONS

The plasmid k14.1_pJS209 (University of Cambridge) was used as the template for the amplification of the yellow reporter gene, *Venus*, using the *Venus* primers (Table 2) whereas plasmid k14.1 (Juhas et al. 2014) was linearised using the k14.1 primers (Table 2). Polymerase chain reaction (PCR) was performed in a total reaction volume of 25 μL using Q5® High Fidelity Polymerase (New England BioLabs) according to the supplier's instructions. The amplicons (k14.1 and *Venus*) were electrophoresed and purified using the Qiaquick Gel Extraction Kit (Qiagen). Gel purification was carried out according to the supplier's instructions with slight modifications. The modifications made to the standard protocol were to replace the single 750 μL wash of Buffer PE with two washes of 600 μL and 400 μL and also, a total of 14 μL of Buffer EB was added instead of 50 μL. Incubation at room temperature was increased to 5 min and this was repeated twice. The concentrations of the amplicons were evaluated on a spectrophotometer (NanoDrop 2000c, Thermo Fisher Scientific Inc.).

GIBSON CLONING TO ASSEMBLE K14.1_*VENUS*

A modified Gibson Assembly was used to ligate the gene of interest into the plasmid backbone (Gibson et al. 2009). For the construction of k14.1_*Venus*, equimolar of k14.1 and *Venus* (3.7×10^{-5} nmol) were added to 4 μL of 1.33× Gibson Master Mix consisting of 5× isothermal buffer, T5 exonuclease, *Taq* ligase, Phusion polymerase

TABLE 2. Primers used in this study

Primers	Sequence (5' ® 3')
k14.1_F	TTTACAGCTAGCTCAGTCCTAGG
k14.1_R	TGAGAATTAATTCCGGGGATC
*eforRED*_F	CTTCGAACTGCAGGTCGACGGATCCCCGGAATTAATTCTCATTCAGAACGCTCGGTTGCC
*eforRED*_R	TAGCATAATACCTAGGACTGAGCTAGCTGTAAAGAGAGCGTTCACCGACAAACAACAGAT
openk14.1_*Venus_eforRED*_F	ATCTGTTGTTTGTCGGTGAACGCTCTCTTTACAGCTAGCTCAGTCCTAGGTATTATGCTA
openk14.1_*Venus_eforRED*_R	GGCAACCGAGCGTTCTGAATGAGAATTAATTCCGGGGATCCGTCGACCTGCAGTTCGAAG
*Venus*_F	CTTCGAACTGCAGGTCGACGGATCCCCGGAATTAATTCTCATTCAGAACGCTCGGTTGCC
*Venus*_R	CTAGCATAATACCTAGGACTGAGCTAGCTGTAAAGAGAGCGTTCACCGACAAACAACAGA
openk14.1_*Venus_cat*_F	TGGTGAAAGTTGGAACCTCTTACGTGCCGAGCGAAACGATCCTCATCCTGTCTCTTGATC
openk14.1_*Venus_cat*_R	ATGAGCAAACTGAAACGTTTTCATCGCTCTATAAGGGGATCTTGAAGTTCCTATTCCGAA
*cat*_F	TTCGGAATAGGAACTTCAAGATCCCCTTATGTCGAATTTGCTTTCGAATTTCTGCCATTC
*cat*_R	GATCAAGAGACAGGATGAGGATCGTTTCGCTCGGCACGTAAGAGGTTCCAACTTTCACCA
*motA*_F	CTCCAAATACACCAAAGCAATGTATATGGATCTGCTGGCTCTGCTTTATCGGTTGATGGCGAA ATCGTATCACGAGGCAGAATTTCAGAT
*motA*_R	TTTCTGACGTAAAACAGTCGCTAATGGGGAAATAAATCCGTAAGCCAATAAAATGCCGAGGA AAGTCGGTTTTAAAGAAAAAGGGCAGG
*flgG*_F	TTTGTCACTAATCCACTACAGGACATTTTATGATCAGTTCATTATGGATCGCCAAAACGGGCCT TGGTATCACGAGGCAGAATTTCAGAT
*flgG*_R	CACCTGAATCATATTGACCAGTTCTTCCGCCACGTTGACGTTAGACGTTTCAACATACCCTTG ATACGGTTTTAAAGAAAAAGGGCAGG
*motA*i_F	GGGTCGTTTATCGTCGGCAATAATGGCAAAGCGATTAAAGGCACGCTGAA
*motA*i_R	GAAAGCAGAGTGACTTTGACGCACTGCATCATTTTGCTGGTTTCGGCGCT
*flgG*i_F	GAAAGGATACATGACAAGTATAAGTTGCCCGATGCGCAAGTTTATCGGGT
*flgG*i_R	CCGGTTAAGCCTTAGAGTTGCGTCAGTTTTTGCAGCATCTGATCGGTGGT
FRT_F	GATTGCAGCATTACACGTCTTGAGCGATTGTGTAGGCTGGAGCTGCTTCG
FRT_R	TGAGAATTAATTCCGGGGATCCGTCGACCTGCAGTT

and nuclease-free water (https://openwetware.org/wiki/Gibson_Assembly). The reaction mixture was incubated at 50°C for 60 min.

