RESEARCH ARTICLE

Identification and Validation of Novel Chromosomal Integration and Expression Loci in *Escherichia coli* Flagellar Region 1

Mario Juhas*, James W. Ajioka

Department of Pathology, University of Cambridge, Cambridge, United Kingdom

* mj417@cam.ac.uk

Abstract

*Escherichia coli* is used as a chassis for a number of Synthetic Biology applications. The lack of suitable chromosomal integration and expression loci is among the main hurdles of the *E. coli* engineering efforts. We identified and validated chromosomal integration and expression target sites within *E. coli* K12 MG1655 flagellar region 1. We analyzed five open reading frames of the flagellar region 1, flgA, flgF, flgG, flgI, and flgJ, that are well-conserved among commonly-used *E. coli* strains, such as MG1655, W3110, DH10B and BL21-DE3. The efficiency of the integration into the *E. coli* chromosome and the expression of the introduced genetic circuit at the investigated loci varied significantly. The integrations did not have a negative impact on growth; however, they completely abolished motility. From the investigated *E. coli* K12 MG1655 flagellar region 1, flgA and flgG are the most suitable chromosomal integration and expression loci.

Introduction

*Escherichia coli* K-12 is a model Gram-negative bacterium used as an intermediate and final destination chassis for a number of biotechnological applications and Synthetic Biology devices [1–5]. Integration and expression of genetic circuits from the *E. coli* chromosome is preferable to their introduction into the genome on plasmids due to the lower metabolic burden and no need for selective pressure [6]. Chromosomal integration strategies differ in their speed and flexibility. Recently developed methods include ‘the one-step clonetegration’ that relies on the bacteriophage integrase-mediated site-specific recombination between phage attachment (*att*) sites [7]. Besides transposons, phages and phage-derived elements [8, 9], Red recombinase of the bacteriophage λ [10] is often used to engineer *E. coli*. The recently developed Red recombinase-based strategies include those based on the yeast mitochondrial homing endonuclease I-SceI [9], knock-in/knock-out (KIKO) vectors [11], and plasmid pSB1K3(FRTK) [12].

The lack of suitable chromosomal integration loci is among the main hurdles of the current *E. coli* engineering efforts. Traditionally phage attachment (*att*) sites have been used as integration targets [7]; however, *att* sequences are not present in all commonly-used *E. coli* strains. Integration target sites should be well-conserved, non-essential, well-characterized and highly
expressed. Previously we have analyzed four genes of the *E. coli* K12 MG1655 flagellar gene region 3a and identified one locus that fulfills all these criteria and supports high efficiency integration [12]. The identification and characterization of the alternative integration sites is crucial for the development of a robust synthetic biology toolkit. Some applications require integrations of a number of genetic circuits into the chromosome (e.g., measuring synergistic effect of multiple genetic circuits on the phenotype or integration of the repressor and other genetic circuit(s) regulated by this repressor). Thus identification and validation of other suitable chromosomal integration and expression target sites would significantly help progress. Here we analyse open reading frames of the *E. coli* flagellar region 1. This region of the *E. coli* chromosome shows high probability of being occupied by RNA polymerase [13, 14] and is therefore potentially suitable for integration and expression of genetic circuits. We integrated a genetic circuit harbouring the thermosensitive λ repressor [15] into five well-conserved open reading frames of the flagellar region 1. We propose *flgA* and *flgG* as the most suitable chromosomal integration and expression loci of the *E. coli* flagellar region 1.

**Materials and Methods**

**Bacterial strains, plasmids, and growth conditions**

Bacterial strains and plasmids used in this analysis are listed in Table 1. *Escherichia coli* strains were routinely grown in Luria-Bertani (LB) medium. When needed, LB broth was supplemented with kanamycin (50 μg/ml) or ampicillin (100 μg/ml). Liquid cultures were grown in LB broth on a rotatory shaker at 200 r.p.m. Solid cultures were grown on agar plates for 24 hours. Depending on the requirements, both liquid and solid cultures were grown at 30°C, 37°C or 42°C.

