A comprehensive library of fluorescent transcriptional reporters for *Escherichia coli*

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***E. coli* is widely used for systems biology research; there exists a need, however, for tools that can be used to accurately and comprehensively measure expression dynamics in individual living cells. To address this we present a library of transcriptional fusions of *gfp* to each of about 2,000 different promoters in *E. coli* K12, covering the great majority of the promoters in the organism. Each promoter fusion is expressed from a low-copy plasmid. We demonstrate that this library can be used to obtain highly accurate dynamic measurements of promoter activity on a genomic scale, in a glucose-lactose diauxic shift experiment. The library allowed detection of about 80 previously uncharacterized transcription units in *E. coli*, including putative internal promoters within previously known operons, such as the *lac* operon. This library can serve as a tool for accurate, high-resolution analysis of transcription networks in living *E. coli* cells.**

Use of *E. coli* as a model organism for systems biology research requires new tools that can provide quantitative blueprints of gene circuitry[1]. One current approach, DNA microarrays, provides comprehensive snapshots of the concentrations of mRNAs in the cell[2]. Microarrays have been used to explain the responses of *E. coli* to many perturbations (reviewed in ref. 3). As microarrays require extraction of mRNA at levels sufficient for hybridization and detection, they cannot be used to easily assay individual cells. Furthermore, time-course analysis requires one microarray experiment per time point, limiting the feasibility of obtaining very high-resolution time courses for multiple conditions.

Another approach is to measure the transcriptional activity of promoters by means of reporter genes. Classic studies used the *lacZ* reporter, and in the last decade GFP has become widely used[4]. An important advance is the development of fast-folding GFP variants, which become fluorescent within minutes after transcription initiation in *E. coli*[5]. These reporters allow high-temporal-resolution measurement of promoter activity in living cells. Promoter activation is easily detected by increased fluorescent signal. Deactivation of promoters can be easily detected by a decrease in the accumulation rate of fluorescence, monitored by differentiating the fluorescent signal with respect to time.

Recent studies have used GFP as a reporter fused to selected promoters to discover detailed temporal expression programs in systems such as DNA repair[6,7], amino-acid biosynthesis pathways[8] and the flagella regulatory circuit[9–11]. GFP reporter strains are also useful as biosensors within ecological environments[12].

Use of selected reporter strains also has enabled the detailed study of the functions of recurring gene circuits called network motifs[1,13]. For example, the function of the feed-forward loop gene circuit has been studied in *E. coli*, to demonstrate its temporal filtering function[10,14] and its response-acceleration function[15]. Reporter strains also have been used to map in detail the *cis*-regulatory input functions of selected promoters[16,17].

In addition to measurements on cell populations, fluorescent reporter strains can be used to assay promoter activity in individual living cells. This has been demonstrated for example in the SOS system[7] where this technology uncovered oscillations in SOS gene expression in response to DNA damage. In short, reporter fusions allow highly accurate measurements of the input-output relationship of gene circuits in living cells, which is very useful in developing quantitative models of these systems.

To allow such studies for virtually any *E. coli* gene system, we present a new resource for *E. coli*, a library of fluorescent transcription fusions that includes over 75% of the promoters of the organism. We demonstrate that the library can provide high-resolution dynamic measurements of promoter activity on a genomic scale. Use of this library allowed us to detect about 80 new putative transcription units in *E. coli*, including internal promoters within previously known operons.

RESULTS

A comprehensive library of fluorescent reporter *E. coli* strains

To construct the library we used a procedure that allowed many of the cloning steps to be performed in parallel in 96-well plates (**Fig. 1a**). First, all intergenic regions larger than 40 base pairs (bp) were identified, based on the open reading frame annotation of the sequenced genome of *E. coli* K12 MG1655 strain[18]. Primers were