TRANSFORMATION OF RECOMBINANT PLASMIDS INTO CHEMICALLY COMPETENT *E. COLI*

Chemically competent *E. coli E. cloni* 10G was prepared using the CCMB80 buffer (Hanahan et al. 1991). One microliter of the Gibson assembly reaction was added into a vial of 100 µL of chemically competent *E. coli* and incubated on ice for 30 min. The vial of cells was heat-shocked at 42°C for 30 s followed by immediate incubation on ice for 2 min. A total of 250 µL of Super Optimal broth with catabolite repression (SOC) medium at room temperature was added into the bacterial mixture and recovered by horizontal shaking at 37°C for 1 h before plating on LB agar with 50 µg/mL kanamycin. The transformants were grown for 16 h at 37°C.

VERIFICATION OF K14.1_*VENUS*

Colonies that grew on the kanamycin selective plates and emited yellow fluorescence when observed under UV light were picked and inoculated into LB with kanamycin. The liquid culture was grown for 16 h at 37°C and 250 r.p.m. Plasmids were prepared using the Qiaprep Spin Miniprep Kit (Qiagen) with the time of incubation at room temperature increased from 1 to 5 min and the incubation was done twice. The recombinant plasmid was then sent to Source Bioscience (UK) for DNA sequencing. Sequence analysis was performed using the A plasmid Editor (ApE) software (Davis 2012).

CONSTRUCTION OF k14.1_*eforRED* AND p*cat_Venus*

PCR amplifications were performed using Q5® High-Fidelity Polymerase (New England Biolabs). The plasmid k14.1_*Venus* was used as the plasmid backbone for the construction of k14.1_*eforRED* and p*cat_Venus*. In the construction of k14.1_*eforRED,* plasmid k14.1_*Venus* was linearised using the openk14.1_*Venus_eforRED* primers (Table 2). The plasmid pJS209_*eforRED* was used as the template for the amplification of the red reporter gene, *eforRED*, using the *eforRED* primers (Table 2). The openk14.1_*Venus_cat* primers (Table 2) were used to linearise k14.1_*Venus* to exclude the kanamycin (*kan*) cassette. Plasmid pLysS was extracted from *E. coli* BL21 STAR™ (DE3) using the Qiaprep Spin Miniprep Kit (Qiagen) and used as template for the amplification of the chloramphenicol acetyltransferase (*cat*) gene using the *cat* primers (Table 2). The amplicons were gel-purified before their concentrations were determined using a spectrophotomer ((NanoDrop 2000c, Thermo Fisher Scientific Inc.).

Equimolar of linearised k14.1 and *eforRED* (3.0 × 10^{-5} nmol) or linearised k14.1_*Venus* (*kan* cassette excluded) and *cat* gene (3.4 × 10^{-5} nmol) were added to 4 µL of 1.33× Gibson Master Mix prior to incubation at 50°C for 60 min. One microliter of each assembly reaction was transformed into *E. coli E. cloni* 10G by heat shock. Transformants were selected on 50 µg/mL kanamycin (k14.1_*eforRED* transformants) or 35 µg/mL chloramphenicol (p*cat_Venus* transformants) plates. Culture plates were grown at 37°C and antibiotic resistant colonies that fluoresced under UV light were subjected to colony PCR using Go*Taq* Polymerase (Promega) according to the supplier's information in a final volume of 25 µL. The PCR programme performed was as recommended by the supplier except that a cell lysis step at 98°C for 10 min was included in the beginning of the protocol. Plasmids of the positive transformants were sequenced and sequences were analysed with the ApE software.

AMPLIFICATION OF *flgG-eforRED* AND *motA-Venus* RECOMBINEERING CASSETTES

Plasmid k14.1_*eforRED* was used as the template to generate the recombineering cassette *flgG-eforRED* whereas plasmid p*cat_Venus* was the template used to amplify the *motA-Venus* cassette. The *flgG* and *motA* flanking homologous sequences for site-directed Red recombinase-based chromosomal integration were fused to the reporter genes using the *flgG* and *motA* primers (Table 2), respectively. PCR was carried out using Q5® High-Fidelity Polymerase (New England Biolabs) and the concentrations of the gel-purified products were determined by spectrophotometry.

TRANSFORMATION OF PCR PRODUCTS INTO ELECTROCOMPETENT *E. COLI*

Electrocompetent *E. coli E. cloni* 10G was prepared using the modified protocol of Juhas et al. (2014). The *E. coli* was electroporated with pKM208 (expressing the λ Red recombinases for chromosomal insertion) in a 2 mm electroporation cuvette (Sigma-Aldrich) in the Bio-Rad micropulser at 2.5 kV, 200 Ω and 25 µF for 5 miliseconds, recovered and selected on plates with 100 µg/mL of carbenicillin at 30°C. A carbenicillin resistant colony was inoculated into LB with 100 µg/mL of carbenicillin and grown overnight at 30°C and 250 r.p.m. One mililiter of the overnight culture was diluted (1:100 dilution) using LB with 100 µg/mL carbenicillin and grown at 30°C and 250 r.p.m. to OD_{600} = 0.2. Isopropyl β-D-1-thiogalactopyranoside (IPTG, 1mM) was added and the culture was grown to an OD_{600} of 0.5. The *E. coli* cells were harvested by centrifugation at 5500 ×g and 4°C for 15 min and washed with 10% (v/v) chilled glycerol. The centrifugation and washing steps were repeated twice prior to resuspending the cells in a final volume of 100 µL of 10% chilled glycerol. Fourteen microliters of the gel-purified *flgG-eforRED* (~4 µg of DNA) was added into a vial of electrocompetent cells and electroporation was carried out using the electroporation settings described above. Transformants were selected on kanamycin and carbenicillin double selection plates and grown at 30°C for 16 h.