**Recombinant DNA methodology**

PCR amplifications of DNA fragments were performed using Dream Taq master mix kit (Thermo Scientific) or Phusion DNA polymerase (Thermo Scientific) in 50 μl volumes according to the supplier’s instructions. Oligonucleotide primers used in this analysis were synthesized by Integrated DNA Technologies (IDT). Plasmid isolations and gel extractions were carried out using Qiaprep Spin Miniprep kit (Qiagen) and Qiaquick Gel Extraction kit (Qiagen), respectively, according to the manufacturer’s instructions. Sequencing of DNA fragments was performed by Source Bioscience (Cambridge, UK). DNA fragments were assembled using

| Characteristics | Reference |
|-----------------|-----------|
| **Strains**     |           |
| K12 MG1655      | *E. coli* wild type [32] |
| Ec:flgAi        | *E. coli* K12 MG1655 with integration in *flgA* This study |
| Ec:flgFi        | *E. coli* K12 MG1655 with integration in *flgF* This study |
| Ec:flgGi        | *E. coli* K12 MG1655 with integration in *flgG* This study |
| Ec:flgIi        | *E. coli* K12 MG1655 with integration in *flgI* This study |
| Ec:flgJi        | *E. coli* K12 MG1655 with integration in *flgJ* This study |
| **Plasmids**    |           |
| pCP20           | FLP recombinase helper plasmid [10] |
| pKM208          | Red recombinase controlled by lacZ [33] |
| pSB1A1(GFP)     | AmpR, GFP under the control of λ promoter [12] |
| pSB1K3(FRTKr)   | KanR, λ repressor [12] |

Data:10.1371/journal.pone.0123007.t001
the modified Gibson Isothermal Assembly method [16] as described previously (http://www.srcf.ucam.org/~wac26/gibson/index.html) [12]. The modified Gibson Isothermal Assembly reaction was incubated at 50°C for 60 min prior to *E. coli* transformation.

**E. coli chromosomal integration**

Electro-competent and chemically-competent *E. coli* were prepared by the modified Miller and Nickoloff [17] and Hannah methods [18], respectively. Integrations into the *E. coli* chromosome were performed by method described previously [12]. Briefly, DNA fragment composed of the thermosensitive λ repressor construct, kanamycin and FRT sites flanked by sequences homologous to the *E. coli* chromosome target genes was amplified by PCR and gel-purified. *E. coli* strain K12 MG1655 was transformed with pKM208 harboring resistance to ampicillin and selected on ampicillin plates at 30°C. Overnight culture (1:100 dilution) of *E. coli* strain K12 MG1655 with pKM208 was inoculated into LB with ampicillin and grown at 30°C to OD$_{600}$ of 0.2. 1 mM IPTG was added at this time point and the bacterial culture was grown to OD$_{600}$ of 0.4–0.6. Cells were washed twice with 10% (v/v) glycerol and resuspended in 100 μl of 10% glycerol per 100 ml of starting culture. Purified DNA fragment was introduced into electro-competent cells by electroporation on a Bio-Rad micropulser. Transformants were selected on kanamycin plates at 37°C. Temperature sensitive pKM208 was cured out by incubation at 42°C. Successful integration into the *E. coli* was confirmed by PCR using the flanking primers.

**Quantitative measurement of GFP fluorescence**

*E. coli* cultures were grown overnight and normalized to OD$_{600}$ of 0.05. 200 μl of these were transferred into flat-bottomed black 96 well plates (Greiner BioOne, UK). The plates were incubated in a Fluostar Omega fluorimeter (BMG Labtech, UK) at 30°C for 3 hours followed by 10 hours at 42°C for the measurement of the GFP fluorescence with an automatically repeated protocol. Excitation filter 485–12, emission filter EM520, gain 1400, double orbital shaking at 200 rpm, and cycle time 60 min were used for the GFP fluorescence measurement.

**Plate reader measurement of the growth rate**

The overnight *E. coli* cultures were normalized to OD$_{600}$ of 0.05. The total volume of 200 μl was transferred into flat-bottomed clear 96 well plates (Seralin Sero-Well, UK). The plates were incubated in a microplate reader (Fluostar Omega) (BMG Labtech) for 24 hours at 37°C and 30°C with an automatically repeated protocol. The following parameters were used for the absorbance measurement (600 nm absorbance filter, cycle time 60 min, double orbital shaking at 500 rpm).

