

## **Supplementary Note**

### **Real-Time PCR measuring mRNA levels of *fliA* and *fliY* in different strains**

#### **Experimental procedure:**

Total RNA was extracted from three different *E. coli* strains: MG1655, RP437, and RP437  $\Delta$ *flhDC*<sup>1,2</sup>. The strains were grown in M9 minimal medium (Sigma) supplemented with 0.2% casamino and 0.4% glucose. When cultures reached mid-exponential growth phase (OD ~ 0.4), RNA protect reagent (Qiagen) was added, and then total RNA was extracted (RNeasy Mini-kit, Qiagen). mRNA was reverse transcribed to cDNA using SuperScriptII reverse transcriptase (Invitrogen). Real time PCR was then used to quantify mRNA levels (ABI PRISM, Applied Biosystems). The primers used for the real time PCR were: for *fliA*, 5' CGCCACTCATCGTAGGAGAAGA and 5' CCAAATGTTGCTCGACACCAAT; for *fliY*, 5' CCGGTTAATTTGCCGTCATCT and 5' AGGAACTTATCCGCCGTTTCAG and for *rpsB* 5' GCTGACCACGAACACATTGCT and 5' CCGGAATACCCAGGTTGTTTG.

All mRNA measurements were normalized according to *rpsB* mRNA levels which were found to be constant in the three strains.

#### **Results :**

In *E. coli*, *fliAZY* operon is known to be activated by *flhDC*<sup>3-5</sup>. In a wild type RP437 strain, we found that both *fliY* and *fliA* showed significant mRNA expression levels. In

RP437  $\Delta flhDC$  strain, in which  $flhDC$  is deleted<sup>1,2</sup>,  $fliA$  is barely detectable, whereas  $fliY$  is expressed in considerable amounts (25% of the wild type RP437 strain, supplementary figure 3). In *E. coli* MG1655 strain, which is defective in its  $flhDC$  expression,  $fliY$  mRNA levels were significantly higher than  $fliA$  mRNA levels (60% of  $fliY$  and ~5% of  $fliA$  relative to  $rpsB$  mRNA levels).

### **Construction of mutated $fliY$ reporter strain**

To generate a mutant  $fliY$  reporter strain (Supplementary figure 4), in which two specific base pairs in the putative  $\sigma_S$  site are changed, we used the QuickChange Site directed Mutagenesis Kit (Stratagene). Two primers were synthesized: 5' GTCTGTTTGCAGTGGGGAGCCATTGTTTGTAACAC and 5'GTGTTTACAAACAATGGCTCCCCACTGCAAACAGAC, and used in a PCR reaction to generate the mutated vector according to Stratagene protocol. The two point mutations were confirmed by sequencing of the resulting vector.

### **Expression profiles of wild type and mutant $fliY$ reporter strains:**

The two reporter strains (Wild type and mutant  $fliY$ ) were inoculated from frozen stocks into M9 Medium + kanamycin 25 $\mu$ g/ml with casamino 0.2% and glycerol 0.5%. Following 16 hours of growth 37°C/ 250rpm the strains were diluted 1:100 into M9 medium + kanamycin 25  $\mu$ g/ml supplemented with either (i) - Glucose 0.5%. (ii) - 0.5% glucose + 50 mM NaCl. (iii) - 0.5% glucose + 50mM Na-Nitrate, or (iv) - Glycerol 0.5%. The diluted strains were grown in 96-well plates which were inserted into Wallac automated fluorimeters.

Fluorescence and absorbance were measured every 5 minutes. The expression profile, denoted by GFP per OD is shown in supplementary figure 4b-e. In the tested conditions, the expression profile of wild type and mutant *fliY* reporter strains was found to be similar, except that the expression levels of the wild type were 4-5 times higher than the expression level of the mutant *fliY*. These results suggest that the putative  $\sigma_S$  consensus site located 31 bp upstream of the translation start site plays an important role in transcription of the *fliY* internal promoter.