VERIFICATION OF CHROMOSOMALLY INSERTED *EFORRED* AND REMOVAL OF ANTIBIOTIC RESISTANCE GENE

The kanamycin and carbenicillin resistant colonies were grown on LB agar without antibiotics at 42°C for 16 h to cure pKM208 and subsequently, tested for kanamycin and carbenicillin sensitivity. Colonies resistant to kanamycin but not carbenicillin were picked for colony PCR. The chromosomally integrated transformants were verified with flanking *flgG* primers (Table 2) using Go*Taq* Polymerase (Promega) and the PCR samples were run on a 2% agarose gel. Successful chromosomally integrated colonies were made electrocompetent as described by the method above, without the addition of 1 mM IPTG. The thermosensitive plasmid pCP20 that harbours the *flp* gene encoding Flip recombinase (FLP) was transformed into the electrocompetent cells. Transformants were selected on carbenicillin plates and grown for 16 h at 30°C. Carbenicillin resistant colonies were grown for 16 h at 42°C to cure pCP20 and tested for sensitivity towards kanamycin and carbenicillin. Colonies that were sensitive to both antibiotics were subjected to colony PCR. A colony PCR with flanking FRT primers (Table 2) using Go*Taq* Polymerase (Promega) was performed and the amplicons were electrophoresed on 2% agarose gel. The chromosomally integrated *eforRED* transformant strain with a successfully knocked-out *kan* was named *flgG*i *eforRED*.

DEVELOPMENT OF THE DUAL REPORTER SYSTEM

The dual reporter system was developed by integrating the *motA-Venus* recombineering cassette into the chromosomal *motA* site (*motA*i *Venus*) of *flgG*i *eforRED* strain and subsequently, *cat* cassette removal using the same method as described above. In parallel, the construction of the marker-free reverse dual reporter system (*motA*i *eforRED* and *flgG*i *Venus*) was attempted following the methods as described before for the development of the dual reporter system.

SWIMMING ASSAY

An overnight bacterial culture of wild-type *E. coli E. cloni* 10G, *flgG*i *eforRED* and DRS were normalised to an OD_{600} of 0.5 before 1 µL of culture was inoculated into the bottom of pre-set swimming agar (0.3%; 10 g tryptone, 5 g NaCl, 0.3% Bacteriological Agar (Pronadisa)) plates. The assay was carried out with three biological replicates. The plates were incubated at 37°C for 48 h and the diameter of the swimming halo formed was measured. Statistical significance (p-value) in the size of halo formed between mutant and wild-type strains was calculated using the paired sample Student's t-test (Microsoft Excel 2013).

MEASUREMENT OF BACTERIAL GROWTH AND VENUS AND EFORRED FLUORESCENCE

Growth and fluorescence were measured based on optical density readings of three biological replicates and three technical replicates each. The wild-type *E. coli E. cloni* 10G and DRS were grown overnight to stationary phase and normalised to OD_{600} of 0.05 using LB broth. One mililiter of the bacterial cultures and LB medium (blank) were loaded into 10 mm cuvettes (Greiner Bio-One). The optical densities of EforRED and Venus were measured at excitation wavelengths of 589 nm and 515 nm, respectively, whereas cell density of the strain was measured at 600 nm. Fluorescence and cell absorbance were measured using spectrophotometry every hour over 10 time-points.

STATISTICAL ANALYSIS OF FLUORESCENCE BETWEEN VENUS AND EFORRED

The optical density readings for each time point were processed using Microsoft Excel 2013. For each fluorescence protein, the average absorbance value (from three biological replicates with three technical replicates each) and corresponding and standard error at every time-point was calculated. The significance between related data (p-value) of each protein at each time-point was calculated using the paired sample Student's t-test (Microsoft Excel 2013).

RESULTS AND DISCUSSION

SUCCESSFUL CONSTRUCTION OF REPORTER PLASMIDS k14.1_*Venus*, k14.1_*eforRED* AND p*cat_Venus*

The plasmid k14.1 (Juhas et al. 2014), also known as pSB1K3(FRTK), was linearised by PCR to produce an amplicon of ~5000 bp (Figure 1(A)). In this study, the mutant Green Fluorescent Protein (GFP) of *Aequorea victoria* was chosen as the reporter protein because it contains the F46L mutation that accelerates the oxidation of the chromophore at 37°C (Nagai et al. 2002).

The *Venus* amplicon of ~1000 bp (Figure 1(B)) and the linearised k14.1 were ligated using a two-way Gibson Assembly to produce the plasmid k14.1_*Venus*. The Gibson Assembly master mix employs three enzymes namely T5 exonuclease, Phusion polymerase and *Taq* DNA ligase. At 50°C, T5 exonuclease degrades the 5' end of dsDNA to generate 3' ssDNA and the enzyme is quickly degraded. The homologous region ends of DNA fragments hybridise to each other at the 3' end and Phusion polymerase adds dNTPs to fill in the missing nucleotides followed by sealing of the DNA gap by *Taq* DNA ligase (Gibson et al. 2009).

The ligated product was transformed into competent *E. coli* and selected on kanamycin. Kanamycin resistant colonies that emit bright yellow fluorescence when viewed using UV light indicated the presence of the recombinant plasmid k14.1_*Venus* (Figure 1(C)). Plasmid k14.1_*Venus* was later harvested and its sequence was validated by DNA sequencing.