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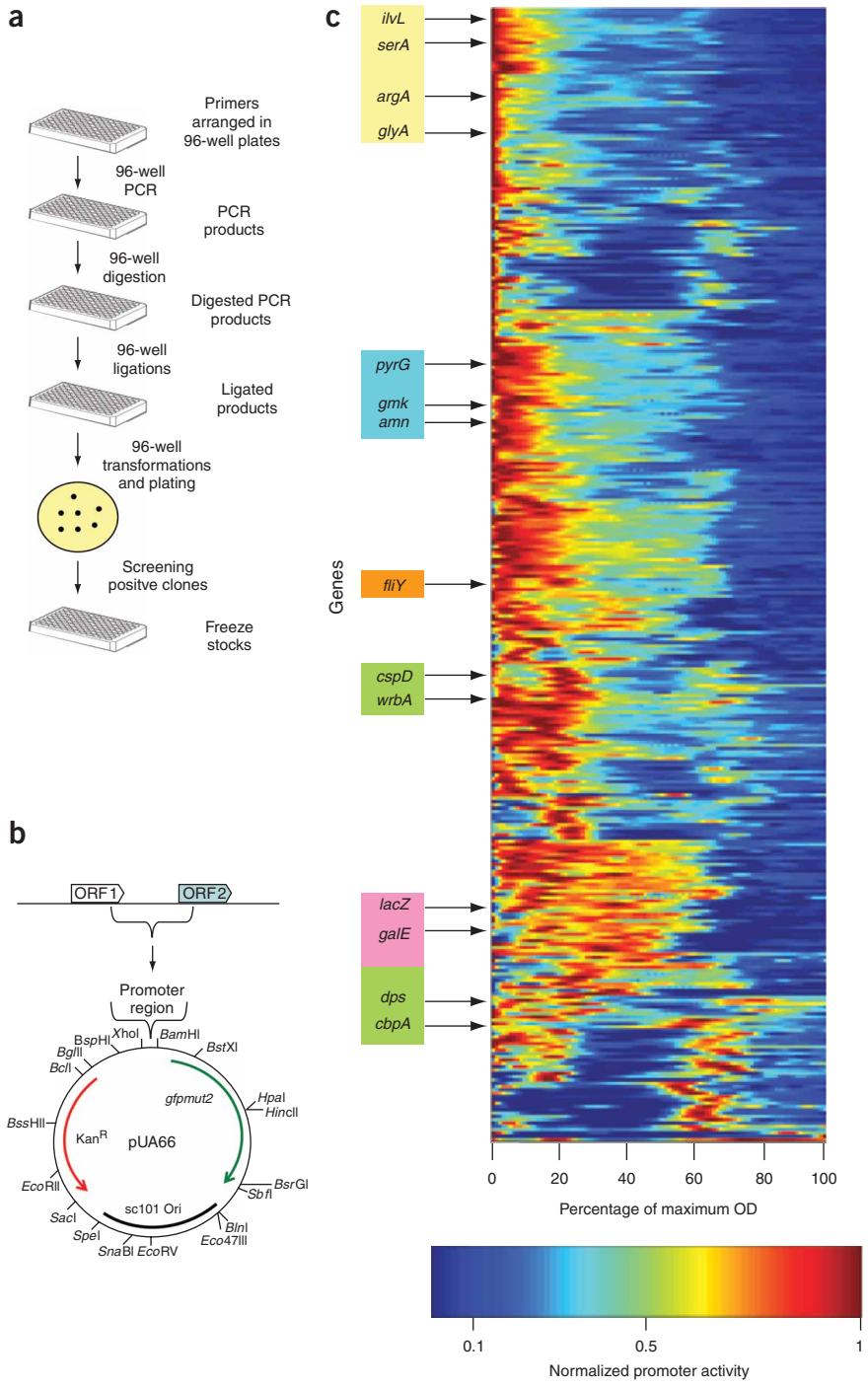

Figure 1 | High-throughput cloning strategy used for the construction of the reporter-strain library, and promoter activity of the reporter strains during a diauxic shift experiment. (**a**) The cloning steps to construct the library were carried out in 96-well plates. (**b**) A map of the low copy-number plasmid, pUA66, used to construct the library. The plasmid contains a kanamycin resistance gene and a fast-folding *gfpmut2* gene as a reporter gene with a strong ribosome binding site. The second plasmid used for the library construction, pUA139, is similar to pUA66, except that the restriction sites *Bam*HI and *Xho*I are switched. (**c**) Promoter activity during growth in a diauxic shift condition. Shown are reporter strains showing detectable GFP signals above background levels. Promoter activity was determined by calculating dGFP/d*t* / OD. The normalized values of promoter activity are shown.

designed to include the entire intergenic region plus about 50–150 bp into each flanking coding region. This primer design was aimed at capturing most of the transcription-regulator binding sites around the promoter (**Fig. 1b** and **Supplementary Fig. 1** online).