**RNA isolation and RT-PCR**

Isolate II RNA Mini Kit (Bioline) was used to isolate the total RNA from 10^9 *E. coli* cells grown into mid-exponential phase (OD$_{600}$ = 0.7). RNA was eluted with 60 μl of RNAse-free H$_2$O and purified from genomic DNA contamination using a TURBO DNA-free Kit (Applied Biosystems) according to supplier’s instructions. cDNA was synthesized from 1 μg of purified total RNA using SuperScript III Reverse Transcriptase (Invitrogen) according to supplier’s instructions. QuantiTect SYBR Green PCR Kit (Qiagen), 7500 Fast Real-Time PCR System (Applied Biosystems) and MicroAmp Fast Optical 96-Well Reaction Plates (Applied Biosystems) were used to measure the expression levels of the target DNA sequences (100–150 bp long). RT-PCR primers were designed using Primer3 Software. The following program was used to amplify target DNA sequences in the 7500 Fast Real-Time PCR System: initial activation (15
min, 95°C), followed by 35–45 cycles of denaturation (15 s 94°C), annealing (30 s 50–60°C) and extension (30 s 72°C). The relative expression was quantified with REST9 Software (Qiagen) employing Pfaffl method [19]. The experiments were carried out in triplicate and the means and standard errors were calculated.

**Motility assay**

Motility assays were performed on motility agar plates. 13 cm motility agar plates composed of 100 ml motility agar (5 g NaCl, 10 g tryptone, 0.25% Bacto-Agar (Difco)) were let to set overnight and pre-warmed at 37°C before inoculation of bacteria. Overnight bacterial cultures (2 μl of the OD600 of 1.0) were spotted into the middle of the motility plates and incubated for 4–6 hours at 37°C.

**Sequence analyses and databases**

The target flagellar genes sequences were obtained from the *E. coli* K-12 project website (http://www.xbase.ac.uk/genome/escherichia-coli-str-k-12-substr-mg1655). The National Center for Biotechnology Information (NCBI) website’s (http://ncbi.nlm.nih.gov) BLASTN and TBLASTX [20] algorithms were used to search for similarities between DNA sequences.

**Results and Discussion**

**Flagellar region 1 as target for chromosomal integration**

The main hurdle of *E. coli* engineering is the shortage of suitable loci that support high efficiency integration and expression of integrated genetic circuits [12]. Previously we showed that *fliT* is the most suitable integration site from the four analysed open reading frames of the *E. coli* K12 MG1655 flagellar region 3a [12]. The identification and validation of the alternative integration sites is crucial, particularly for applications requiring integrations of multiple genetic circuits into the chromosome. Here we investigate flagellar region 1 as potential target for chromosomal integrations. ChIP-seq data [13, 14] indicate high probability of the flagellar region 1 being occupied by RNA polymerase (Fig. 1), suggesting that genetic circuits integrated here would be highly expressed. Chromosomal integration sites should be well-conserved. BLAST search revealed that out of the 14 open reading frames of the *E. coli* K12 MG1655 flagellar gene region 1 (*flgA-L*), nine (*flgB, C, D, E, H, K, L, M, N*) were either missing or only partially conserved in some *E. coli* strains. The remaining five genes (*flgA, F, G, I, J*) are well-conserved among commonly-used *E. coli* strains, including MG1655, W3110, DH10B and BL21-DE3. *flgA* and *flgI* encode proteins for the flagellar basal-body periplasmic P ring assembly [21–24]. *flgF* and *flgG* encode flagellar components of the cell-proximal and the cell-distal part of basal body, respectively [25]. *flgJ* encodes B-N-acetylglucosaminidase that is essential for the bacterial flagella biogenesis [26]. The location of *flgA, flgF, flgG, flgI*, and *flgJ* in the *E. coli* K12 MG1655 flagellar region 1 are shown in Fig. 1. We used our streamlined Red recombinase-based chromosomal integration protocol [12] to integrate a genetic circuit (Repr-ts-1) consisting of the thermosensitive λ repressor expressed using a strong constitutive promoter, a RBS and a terminator taken from the iGEM Parts Registry into the investigated target genes *flgA, flgF, flgG, flgI*, and *flgJ* of the *E. coli* flagellar region 1.