## **Real-Time PCR measuring mRNA levels of *lacZ* and *lacY* in different strains and various conditions**

### **Experimental procedure:**

Total RNA was extracted from different *E. coli* strains grown under different conditions as described in supplementary figure 5a. WT (Wild type MG1655), Mut1 (a mutant MG1655 defective in *lacZ* expression<sup>6</sup>), and Mut2 (BW25113 *E. coli* strain known to be a *lacZ* mutant<sup>7</sup>). In addition, MG1655 transformed with pREP4 (in which LacI is constitutively expressed) was also included in the study. The strains were grown in minimal medium (M9, Sigma) supplemented with 0.2% casamino and glycerol 0.2%. IPTG (1 $\mu$ M) was added to selected growth media. When cultures reached mid-exponential growth phase (OD ~ 0.4), RNA was first stabilized using bacterial RNA protect reagent (Qiagen), and then total RNA was extracted (RNeasy Mini-kit, Qiagen). The mRNA was reverse transcribed to cDNA using SuperScriptII reverse transcriptase (Invitrogen). Real time PCR was then used to quantify mRNA levels (ABI PRISM, Applied Biosystems). The primers used for the real time PCR were: for *lacZ*, 5' CGCTGACGGAAGCAAAACA and 5' GCCCGGATAAACGGAAGT; for *lacY*, 5' TGCCACGGTTGCCAATG and 5' TTAAGGCTAAATGCCGAATGGT. All mRNA measurements were normalized according to *rpsB* mRNA levels which were found to be constant in the three strains. *rpsB* primers were the same as mentioned above for the *fliA* and *fliY* mRNA measurements.

## **Results :**

Real-time PCR results measuring *lacZ* and *lacY* mRNA levels are summarized in supplementary figure 5.

We found that *lacY* is expressed in strains where *lacZ* activity is impaired. For example, a mutant MG1655 strain, which is defective in its *lacZ* expression, was found to have considerable *lacY* mRNA levels (similar to the WT MG1655), although *lacZ* mRNA levels were 10 times lower. Addition of IPTG did not increase the mRNA levels of both *lacZ* and *lacY* mRNA levels (in the mutant), while mRNA of both genes was significantly increased in the WT MG1655 (as expected). This implies that *lacY* is transcriptionally independent of *lacI* repressor. The same observations were found when we used another strain, BW25113, also impaired in LacZ production<sup>7</sup>. These results support the existence of an internal promoter in the intergenic region between *lacZ* and *lacY*.

### **Expression profiles of *lacZ* and *lacY* reporter strains:**

*lacZ* and *lacY* reporter strains were inoculated from frozen stocks into M9 Medium +kanamycin 25 µg/ml with casamino 0.2% and glycerol 0.5%. Following 16 hours of growth at 37°C/ 250rpm strains were diluted 1:100 into M9 medium + kanamycin 25 µg/ml supplemented with either lactose 0.5% alone, or with glucose 0.5% and lactose 0.5%. Cultures were grown in 96-well plates which were put in the Wallac<sup>2</sup> automated fluorimeters.

Fluorescence and absorbance were measured every 5 minutes. The expression profile, denoted by GFP per OD is given in supplementary figure 5b-c for the two mediums. We found that *lacZ* expression was induced after 5 hours at the time of diauxic shift

(supplementary figure 5b). In conditions where lactose was the sole carbon, expression of *lacZ* was induced immediately at the beginning of the growth (supplementary figure 5c). In contrast, *lacY* expression was much lower and did not depend on the presence of lactose, or on glucose depletion from the medium. Note that in figures 5b and 5c, *lacY* GFP-fusion expression is measured only from the intergenic promoter region between 3' *lacZ* and 5' *lacY*. In contrast, *lacY* mRNA levels measured by RT-PCR reflect the summed transcription driven by upstream *lacZ* promoter (which is induced by lactose) and the transcription initiated from the intergenic region between 3' *lacZ* and 5' *lacY*. The data suggests that the intergenic promoter between 3' *lacZ* and 5' *lacY* is not induced by lactose.

## **Growth in automated fluorimeter**

***Diauxic shift experiment:*** The reporter strains were directly inoculated from frozen stocks to a medium containing M9 salts with glucose 0.4%, casamino 0.2% and kanamycin 25µg/ml. The strains were grown in high brim 96-well plates at 37°C for 16h, and then diluted 1:100 into minimal M9 medium supplemented with 0.1% lactose and 0.03% glucose to a final volume of 150µl per well in flat bottom 96-well plates (Nunc). Note that glucose is depleted during the first phase of growth, as indicated by experiments under very similar conditions<sup>8,9</sup>. A 100µl layer of mineral oil was added on top of each well to avoid evaporation. The oil layer was found not to significantly affect growth rate, and did not cause high expression of anaerobic promoters<sup>10-12</sup>. The cells were grown in a Wallac Victor<sup>2</sup> multi-well fluorimeter (1420 multilabel counter model, PerkinElmer). Time between repeated measurements was 7 minutes and orbital shaking was applied during the measurement intervals. OD was measured in 600nm and fluorescence emission in 535nm. The temperature in the multi-well fluorimeter was set to 30°C. We find that OD and GFP measurements were linear throughout the experiment. This was assayed by diluting cultures and noting the change in GFP and OD. We repeated the experiment for several 96-well plates and found a ~10% standard error between replicate experiments. This is in agreement with our previous experiments<sup>12</sup>. In addition, we find similar dynamics of growth and expression for micro-titer plates and in flasks<sup>6</sup>.