We amplified the intergenic regions flanked by 50–150 bp into the adjacent coding regions, which we termed 'promoter regions'. We cloned the products into one of two reporter plasmids according to their orientation in the chromosome (**Fig. 1b**). The plasmids are low copy with a pSC101 origin and include *gfpmut2* with a strong ribosome binding site as the reporter gene. We used a cloning site upstream of *gfpmut2* to clone the promoter region, so that the cloned promoter controls the transcription of *gfpmut2*. The GFP variant used becomes fluorescent within less than 5 min of transcription initiation[11], and is highly stable and nontoxic in *E. coli*. As a result, the accumulation of GFP fluorescence in these strains serves as a reporter for the rate of transcription initiation from the promoter region. Note that in contrast, DNA microarrays report mRNA concentration, which is a balance of mRNA production and degradation processes.

We sequenced over 90% of the plasmids in the library to verify the insert identity. The fraction of strains with the wrong insert region or no insert was about 5%; we removed these erroneous constructs from the library. Thus, the identity of the promoter regions in the verified library is expected to be accurate in over 99% of the reporter strains. The resulting library includes 1,820 different promoter regions.

High-resolution expression dynamics during diauxic shift

We used the library to measure promoter activity on a genomic scale during growth in a diuaxic shift experiment[19,20]. In this classic experimental condition, cells grow on a medium with limited glucose (0.03%) and abundant lactose (0.1%). The bacteria first grow on glucose without using lactose. When glucose is expended, growth is slowed for about 30 min. The cells then resume growth using lactose. Finally the cells reach stationary phase in which growth stops.

We assayed the promoter activity of the different promoters in our library by growing cells in 96-well plates within a multiwell fluorimeter-photometer (denoted automated fluorimeter; for details see **Supplementary Note** online). The automated fluorimeter allowed growth with shaking and temperature control, and measured cell density (OD at 600 nm) and GFP fluorescence (535 nm) every 7 min over ~20 h of growth, resulting in 178