**Chromosomal integrations into flgA and flgG occur with the highest frequency**

Chromosomal integration efficiency differs significantly between integration sites, suggesting that to find the most suitable loci, the integration efficiencies have to be determined.
Genetic circuit Repr-ts-1 was integrated into five targeted open reading frames of the *E. coli* flagellar region 1: *flgA* (flgAi), *flgF* (flgFi), *flgG* (flgGi), *flgI* (flgIi), and *flgJ* (flgJi). Integration into the chromosome was verified by diagnostic PCR with flanking primers (S1 Fig.) and DNA sequencing. Primer sequences homologous to the target genes that were used to amplify Repr-ts-1 and the diagnostic flanking primers are listed in S1 Table. The integration efficiencies for the five targeted integration loci differed significantly. The integration of Repr-ts-1 into *flgA* and *flgG* occurred with the highest efficiency (Fig. 2). The integrations into both *flgA* and *flgG* generated two times and four times more recombinants per μg DNA than integrations into *flgF*, *flgI*, and *flgJ*, respectively. As *flgF*, *flgG*, *flgI*, and *flgJ* are part of one operon, while *flgA* is located in another operon, the observed differences in the integration efficiencies are not operon-specific. Notably, integrations into the analyzed genes of the *E. coli* flagellar region 1 occurred with the lower efficiency than integrations into the best locus of the previously analyzed region 3a, *fliT* [12]. Integrations into *fliT* yielded approximately twice the number of recombinants than integrations into *flgA* and *flgG* (Fig. 2).
Effect of integration into flgA, flgF, flgG, flgI and flgJ on motility and cell growth

Integration of the genetic circuit into the five target loci of the flagellar region 1 (flgAi, flgFi, flgGi, flgIi, and flgJi) completely abolished the motility of the engineered strains (Fig. 3). This is in contrast with the previously analysed flagellar region 3a where integrations into two of the four analysed loci had only a minor effect on motility [12]. As integrations should not inhibit growth, the target loci can not be located within essential genes [1, 5, 27–29]. The growth rates of the engineered strains harboring Repr-ts-1 integrations (flgAi, flgFi, flgGi, flgIi, and flgJi) and E. coli K12 MG1655 wild type were measured with the microplate reader (Fluostar Omega). Integrations into all five target loci did not have a negative impact on the growth at both the permissive (30°C) and the restrictive (37°C) conditions for the Repr-ts-1-bourne thermosensitive repressor (Fig. 4). Some of the engineered strains (flgAi, flgFi, flgGi, flgIi) grew to a higher final density than the wild type strain at permissive temperatures, while the growth of flgIi and the wild type strain was similar at both conditions (Fig. 4). This result is similar to that obtained for flagellar region 3a where strains with integrations grew to a higher density than the wild type strain [12]. The abolished motility and good growth (particularly of flgAi, flgFi, flgGi, flgIi) confirm the suitability of the tested loci for chromosomal integrations.
Expression from \( \text{flgA} \), \( \text{flgF} \), \( \text{flgG} \), \( \text{flgI} \) and \( \text{flgJ} \) of the flagellar region 1