***Measurement of gene expression for internal promoters:*** The reporter strains were directly inoculated from frozen stocks and grown ~16h in medium containing glucose 0.4%, casamino 0.2%, and kanamycin 25µg/ml at 37°C in a high-brim 96-well plates.

Next, the strains were diluted 1:100 into 96-well plates (Nunc) containing minimal medium with either 0.4% arabinose, 0.4% succinate, 0.4% glucose or 0.2% casamino to a final volume of 150 $\mu$ l. The plates were covered with oxygen permeable film (Greiner), and incubated in a shaker (250rpm, 37°C). After 16h of growth the plates were assayed in a multi-well fluorimeter for OD and GFP.

### **Analysis of gene expression profiles**

The raw data of GFP and OD measurements was background subtracted. First, OD data points of a well containing medium with no cells were subtracted from the OD data points of the reporter strains. Next, The GFP data points of the promoterless plasmids, pUA66 and pUA139, were averaged and subtracted from the GFP data points of the different reporter strains. Each background GFP value was subtracted from the reporter strain GFP value at the same OD (and not necessarily at the same time point).

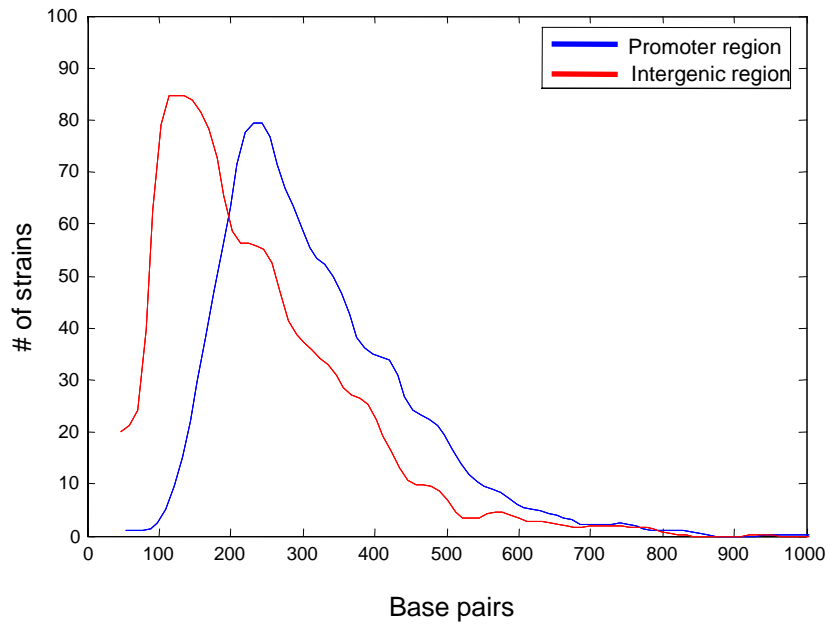
To analyze the expression profiles, OD and GFP curves were time shifted so that all OD curves reached 20% of their maximal OD at the same time point. Expression profiles were calculated by dividing GFP by OD, and promoter activity by taking the time derivative of GFP divided by OD ( $dGFP/dt / OD$ ). Normalized promoter activity was obtained by dividing promoter activity of each reporter strain by its maximal promoter activity value (Fig 2d-h). We then produced scaled expression values (GFP/OD and  $dGFP/dt/OD$ ) as follows: We normalized the OD by dividing it to its maximal value across all time points. Next, for each normalized OD range we averaged the expression values at the time-points at which the strain displayed this OD (Fig 1c). All analysis steps