The plasmid k14.1_*Venus* was then amplified and the *Venus* gene was excluded using the openk14.1_*Venus*_*eforRED* primers (Table 2) to enable substitution with

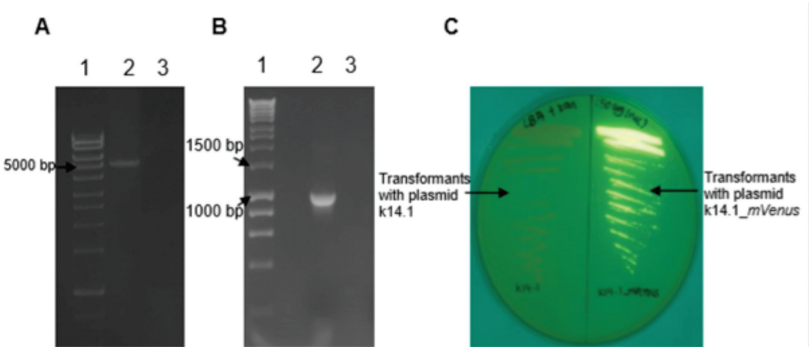

FIGURE 1. The development of the yellow reporter plasmid, k14.1_*Venus*

(A) Amplification of linearised k14.1 using the k14.1 primers. Lane 1: Hyperladder 1 kb (Bioline); Lane 2: Linearised k14.1 amplicon and Lane 3: Negative control. (B) The *Venus* amplicon generated using the *Venus* primers. Lane 1: Hyperladder 1 kb (Bioline); Lane 2: *Venus* amplicon and Lane 3: Negative control and (C) Comparison of yellow fluorescence emitted by transformants with k14.1 and k14.1_*Venus*

the *eforRED* gene. The expected size of the linearised plasmid is 5328 bp and a band sized in between 5000 bp and 6000 bp was observed (Figure 2(A)). The *eforRED* fragment (~1001 bp) (Figure 2(B)) was amplified using the *eforRED* primers (Table 2). These fragments were joined using the Gibson protocol and the recombinant plasmid (k14.1_*eforRED*) was transformed into *E. coli* before validating by colony PCR. Colony PCR showed that the amplified *eforRED* gene had the expected size of ~1001 bp (Figure 2(C)). The plasmid was extracted and the DNA sequence was verified by sequencing.

The plasmid k14.1_*Venus* was linearised to exclude the *kan* cassette and to create a ligation site for the insertion of the *cat* gene using the openk14.1_*Venus_cat* primers. The expected size of the linearised k14.1_*Venus* backbone without *kan* is ~4682 bp while the *cat* gene amplified from pLysS had the expected size of ~920 bp (Figure 2(E)). Both k14.1_*Venus* (without *kan*) and *cat* were assembled