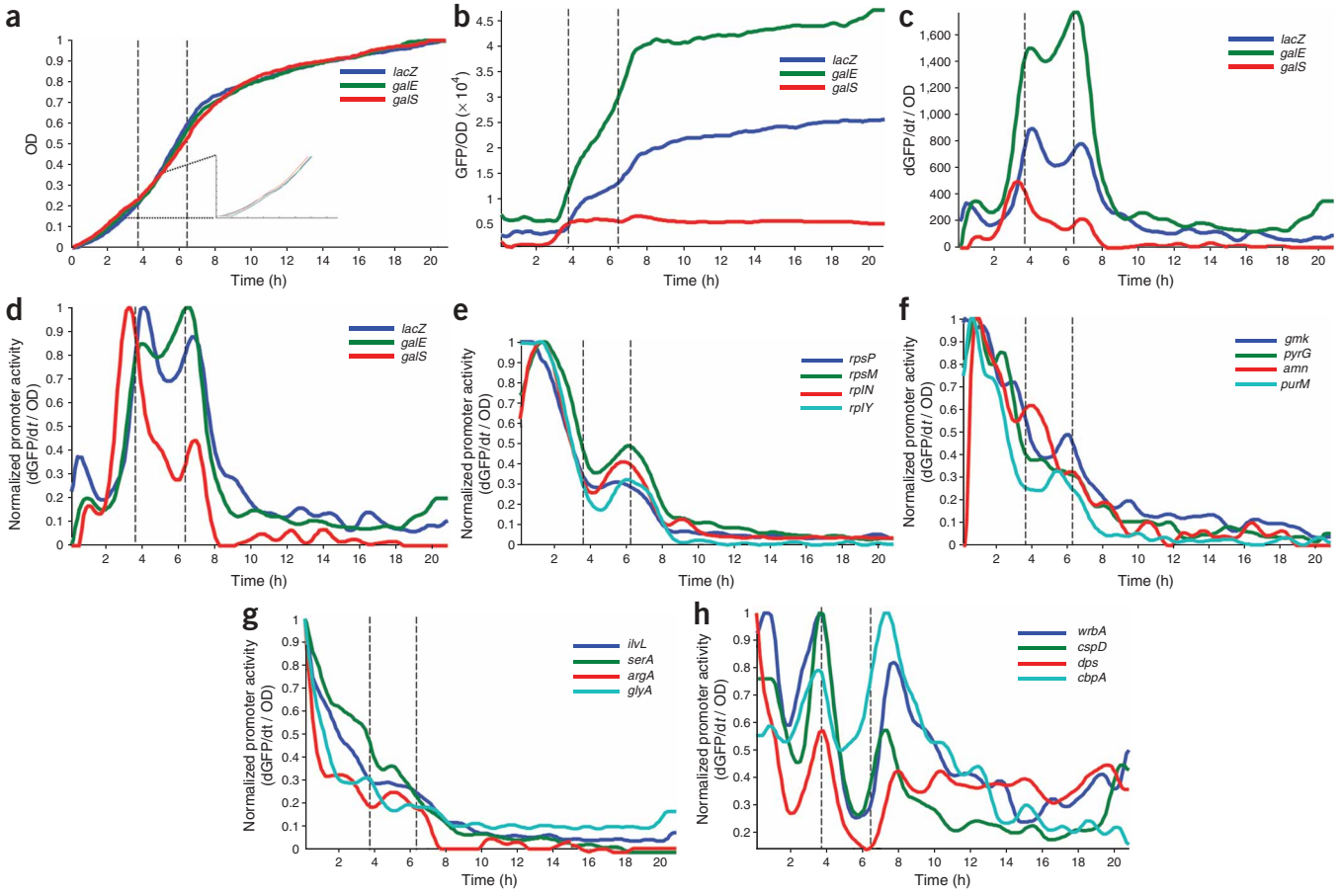

Figure 2 | Gene expression dynamic profiles of reporter strains from diverse systems as measured during the diauxic shift experiment. (**a**–**d**) Measurements of the three strains *lacZ, galS* and *galE* for OD normalized to the maximal level of each growth curve (**a**). Inset is a zoom-in of the diauxic shift time period (~3.5 h). GFP/OD (**b**). Promoter activity (dGFP/d*t* / OD; **c**). Promoter activity (dGFP/d*t* / OD) normalized by the maximal level (**d**). (**e**–**h**) Normalized promoter activities of representative reporter strains from different cellular systems: ribosomal protein genes (**e**), nucleotide biosynthesis genes (**f**), amino-acid biosynthesis genes (**g**) and stationary phase genes (**h**). The vertical dashed lines indicate the time of diauxic shift (~3.5 h) and time of entry into stationary phase (~6.5 h).

time points per reporter strain. The entire data set for 1,820 strains includes over 300,000 data points.

We assayed day-to-day reproducibility by repeating the experiment on two separate days. This repeat experiment shows mean day-to-day errors of about 10% (data not shown), in agreement with previous measurements on 80 strains from the library[8]. We found that the OD signal of the different reporter strains was very similar (**Supplementary Fig 2** online). After a short lag phase (not shown), the cells grew exponentially with a growth rate of 0.8 ± 0.15 doublings per hour. Then, at about 3.5 h of growth, the cells stopped growing for about 30 min (diauxic shift), and resumed exponential growth until they entered stationary phase at about 6 h. We allowed the cells to grow for an additional 14 h to include deeper stationary phase conditions.

About 60% of the reporter strains showed detectable fluorescence levels above background throughout all time points under this experimental condition. The temporal resolution of this experiment allows measurement of the promoter activity, defined as the rate of GFP production from the promoter region. This is given by the time derivative of the fluorescence signal, normalized by the OD, $\beta = \mathrm{dGFP}/\mathrm{d}t \,/\, \mathrm{OD}$, where GFP is the background-subtracted fluorescence signal and OD is the background-subtracted OD signal. **Figure 1c** shows the promoter activity of reporter strains which exhibited substantial expression levels during the diauxic shift experiment. The promoter activity profiles were clustered (see **Supplementary Note**). We found that many genes have a strong promoter activity during early and midexponential phase. A substantial number of promoters (~25%) show considerable activity at the transition between exponential and stationary phase[21].