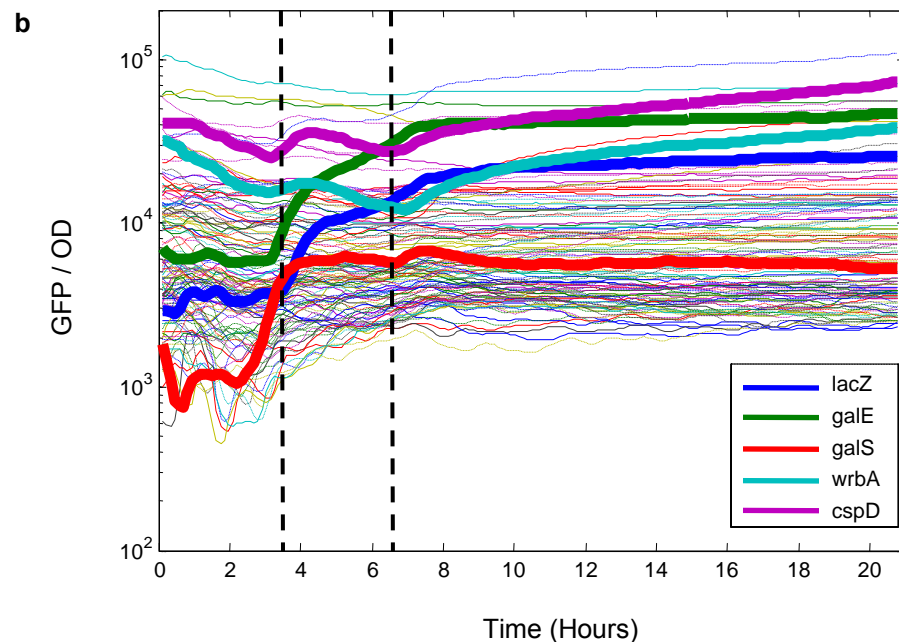
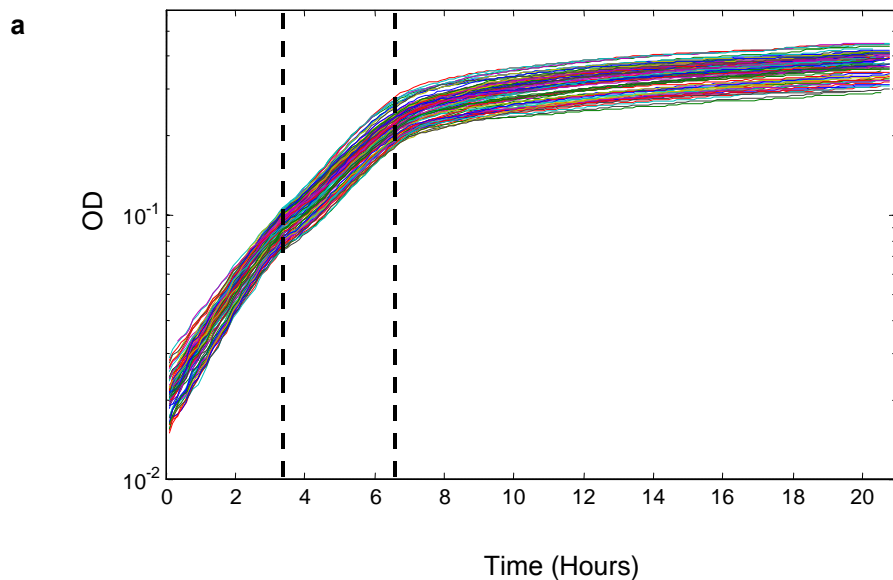
were performed automatically using custom Matlab software. A Matlab hierarchical clustering algorithm (Euclidean distances, average linkage) was applied to cluster the normalized promoter activities shown in Figure 1c.