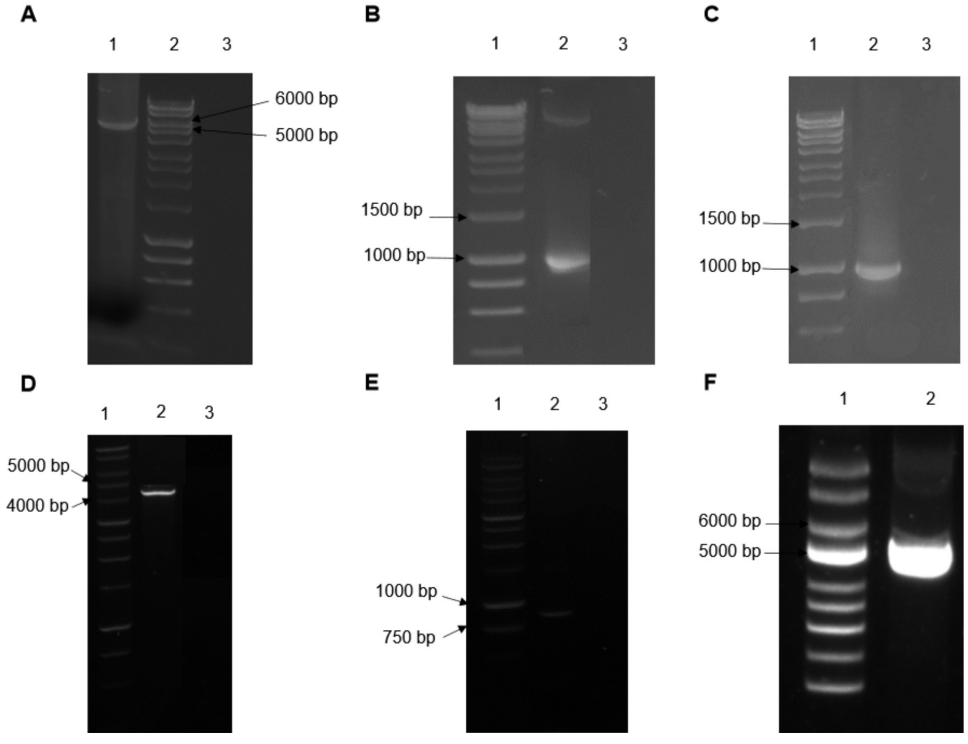

FIGURE 2. The construction and validation of k14.1_*eforRED* and p*cat_Venus*

(A) Linearised k14.1 amplicon without *Venus* using the openk14.1_*Venus_efor RED* primers. Lane 1: Linearised k14.1 amplicon; Lane 2: Hyperladder 1 kb (Ladder) and Lane 3: Negative control. (B) The *eforRED* amplicon amplified using the *eforRED* primers. Lane 1: Hyperladder 1 kb (Bioline); Lane 2: *eforRED* amplicon and Lane 3: Negative control. (C) Colony PCR of the transformant with k14.1_*eforRED*. Lane 1: Hyperladder 1 kb (Bioline); Lane 2: Amplified *eforRED* gene using the *eforRED* primers and Lane 3: Negative control. (D) The k14.1_*Venus* backbone without *kan* amplified with openk14.1_*Venus_cat* primers. Lane 1: 1 kb DNA Ladder (Promega); Lane 2: k14.1_*Venus* without kanamycin and Lane 3: Negative control. (E) The *cat* amplicon generated using the *cat* primers. Lane 1: 1 kb DNA Ladder (Promega); Lane 2: *cat* amplicon and Lane 3: Negative control and (F) Extracted p*cat_Venus*. Lane 1: Supercoiled DNA Ladder (NEB) and Lane 2: p*cat_Venus*

and transformed into *E. coli*. The extracted plasmid DNA had the expected size of ~5482 bp (Figure 2(F)) and presence of the p*cat_Venus* was validated by sequencing. Both k14.1_*eforRED* and p*cat_Venus* were then used for the construction of DRS using the Red recombineering and FLP/FRT recombination techniques.

RED RECOMBINEERING FOR CHROMOSOMAL INTEGRATION AND FLP/FRT RECOMBINATION FOR ANTIBIOTIC GENE REMOVAL

Chromosomal integration of heterologous genes in a surrogate host has been reported to increase recombinant protein production. In an earlier report, the chromosomal integration strategy was adopted to integrate *Zymomonas mobilis* genes involved in the ethanol production pathway, into the *adhE* operon of *E. coli* K12 to create ethanologenic *E. coli* strains. The promoterless cassette was used to disrupt the *adhE* operon to create an operon fusion and relied on the *adhE* promoter to increase the production of ethanol (Martinez-Morales et al. 1999). In addition to *E. coli*, integration of genes has also been successful in *S. cerevisiae* where genes encoding for endo/exoglucanase and β-glucosidase were chromosomally inserted into the yeast host to enable the production of ethanol in media containing cellulose (Cho et al. 1999).

To allow a large DNA cassette to be integrated into the host chromosome, many different chromosomal integration methods can be employed and one such method is the Red recombinases system. The Red recombinase-based chromosomal integration strategy was shown previously to introduce large DNA constructs into the *E. coli* chromosome (Kuhlman & Cox 2010; Sabri et al. 2013; Ublinskaya et al. 2012). One of these strategies is the two-step Red-recombinase integration method that requires the insertion of a landing pad on chromosome, followed by excision of the pad via I-SceI (yeast endonuclease) cleavage and subsequently, second integration of the gene of interest into the integrated pad (Kuhlman & Cox 2010). However, the I-SceI cleavage site is not found in the *E. coli* genome (Sabri et al. 2013). In our study, we employed the one-step Red recombineering technique that targets the *E. coli* chromosomal regions to develop the DRS (Juhas et al. 2014). This technique is similar to the knock-in/knock-out (KIKO) vector-based method (Sabri et al. 2013) but simpler. Figure 3 depicts the key steps involved in the development of the DRS.

Our study compared two previously identified nonessential *E. coli* chromosomal insertion target sites, *flgG* (Juhas & Ajioka 2015a) and *motA* (Juhas & Ajioka 2015b). In this work, the recombineering cassettes *flgG-eforRED* and *motA-Venus* were generated with the *flgG* and *motA* flanking primers using PCR where a 66 bp region (homologous *flgG* or *motA* region) was added to the 5' and 3' ends of the recombineering cassettes, respectively, to facilitate homologous recombination (Figure 3(A) and 3(B)). Each cassette harbours the reporter gene regulated by a constitutive penicillinase promoter, pPen from *Bacillus licheniformis* (Yansura & Henner 1984) and an antibiotic resistance gene, flanked by the FLP Recognition Target (FRT) (Figure 3(B)).