Promoter activity measurements reveal detailed gene-expression dynamics. Here we report several features of the data; additional analysis will appear elsewhere. In the *lac* and *gal* sugar utilization systems, we found that promoter activity increases before the cells begin slow growth owing to the diauxic shift phase (time of upregulation is ~2 h, whereas cells begin slow growth at ~3.5 h; **Fig. 2a**). In fact, promoter activity reached its maximal levels at about the time that growth begins to slow down at 3.5 h. The present measurement thus suggests that the cells might anticipate the shift as glucose levels decrease but before growth stops.

Other gene systems, such as translation apparatus genes, nucleotide biosynthesis genes and amino acid biosynthesis genes, showed expression that was high in the rapid growth phases of the experiment (before and after the shift) and low in stationary

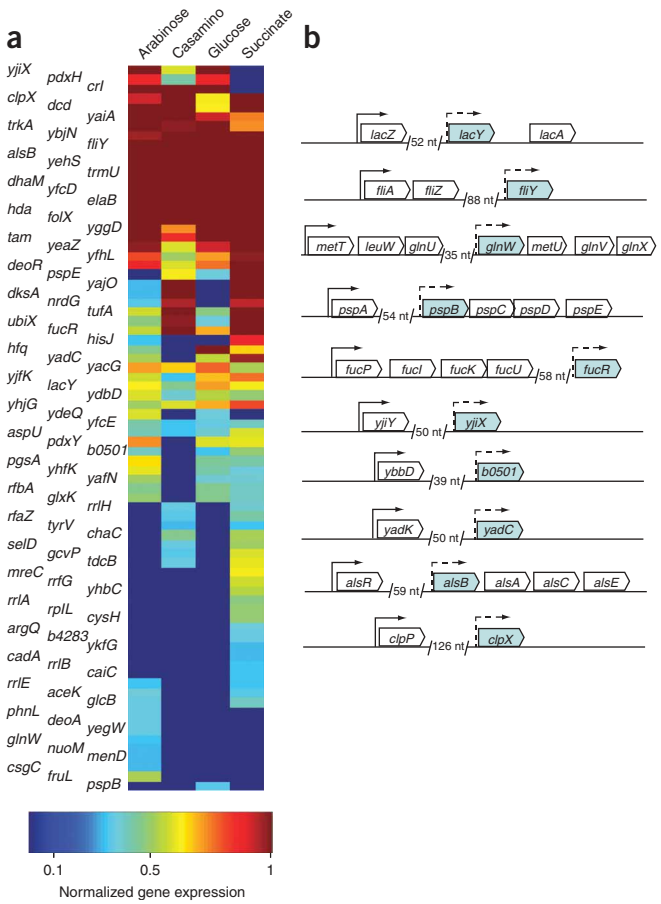

Figure 3 | Identification of previously uncharacterized promoters. (**a**) Expression levels of selected reporters for previously uncharacterized promoters. The reporter strains were grown in minimal medium supplemented with either 0.4% arabinose, 0.2% casamino acids, 0.4% glucose or 0.4% succinate for 16 h, and then assayed for OD and GFP levels. (**b**) Structure of operons (according to EcoCyc Version 9.0 database[22]), in which new internal promoter activity was found. Solid arrows indicate the known promoter of the operon, and dashed arrows indicate the location of the previously uncharacterized internal promoter (upstream of the genes highlighted in blue).

phase (**Fig. 2e–g**). Genes coding for ribosomal proteins showed a moderate increase in promoter activity at the second exponential growth (after the diauxic shift), which peaked at the time of entry to stationary phase (**Fig. 2e**). Other genes, such as stress genes and stationary phase genes, showed relatively high promoter activity in stationary phase (**Fig. 2h**). Many of these genes were also activated upon the transient growth arrest during diauxic shift, suggesting that this pause in growth exhibits stationary phase–like expression patterns[21]. This result agrees with a 17-timepoint microarray experiment under similar diauxic shift conditions[19].