## References:

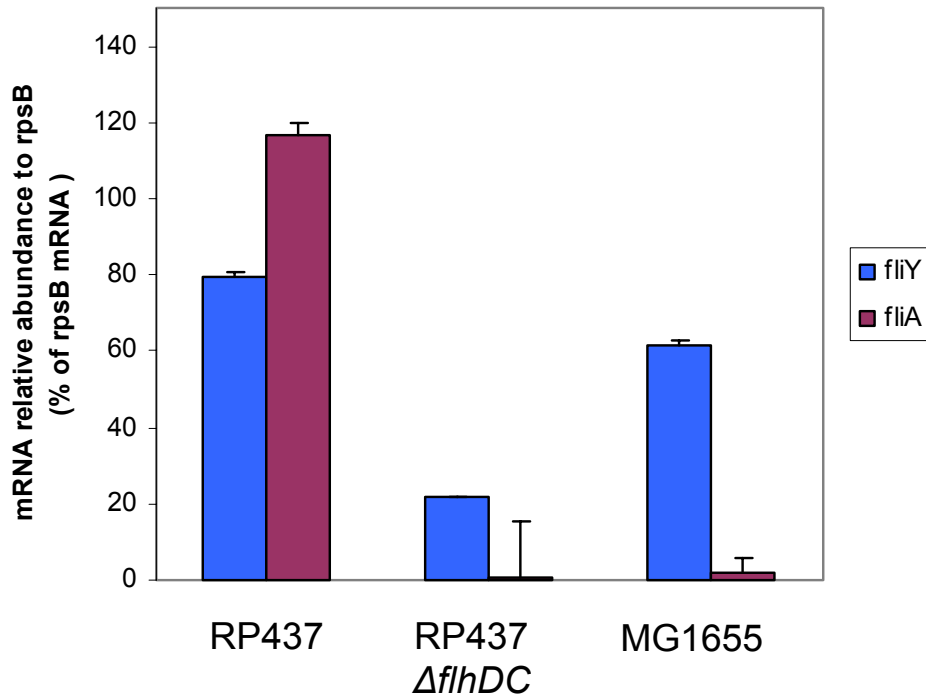
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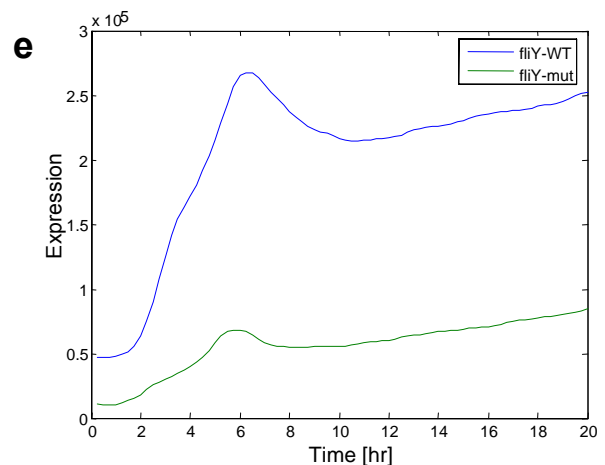
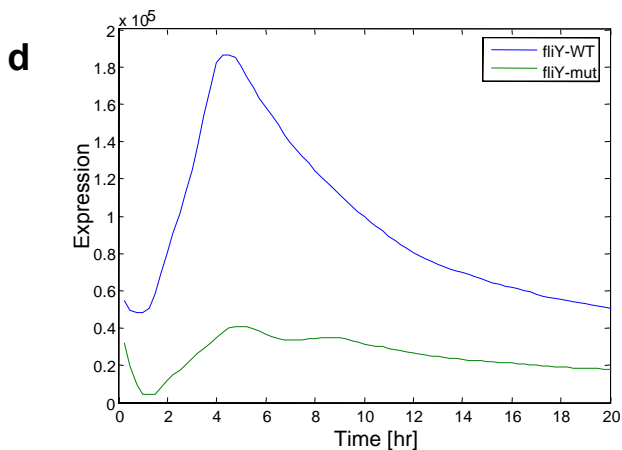
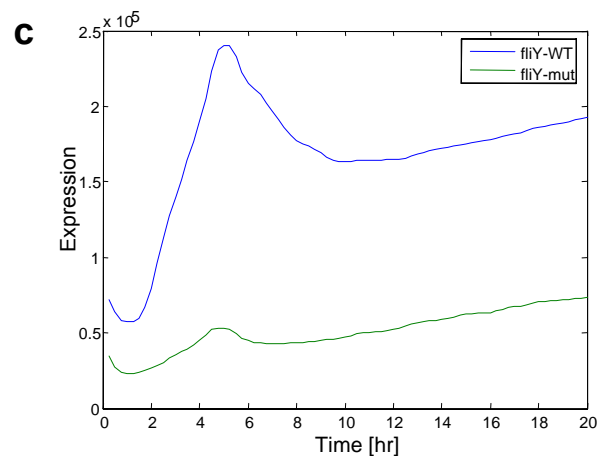
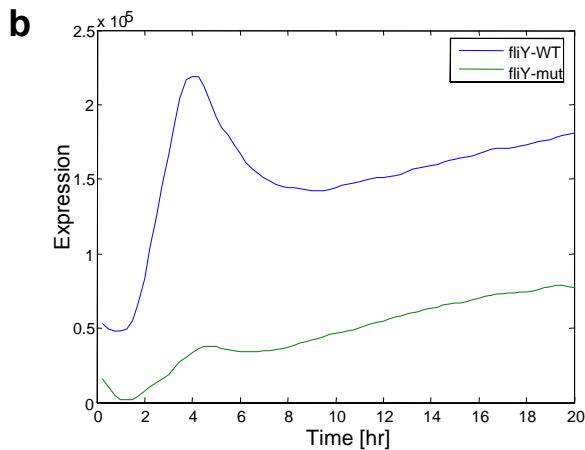
**Supplementary Figure 1:** Distribution of intergenic regions length in *E. coli*, and the distribution of the length of the promoter regions used to construct the library.