The high probability of the \( E. \ coli \) K12 MG1655 flagellar region 1 being occupied by RNA polymerase (Fig. 1) suggests that genetic circuits integrated here would be strongly expressed. We verified this by measuring relative expression of \( \text{flgA} \), \( \text{flgF} \), \( \text{flgG} \), \( \text{flgI} \), and \( \text{flgJ} \) by RT-PCR (Fig. 5A). The relative expression of the two integration target loci, \( \text{flgF} \) and \( \text{flgG} \), was higher (2–3 fold) than the mean expression of the housekeeping genes \( arcA \) and \( rpoD \) [30, 31]. The relative expression of \( \text{flgI} \) and \( \text{flgJ} \) was lower, while the relative expression of \( \text{flgA} \) was not different from the mean expression of the housekeeping genes (Fig. 5A). RT-PCR was also employed to assess the relative expression of the genetic circuit integrated into \( \text{flgA} \), \( \text{flgF} \), \( \text{flgG} \), \( \text{flgI} \), and \( \text{flgJ} \). Repr-ts-1 was expressed strongly in all five target loci of the \( E. \ coli \) flagellar region 1 (Fig. 5B). The highest expression of Repr-ts-1 was detected when integrated into \( \text{flgA} \) and \( \text{flgF} \). The expression at these sites was 4–5 fold higher than the mean expression of the housekeeping genes (Fig. 5B). Notably, the expression of Repr-ts-1 was significantly higher (3–4 fold) at the best locus of the previously analyzed flagellar region 3a, \( fliT \) [12] (Fig. 5B). This could be due to the...
higher probability of the *E. coli* K12 MG1655 flagellar region 3a being occupied by RNA polymerase [12–14]. To assess the expression of the integrated genetic circuit we measured the repressive activity of the Repr-ts-1-bourne thermosensitive repressor on the GFP expression (Fig. 6). We introduced plasmid pSB1A1(GFP) into *E. coli* harboring chromosomal integrations flgAi, flgFi, flgGi, flgIi, and flgJi. pSB1A1(GFP) harbours GFP-encoding gene
downstream of the pR promoter that is controlled by the Repr-ts-1-bourne repressor. The repressive activity of the integrated genetic circuit was quantified by measuring GFP expression over time with the multiwell plate fluorimeter (FluoStar Omega). At 30°C GFP was not expressed, while increased temperature (42°C) led to GFP expression (Fig. 6).
Conclusions

The *E. coli* flagellar genes are suitable targets for integration of genetic circuits. Previous investigation of the *E. coli* K12 MG1655 flagellar region 3a led to the identification of one locus that supports both high integration efficiency and strong expression of the integrated genetic circuit [12]. The identification and validation of other suitable loci would significantly aid progress.
The aim of this analysis was to identify and validate suitable integration and expression sites within *E. coli* flagellar region 1, which has good transcriptional profile and high probability of being occupied by RNA polymerase. We integrated genetic circuit Repr-ts-1 into five open reading frames of the flagellar region 1 (flgA, flgF, flgG, flgI, and flgJ) that are well-conserved among commonly-used *E. coli* strains. The five targeted genes of the flagellar region 1 varied significantly in the efficiency of integration and expression of the integrated genetic circuit. The integrations did not have a negative impact on growth; however, they all abolished motility. This analysis shows that *flgA* and *flgG* are the most suitable chromosomal integration sites of the investigated *E. coli* K12 MG1655 flagellar region 1 due to high efficiency of integration and strong relative expression. Expression of the integrated genetic circuit at *flgF* is higher than expression at *flgG*; however, the integration efficiency at *flgF* is only half of that measured at *flgA* and *flgG*. In conclusion, the presented study of the *E. coli* flagellar region 1 contributes two novel sites for the robust integration and expression of synthetic gene circuits.

Supporting Information

**S1 Fig. Confirmation of the integration into the *E. coli* flagellar region 1.** Figure depicts chromosomal integration into the target genes of the *E. coli* K12 MG1655 flagellar region 1 (*flgA* (flgAi), *flgF* (flgFi), *flgG* (flgGi), *flgI* (flgIi), and *flgJ* (flgJi)). Flanking primers were used for the verification of successful integration and the HyperLadder 1kb (Bioline) has been used as the molecular weight marker. Wt (wild type), +i (integrated DNA fragment). (TIF)

**S1 Table. Primers used in this study.**

(DOC)

**S2 Table. Raw plate reader data of the growth rates of strains with chromosomal integrations.**

(DOC)

Acknowledgments

We thank Dr. Kerstin Ewen for providing us with the plasmid pKM208 and members of the James Ajioka and Gillian Fraser laboratories for helpful discussions.

Author Contributions

Conceived and designed the experiments: MJ JWA. Performed the experiments: MJ. Analyzed the data: MJ JWA. Wrote the paper: MJ JWA.