Previously uncharacterized promoters in *E. coli*

The library also allowed detection of new putative promoters. The library was constructed systematically to include intergenic regions longer than 40 bp. As a result, the library includes 154 intergenic regions, which are not known to correspond to separate transcription units according to the EcoCyc Version 9.0 database[22]. We found that 78 of these previously uncharacterized promoter regions show detectable fluorescence in at least one out of the four conditions that we tested (**Fig. 3a**). The fluorescence was well above the background levels determined by use of a promoterless control vector. Ten of these correspond to regions between genes within a previously characterized operon (**Fig. 3b**). Many of the putative promoters were differentially active when grown on different media (**Fig. 3a**).

For example, we found strong promoter activity from a region of 88 bp between *fliZ* and *fliY* within the *fliAZY* operon (**Fig. 3b**), an operon related to flagella synthesis. This reporter plasmid is termed *fliY*. The expression of the *fliAZY* operon is known to be regulated by the flagella master regulator *flhDC* in a promoter upstream of *fliA*[23,24]. We used real-time PCR to measure endogenous mRNA levels of both *fliA* and *fliY*. We found that *fliY* mRNA is expressed in strains and conditions in which *fliA* mRNA is not (**Supplementary Fig. 3** online), supporting the existence of an internal promoter in this operon. Inspection of the 88-bp intergenic region revealed a short sequence (31 bp from start of translation), which differs only by one base from the σ_S consensus sequence (**Supplementary Fig. 4** online) that characterizes genes regulated under stress conditions. Two point mutations in this site on the reporter plasmid resulted in a fourfold reduction in expression level across different conditions when compared to the wild-type reporter plasmid (**Supplementary Fig. 4**). Such an internal *fliY* promoter was characterized in the *Salmonella typhimurium fliAZY* operon[25].

An additional example is detection of promoter activity in an internal region upstream of *lacY* within the *lacZYA* operon[20]. Real-time PCR confirmed the independent production of *lacY* mRNA in mutant strains, which are defective in their *lacZ* transcriptional activity (**Supplementary Fig. 5** online). Measurements of promoter activity of both *lacZ* and *lacY* under different conditions (**Fig. 3a** and **Supplementary Fig. 5**), revealed differential promoter activity dynamics. In contrast, we detected no transcriptional activity for a 66-bp region between *lacY* and *lacA* (data not shown). These results suggest that a previously unknown internal promoter may exist within the well-studied *lac* operon.

DISCUSSION

This study presents a library of fluorescent reporter strains in which GFP is used to assay promoter activity in *E. coli*. This library can be used to obtain accurate high-resolution dynamics of promoter activity in living cells. In addition, the library enables the identification of many new transcriptional regulatory regions, as well as internal promoters in well-characterized operons, such as the *lac* operon. Moreover, the fact that selected DNA regions on a plasmid can be easily mutated enables the identification of *cis*-regulatory sites in the promoter regions.

It is important to note that the new transcriptional units revealed in this study are based on GFP fusion constructs. Although we provided additional supporting data for two promoters, *fliY* and *lacY*, each of the new promoters reported herein should be studied by additional analysis. The new promoters should therefore be considered as putative promoters.

The reporter library might report for additional effects beyond the activity of individual promoters. Some of the fusions contain 5′ untranslated region (UTR) segments of the original operons. Therefore, the reported fluorescence may reflect some

post-transcriptional regulation (for example, if there are mRNA stability determinants in the 5′ UTR). Furthermore, the intergenic regions sometimes contain multiple promoters, and thus, the reported fluorescence reflects their combined effects.

It would be interesting to integrate our library with complementary genomic techniques in *E. coli*. The recently constructed knockout library of *E. coli* genes is a good example (http://ecoli.aist-nara.ac.jp/). Such studies will contribute to the goal of understanding *E. coli* on the systems level[22,26]. It would also be interesting to use the present high-throughput cloning approach to construct a library of translation fusions for *E. coli*. A library of fluorescent translational fusions was recently reported for the yeast *S. cerevisiae*[27].

The present library also allows genome-scale measurements of promoter activity in individual cells. High-throughput flow cytometry can be used to obtain measurements of cell-to-cell variability in gene expression[28]. High-throughput microscopy can also be used to assay dynamics in individual cells, as was demonstrated for the SOS DNA repair system[7]. Such studies can help us to understand the stochastic elements of the behavior of gene networks in individual cells[29,30]. The present approach can be used to construct libraries also for other bacteria.