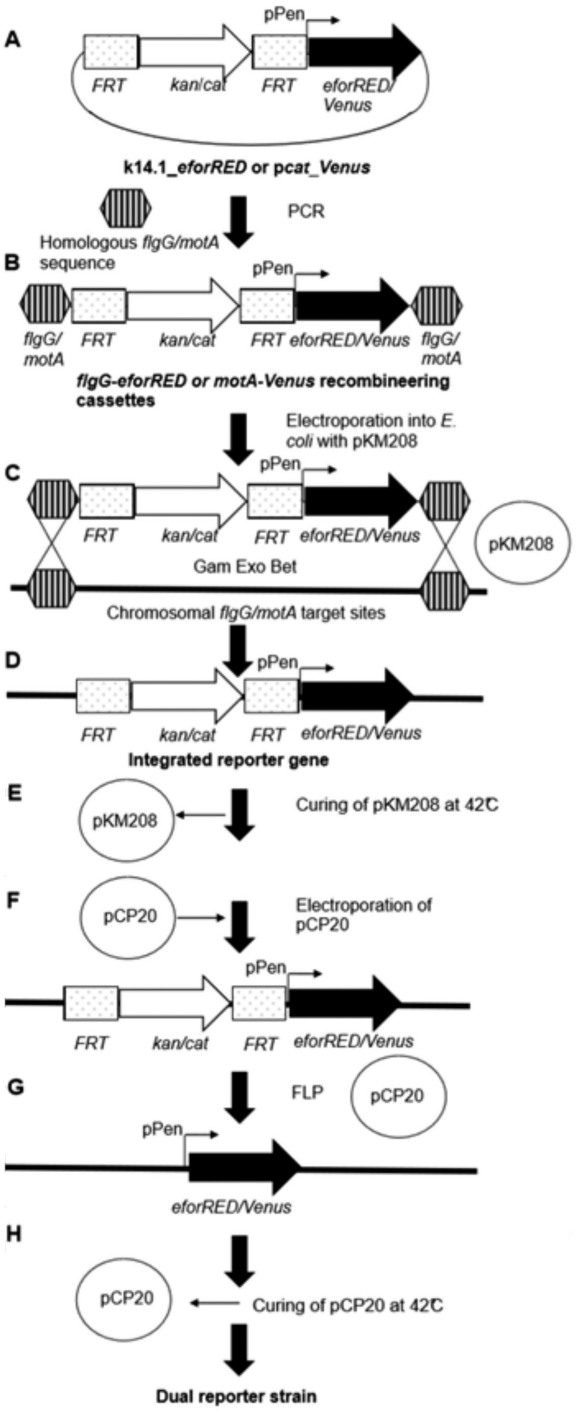

FIGURE 3. Key steps in developing the marker-free dual reporter strain

(A) Amplification of *flgG-eforRED* and *motA-Venus* recombineering cassettes from k14.1_*eforRED* and p*cat_Venus*, respectively. (B) The cassettes consist of reporter and antibiotic resistance genes and were electroporated into *E. coli* with pKM208 upon induction with 1 mM IPTG. (C) Homologous recombination at chromosomal *flgG* or *motA* target site with the help of Gam, Exo and Bet proteins expressed by pKM208. (D) Integrated recombineering cassette (E) Curing of the thermosensitive pKM208 at 42°C. (F) Electroporation of pCP20 that expresses FLP constitutively to excise the antibiotic resistance gene. (G) Removed antibiotic gene from recombineering cassette and (H) Curing of thermosensitive pCP20 at 42°C to generate the dual reporter strain

Amplification of *flgG eforRED* and *motA Venus* produced fragments corresponding to the expected sizes of ~3432 bp and ~3395 bp, respectively (Figure 4). Approximately 4 µg of DNA was electroporated into electrocompetent *E. coli*. The pKM208-plasmid borne Red recombineering system of the λ bacteriophage was employed in this study as the chromosomal integration strategy to allow insertion of recombineering cassettes into the *E. coli* chromosome. Regulated by *lacZ* with the induction of 1 mM IPTG, plasmid pKM208 harbours *gam, exo* and *bet* that code for the 16 kDa polypeptide, the 24 kDa exonuclease Exo and the ssDNA-binding Bet (Muniyappa & Radding 1986). Gam inhibits host RecBCD exonuclease V and SbcCD nuclease (Kulkarni & Stahl 1989), which would otherwise degrade the exogenous dsDNA. Then, Exo degrades a single strand of DNA of the exogenous dsDNA in entirety, generating a ssDNA (Mosberg et al. 2010) followed by the binding of Bet to ssDNA and directing the recombineering cassette to the site of integration where homologous recombination is then mediated (Datsenko & Wanner 2000) (Figure 3(C)).

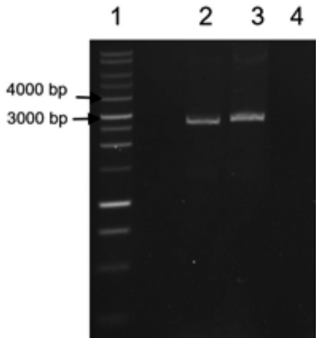

FIGURE 4. Generation of recombineering cassettes using the *flgG* and *motA* primers. Lane 1: 1 kb DNA Ladder (Promega); Lane 2: *flgG eforRED* amplicon; Lane 3: *motA Venus* amplicon and Lane 4: Negative control

Initially, the DRS was developed through the disruption of *flgG* in flagellar region 1 by *eforRED*. Colony PCR using the flanking *flgG*i primers showed that the *flgG*i *eforRED* amplicon has the expected size of ~3658 bp whereas *E. coli* without chromosomal insertion has the expected amplified *flgG* of ~963 bp (Figure 5(A)). The plasmid pKM208 carries the pSC101 origin of replication (ori) that is thermosensitive (Murphy & Campellone 2003) and is easily cured from the bacterial host at 42°C (Figure 3(E)). The plasmid pCP20 that harbours the *S. cerevisiae flp* gene was then introduced into *E. coli* to express FLP constitutively (Figure 3(F)). Therefore, no protein induction was required for strains with pCP20. The k14.1 plasmid backbone has the FRT sites that flank the antibiotic resistance genes (*kan* and *cat*). This enables the removal of these genes via FLP/FRT recombination to generate marker-free strains with integrated reporter genes.

The FLP/FRT recombination functions well in both fungi (Bloemendal et al. 2014) and bacteria (Ishikawa & Hori 2013). Although FLP can also be used to insert DNA into chromosomes (Gu et al. 2015), the preferred direction of FLP is excision (Ringrose et al. 1998). The FRT sites normally have a minimal sequence of 5'-GAAGTTCCTATTCtctagaaaGAATAGGAACTTC-3' with two flanking sequences (5'-GAAGTTCCTATTC-3') and an 8 bp core (5'-tctagaaa-3'). The FLP protein binds to the flanking sequences and cleaves ahead of the core region (Zhu & Sadowski 1995). This results in the excision of the antibiotic resistance gene from the integrated recombineering cassette (Figure 3(G)).