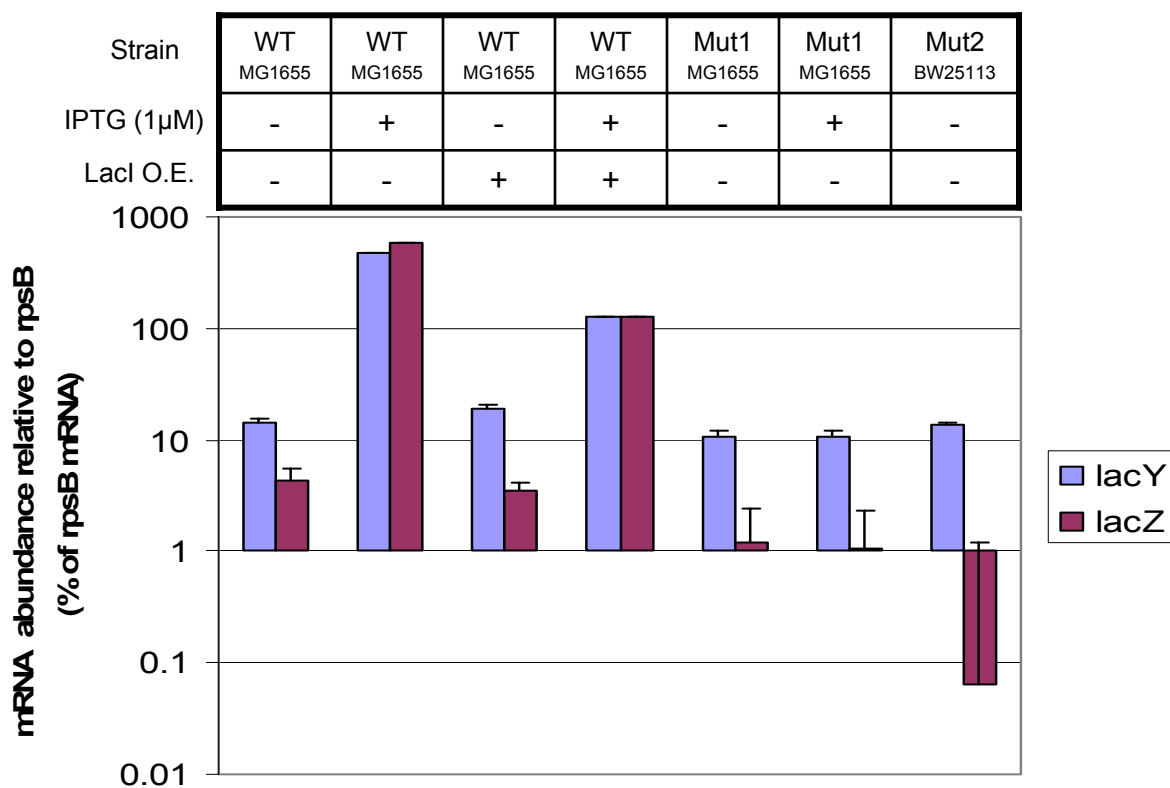
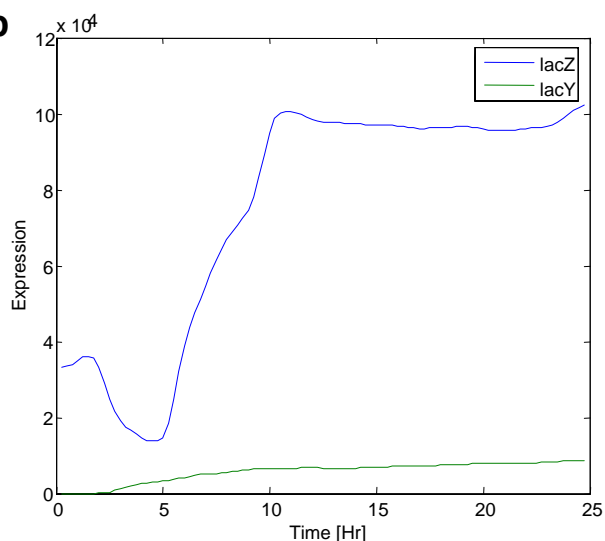
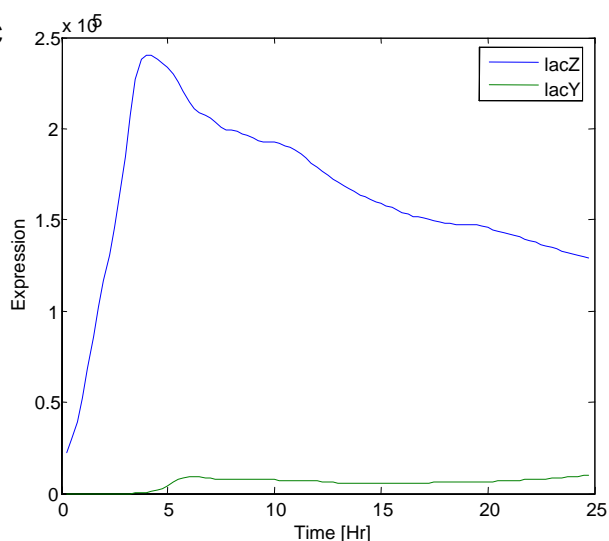
**Supplementary Figure 2: Representative 100 strains measured during a diauxic shift experiment. (a) OD measurements (b) GFP divided by OD (GFP/OD) levels. *lacZ*, *galE* and *galS* reporter strains are highlighted to demonstrate the up-regulation of these genes at the time of the diauxic shift phase. *wrbA* and *cspD* reporter strains known to be stationary phase genes are also highlighted demonstrating their increased expression during the diauxic shift and stationary phases. The vertical dashed lines indicate the time of diauxic shift (~3.5 h), and the entry to stationary phase (~6.5 h).**