References

1. Juhas M, Reuß DR, Zhu B, Cornichau FM. Bacillus subtilis and Escherichia coli essential genes and minimal cell factories after one decade of genome engineering. Microbiology. 2014; 160(Pt 11):2341–51. doi:10.1099/mic.0.079376-0 PMID: 25092907

2. Juhas M, Davenport PW, Brown JR, Yarkoni O, Ajioka JW. Meeting report: The Cambridge BioDesign TechEvent—Synthetic Biology, a new “Age of Wonder”? Biotechnol J. 2013. doi:10.1002/biot.201300042

3. Ajikumar PK, Xiao WH, Tyo KE, Wang Y, Simeon F, Leonard E, et al. Isoprenoid pathway optimization for Taxol precursor overproduction in Escherichia coli. Science. 2010; 330(6000):70–4. doi:10.1126/science.1191652 PMID: 20929806

4. Yim H, Haselbeck R, Niu W, Pujol-Baxley C, Burgard A, Boldt J, et al. Metabolic engineering of Escherichia coli for direct production of 1,4-butanediol. Nat Chem Biol. 2011; 7(7):445–52. doi:10.1038/nchembio.580 PMID: 21602812
5. Juhas M. On the road to synthetic life: the minimal cell and genome-scale engineering. Crit Rev Biotechnol. 2015;1:–. doi:10.3109/07388551.2014.989423

6. Cunningham DS, Koepsel RR, Ataai MM, Domach MM. Factors affecting plasmid production in Escherichia coli from a resource allocation standpoint. Microb Cell Fact. 2009; 8:27. doi:10.1186/1475-2859-8-27 PMID: 19463175

7. St-Pierre F, Cui L, Priest DG, Endy D, Dodd IB, Shearwin KE. One-step cloning and chromosomal integration of DNA. ACS Synth Biol. 2013; 2(9):537–41. doi: 10.1021/sb400021j PMID: 24050148

8. Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, et al. Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol. 2006; 2:2006.0008. msb4100050 [pii] doi:10.1038/msb4100050 PMID: 16738554

9. Ublinskaya AA, Samsonov VV, Mashko SV, Stoynova NV. A PCR-free cloning method for the targeted φ80 Int-mediated integration of any long DNA fragment, bracketed with meganuclease recognition sites, into the Escherichia coli chromosome. J Microbiol Methods. 2012; 89(3):167–73. doi:10.1016/j.mimet.2012.03.013 PMID: 22484061

10. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A. 2000; 97(12):6640–5. doi:10.1073/pnas.120163297 PMID: 10829079

11. Sabri S, Steen JA, Bongers M, Nielsen IK, Vickers CE. Knock-in/Knock-out (KIKO) vectors for rapid integration of large DNA sequences, including whole metabolic pathways, onto the Escherichia coli chromosome at well-characterised loci. Microb Cell Fact. 2013; 12:60. doi:10.1186/1475-2859-12-60 PMID: 23799955

12. Juhas M, Evans LD, Frost J, Davenport PW, Yarkoni O, Fraser GM, et al. Escherichia coli Flagellar Genes as Target Sites for Integration and Expression of Genetic Circuits. PLoS One. 2014; 9(10): e111451. doi:10.1371/journal.pone.0111451 PMID: 25350000

13. Vora T, Hottes AK, Tavazoie S. Protein occupancy landscape of a bacterial genome. Mol Cell. 2009; 35(2):247–53. doi:10.1016/j.molcel.2009.06.035 PMID: 19647521

14. Kahramanoglou C, Seshasayee AS, Prieto AI, Ibberson D, Schmidt S, Zimmermann J, et al. Direct and indirect effects of H-NS and Fis on global gene expression control in Escherichia coli. Nucleic Acids Res. 2011; 39(6):2073–91. doi:10.1093/nar/gkq934 PMID: 21097887

15. Mieschendahl M, Müller-Hill B. F’-coded, temperature-sensitive lambda cI857 repressor gene for easy construction and regulation of lambda promoter-dependent expression systems. J Bacteriol. 1985; 164(3):1366–9. PMID:2999084

16. Gibson D, Young L, Chuang R, Venter J, Hutchison Cr, Smith H. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods. 2009; 6(5):343–5. doi:10.1038/nmeth.1318 PMID: 19363495

17. Miller EM, Nickoloff JA. Escherichia coli electrotransformation. Methods Mol Biol. 1995; 47:105–13. doi:10.1385/0-89603-310-4:105 PMID: 7550724