Colony PCR using the flanking FRT primers showed that the *kan* knocked-out amplicon from the *flgG*i *eforRED* strain had the expected size of ~112 bp whereas the *flgG*i *eforRED* amplicon, in the absence of pCP20, was sized at between 1000 bp and 1500 bp (expected size of amplified *kan* is ~1342 bp) (Figure 5(B)). Similar to pKM208, the thermosensitive pCP20 is cured from the bacterial host at 42°C (Figure 3(H)).

The complete DRS was obtained when *Venus* was integrated at the *motA* site which was validated by PCR. The amplified *motA*i *Venus* was approximately 3519 bp whereas the non-integrated *motA* amplicon had the expected size of ~614 bp (Figure 5(C)). Using FLP/FRT recombination, the *cat* gene was removed from the *Venus* cassette and this was confirmed by colony PCR using the flanking FRT primers (Figure 5(D)). To further validate the accuracy of the developed dual reporter system in determining the best integration site for synthetic DNA to enable efficient protein expression, we attempted to construct a reverse reporter system (*motA*i *eforRED* and *flgG*i *Venus*-reporter genes with switched integration sites) but integration of *Venus* at *flgG* was unsuccessful.

In this study, the *flgG* and *motA* sites were selected as target sites for DNA insertion because they are located away from each other in different chromosomal regions (Juhas et al. 2014). This eliminates the possibility of excising the entire intervening region in the event of FLP/FRT recombination during removal of the antibiotic-resistance genes.

THE *E. COLI flgG*i *eforRED* AND DUAL REPORTER MUTANT STRAINS HAVE ABOLISHED MOTILITY

To confirm the successful integration of *eforRED* and *Venus* at the respective target sites, *flgG*i *eforRED* and DRS (*flgG*i *eforRED* - *motA*i *Venus*) strains were tested using the swimming assay. Both *flgG* and *motA* encode for bacterial proteins that contribute towards motility and disruption of these genes could foil or reduce swimming motility (Juhas & Ajioka 2015a, 2015b). All the reporter strains showed a reduced capacity ($p<0.001$) to swim when compared to the parental strain *E. coli E. cloni* 10G (Table 3). The diameter of the swimming halo formed by the DRS is similar to the strain bearing the single insertion at *flgG* (Table 3). FlgG forms the basal body of the bacterial

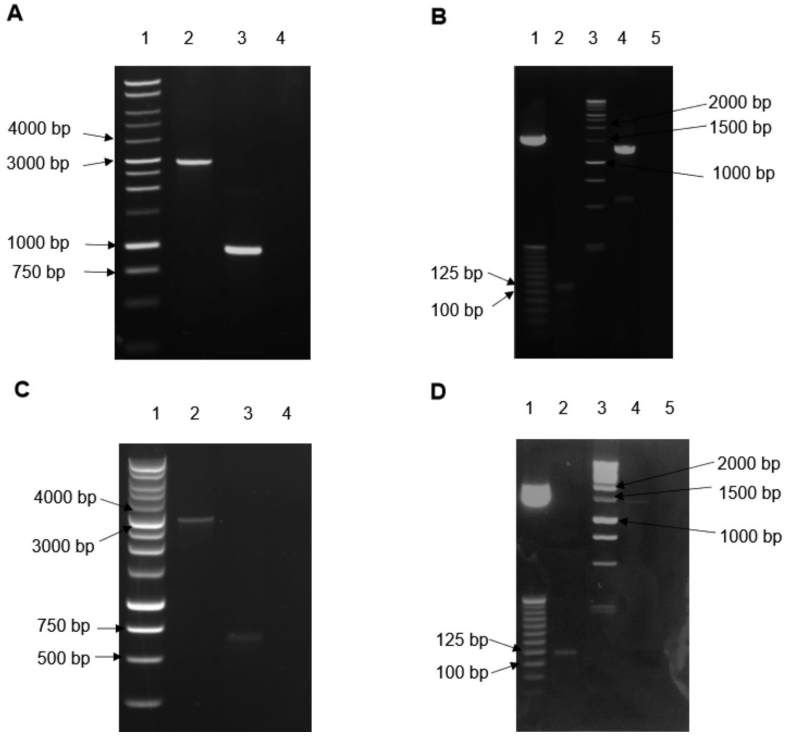

FIGURE 5. Development and validation of the dual reporter system

(A) Colony PCR of the integrated *eforRED* reporter gene at *flgG* site. Lane 1: 1 kb DNA Ladder (Promega); Lane 2: *flgG*i *eforRED* amplicon; Lane 3: *flgG* amplicon and Lane 4: Negative control. (B) Colony PCR of *kan* knocked-out *flgG*i *eforRED* strain. Lane 1: 25 bp DNA Step Ladder (Promega); Lane 2: Flanking FRT amplicon; Lane 3: 1 kb DNA Ladder (Promega); Lane 4: Flanking FRT with *kan* cassette amplicon and Lane 5: Negative control (C) Colony PCR of the integrated *Venus* reporter gene at *motA* site. Lane 1: 1 kb DNA Ladder (Promega); Lane 2: *motA*i *Venus* amplicon, Lane 3: *motA* amplicon and Lane 4: Negative control. (D) Colony PCR of *cat* knocked-out *flgG*i *eforRED motA*i *Venus* strain. Lane 1: 25 bp DNA Step Ladder (Promega); Lane 2: Flanking FRT amplicon; Lane 3: 1 kb DNA Ladder (Promega); Lane 4: Flanking FRT with *cat* cassette amplicon and Lane 5: Negative control