**Supplementary Figure 3: mRNA levels of *fliY* and *fliA* as measured by real-time PCR.** Three *E. coli* strains were measured for their endogenous *fliY* and *fliA* mRNA levels: RP437, RP437  $\Delta$ *flhDC* and MG1655. The mRNA levels were normalized by dividing to mRNA levels of *rpsB*, which were found to be constant in the three strains. Error bars represent the standard error of the mean based on two experiments.

**a** $\sigma_S$  consensus**CTATACT***fliY* WT promoter**CTACACT**---31bp---**ATGAAATTA***fliY* mutated promoter**CCCCACT**---31bp---**ATGAAATTA**

**Supplementary Figure 4: Expression profiles of wild type and mutant *fliY* reporter strains.** (a) shown are the sequence of the putative  $\sigma_S$  regulatory region which is located in the promoter region of *fliY*. The mutant *fliY* reporter strain was generated by substituting TA for CC. Note the high homology between the wild type region and the  $\sigma_S$  consensus region, differing by only a single base-pair. (b)-(e) Expression profiles of the wild type and mutant *fliY* reporter strains when grown on different media: M9 minimal medium supplemented with (b) Glucose 0.5%. (c) - 0.5% glucose + 50 mM NaCl. (d) - 0.5% glucose + 50mM Na-Citrate, and (e) - Glycerol 0.5%.

**a****b****c**

### Supplementary Figure 5: Real-time PCR and expression profiles of *lacY* and *lacZ*

**(a)** mRNA levels of *lacZ* and *lacY* as measured by real-time PCR: The strains which were used are wild-type MG1655, and two LacZ defective mutants: mutant MG1655, and BW25113. MG1655 was also transformed with pREP4 vector, providing constitutive *lacI* over expression (O.E). Shown are the mean of 3 experiments. Note the logarithmic scale of the vertical axis. **(b)-(c)** Expression profiles (GFP/OD) of *lacZ* and *lacY* reporter strains in two mediums: M9 minimal media supplemented with **(b)** Glucose 0.5% + lactose 0.5% (diauxic shift is observed after 5 hours), and **(c)** Lactose 0.5%.