We hope that the present resource will stimulate research aimed at understanding *E. coli* both on the level of individual systems and network motifs, and on the level of computation and regulation across the entire organism.

METHODS

Primer design. We systematically constructed the present library by amplifying each promoter region and fusing it to a GFP reporter, unlike random reporter insertion approaches. We designed primers to flank intergenic regions longer than 40 bp. The *E. coli* MG1655 K12 open reading frame (ORF) coordinates were based on the sequenced genome[18]. We designed the primers using the Primer3 software to amplify the regions between two adjacent ORFs with an extension of 50–100 bp into each of the two ORFs. If we found no suitable primers, we extended the amplified region to 300 bp into each of the ORFs. We designed each of the two primers with either *Xho*I or *Bam*HI restriction site–containing tails. In case the target genomic promoter region contained *Xho*I or *Bam*HI sites, we used other restriction enzymes that leave the same overhang, *Sal*I or *Bgl*II, respectively. We designed all the primers to have a melting temperature of 60 °C to allow high-throughput multiwell PCR.

High-throughput cloning strategy. The vectors used for the reporter strain library construction contain a reporter gene, *gfpmut2* (ref. 5). We used two versions of the vectors: pUA66 (**Fig. 1b**) and pUA139. pUA139 is the same as pUA66 except that the order of the two restriction sites, *Bam*HI and *Xho*I is reversed. We used pUA66 for cloning intergenic regions upstream of genes expressed from the plus strand, and pUA139 for cloning intergenic regions upstream of genes expressed from the minus strand. We PCR-amplified intergenic regions that lie between two open reading frames pointing in opposite directions and cloned them into both vectors. We purified the plasmids using the Qiagen Plasmid Maxi kit from MG1655 K12 cells, digested the plasmids with *Xho*I for 7 h at 37 °C, purified the DNA and digested with *Bam*HI for 5 h at 37 °C. We assessed the efficiency of the digested vectors by self ligation followed by transformation.

We arranged the primers (Sigma) in 96-well plates (**Fig. 1a**) and carried out 96-well PCR (Symport). We used *E. coli* K-12 MG1655 genomic DNA as template and the Expand High-Fidelity PCR system (Roche). PCR conditions were 95 °C for 10 min, then 24 cycles of 95 °C for 30 s, 60 °C for 1 min and 72 °C for 1 min, and a final step at 72 °C for 5 min. We verified product size on 96-lane 1% agarose gel. Next we purified PCR products using a 96-well PCR purification kit (Qiagen), and double digested the PCR products for 6 h at 37 °C with *Xho*I and *Bam*HI. In cases in which the restriction site of the primers was designed to be *Sal*I or *Bgl*II, we digested the products in two steps. All digestions steps were done in 96-well plates (Symport). We purified the digested PCR products using a 96-well PCR purification kit (Qiagen).

We ligated purified PCR products (T4 ligase; Roche) overnight at room temperature (18–25 °C) with the corresponding vectors in 96-well plates (**Fig. 1a**). Transformation was done in high-brim 96-well plates to $CaCl_2$ competent MG1655 cells. We plated the transformants on 9-cm LB-agar plates containing 25 μg/ml kanamycin. We screened for positive colonies by colony PCR in 96-well plates using primers designed for the upstream and the downstream region of the two restriction sites, *Bam*HI and *Xho*I. Primer 1, 5′-CCATTAACATCACCATCTAA-3′, and primer 2, 5′-CCAGCTGG CAATTCCGACGT-3′. We loaded the products on a 96-well agarose gel to verify the size of the corresponding inserts. We prepared frozen stocks (25% glycerol) of the reporter strains in 96-well plates. The reporter library can be obtained from Open Biosystems.

Additional methods. Descriptions of real-time PCR measurements, construction and expression of reporter strains, growth in the automated fluorimeter and analysis of gene expression profiles are available in the **Supplementary Note**.

URL. A complete list of reporter strains and data are available (http://www.weizmann.ac.il/mcb/UriAlon/index.html).

Note: Supplementary information is available on the Nature Methods website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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