TABLE 3. Average diameter of the swimming halo formed by *E. coli* and mutant strains on swimming agar

Bacterial strain	Average diameter of swimming halo formed (cm, ± SE)
E. coli E. cloni 10G	7.9 ± 0.07
*flgG*i *eforRED*	0.4 ± 0.03
Dual reporter	0.5 ± 0.06

flagellar rod (Macnab 2003) while MotA normally forms a hetero-hexameric complex with MotB (MotA tetramer and MotB dimer) to become the stator which will then interact with the rotor to drive the rotation of the bacterial flagellar motor (Takekawa et al. 2016). Hence, disruption of the associated genes resulted in the loss of flagellar function.

CHROMOSOMAL INSERTION DOES NOT CAUSE METABOLIC BURDEN UPON THE HOST AND THE *MOTA* FLAGELLAR GENE IS THE BETTER INTEGRATION TARGET SITE COMPARED TO *flgG*

Overexpression of heterologous proteins usually contribute heavy metabolic burden to the recombinant host (Bhattacharya & Dubey 1995; Carneiro et al. 2013) leading to cell growth arrest and subsequently, reduced heterologous protein synthesis (Bentley & Kompala 1990; Glick 1995). Although many different strategies have been employed to improve recombinant protein production, this was achieved at the expense of reduced cell biomass (Carneiro et al. 2013). The dual reporter system developed in this study was severely impeded in terms of motility (Table 3) but the growth rate of this strain was unaffected when compared to the parental strain (Figure 6(A)).

The reporter proteins EforRED and Venus have maximum absorption at 589 nm (Alieva et al. 2008) and 515 nm (van der Krogt et al. 2008), respectively. The optical densities of the two chromoproteins as expressed by the DRS were evaluated at the respective excitation wavelengths (Figure 6(B)). The absorbance values for Venus were significantly higher compared to EforRED ($p < 0.05$) over the period of evaluation. Juhas and Ajioka (2015a, 2015b) also reported that transcription from *motA*i was 4 times higher compared to *flgG*i. Furthermore, it

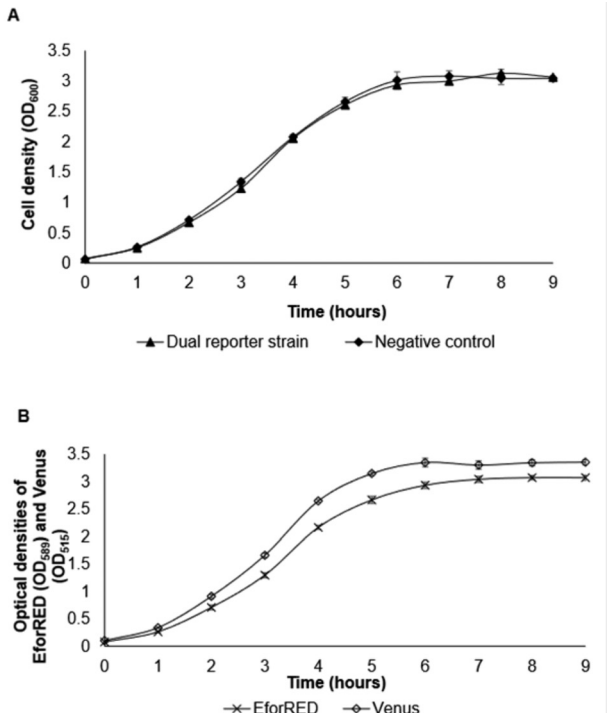

FIGURE 6. Real-time measurement of bacterial cell density (OD_{600}),Venus (OD_{515}) and EforRED (OD_{589}) over 10 time-points

(A) The cell density (OD_{600}) of the dual reporter strain and negative control and (B) Comparison of the optical densities of Venus (OD_{515}) and EforRED (OD_{589}) in the dual reporter strain. Values represent the means and standard errors from three biological replicates with three technical replicates each

was also reported that the primary criterion in selecting the best integration site was the transcription rate at the targeted sites (Bai Flagfeldt et al. 2009). Although the reverse system was not successfully built to further validate the integration sites, the results as obtained by the dual reporter system is adequate for the context of this study. Taken together, the genes inserted into the bacterial chromosome and subsequent expression (as represented by the fluorescence of the reporters) did not present any metabolic burden onto the host. This agrees with the reports by Juhas and Ajioka (2015a, 2015b) and Sabri et al. (2013) that hosts with chromosomally inserted genes had unaffected growth. Since *Venus* was inserted at *motA* whereas *eforRED* was integrated at *flgG*, we propose that the *motA* flagellar gene is the better target locus for future synthetic biology applications.

CONCLUSION

Flagellar genes are suitable non-essential target genes for chromosomal insertion in the design of an *E. coli*-based synthetic biology toolkit. The *flgG* and *motA* target loci were compared using the DRS (*flgG*i *eforRED* - *motA*i *Venus*) that expressed the EforRED and Venus reporter proteins. Gene disruption at the flagellar region completely abolished motility of the host but did not affect its growth. The fluorescence emitted by Venus was significantly higher than EforRED, indicating that *motA* is the more suitable target locus compared to *flgG*. This study rationalises the choice of target site for chromosomal insertion for future synthetic biology applications.

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