18. Hanahan D, Jessse J, Bloom FR. Plasmid transformation of Escherichia coli and other bacteria. Methods Enzymol. 1991; 204:63–113. PMID: 1943786

19. Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 2002; 30(9):e36. PMID: 11972351

20. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 1997; 25(17):3389–402. Epub 1997/09/01. doi: gka562 [pii] PMID: 9254694

21. Matsunami H, Samatey FA, Nagashima S, Imada K, Namba K. Crystallization and preliminary X-ray analysis of FlgA, a periplasmic protein essential for flagellar P-ring assembly. Acta Crystallogr Sect F Struct Biol Cryst Commun. 2012; 68(Pt 3):310–3. doi: 10.1107/S1744309112001327 PMID: 22442230

22. Hizukuri Y, Yakushi T, Kawagishi I, Homma M. Role of the intramolecular disulfide bond in FlgI, the flagellar P-ring component of Escherichia coli. J Bacteriol. 2006; 188(12):4190–7. doi:10.1128/JB.01896-05 PMID: 16740925

23. Hizukuri Y, Kojima S, Yakushi T, Kawagishi I, Homma M. Systematic Cys mutagenesis of FlgI, the flagellar P-ring component of Escherichia coli. Microbiology. 2008; 154(Pt 3):810–7. doi: 10.1099/mic.0.2007/013854-0 PMID: 18310027

24. Hizukuri Y, Kojima S, Homma M. Disulphide cross-linking between the stator and the bearing components in the bacterial flagellar motor. J Biochem. 2010; 148(3):309–18. doi: 10.1093/jb/mvq067 PMID: 20576620
25. Saijo-Hamano Y, Uchida N, Namba K, Oosawa K. In vitro characterization of FlgB, FlgC, FlgF, FlgG, and FliE, flagellar basal body proteins of Salmonella. J Mol Biol. 2004; 339(2):423–35. doi:10.1016/j.jmb.2004.03.070 PMID: 15136044

26. Herlihey FA, Moynihan PJ, Clarke AJ. The Essential Protein for Bacterial Flagella Formation FlgJ Functions as a β-N-Acetylglucosaminidase. J Biol Chem. 2014; 289(45):31029–42. doi:10.1074/jbc.M114.603944 PMID: 25248745

27. Juhas M, Stark M, von Mering C, Lumjiaktase P, Crook DW, Valvano MA, et al. High confidence prediction of essential genes in Burkholderia cenocepacia. PLoS One. 2012; 7(6):e40064. doi:10.1371/journal.pone.0040064 PMID: 22768221

28. Juhas M, Eberl L, Glass JI. Essence of life: essential genes of minimal genomes. Trends Cell Biol. 2011; 21(10):562–8. doi:10.1016/j.tcb.2011.07.005 PMID: 21889892

29. Juhas M, Eberl L, Church GM. Essential genes as antimicrobial targets and cornerstones of synthetic biology. Trends Biotechnol. 2012; 30(11):601–8. doi:10.1016/j.tibtech.2012.08.002 PMID: 22951051

30. Minty JJ, Lesnafsky AA, Lin F, Chen Y, Zaroff TA, Veloso AB, et al. Evolution combined with genomic study elucidates genetic bases of isobutanol tolerance in Escherichia coli. Microb Cell Fact. 2011; 10:18. doi:10.1186/1475-2859-10-18 PMID: 21435272

31. Jandu N, Ho NK, Donato KA, Karmali MA, Mascarenhas M, Duffy SP, et al. Enterohemorrhagic Escherichia coli O157:H7 gene expression profiling in response to growth in the presence of host epithelia. PLoS One. 2009; 4(3):e4889. doi:10.1371/journal.pone.0004889 PMID: 19293938

32. Hayashi K, Morooka N, Yamamoto Y, Fujita K, Isono K, Choi S, et al. Highly accurate genome sequences of Escherichia coli K-12 strains MG1655 and W3110. Mol Syst Biol. 2006; 2:2006.0007. doi:10.1038/msb4100049 PMID: 16738553

33. Murphy KC, Campbellone KG. Lambda Red-mediated recombinogenic engineering of enterohemorrhagic and enteropathogenic E. coli. BMC Mol Biol. 2003; 4:11. doi:10.1186/1471-2199-4-11 PMID: 14672541