

GreA and GreB enhance *Escherichia coli* RNA polymerase transcription rate in a reconstituted transcription-translation system

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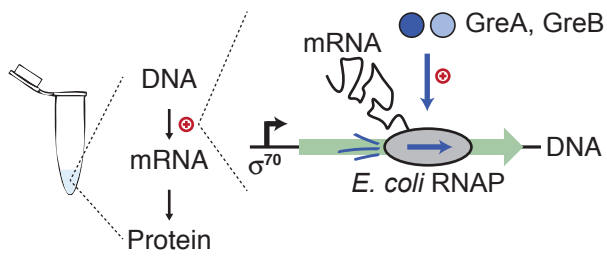
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Abstract:

Cell-free environments are becoming viable alternatives for implementing biological networks in synthetic biology. The reconstituted cell-free expression system (PURE) allows characterization of genetic networks under defined conditions but its applicability to native bacterial promoters and endogenous genetic networks is limited due to the poor transcription rate of *Escherichia coli* RNA polymerase in this minimal system. We found that transcription elongation factors GreA and GreB increased transcription rates of *E. coli* RNA polymerase from sigma factor 70 promoters up to 6-fold in an enhanced PURE system (ePURE). Furthermore, we reconstituted activation of natural *E. coli* promoters controlling flagella biosynthesis by the transcriptional activator FlhDC and sigma factor 28. Addition of GreA/GreB to the PURE system allows efficient expression from natural and synthetic *E. coli* promoters and characterization of their regulation in minimal and defined reaction conditions making the PURE system more broadly applicable to study genetic networks and bottom-up synthetic biology.

Keywords: *E. coli* RNA polymerase, transcription, cell-free protein synthesis, *E. coli* promoters, PURE system, bottom-up synthetic biology

Graphical abstract



Introduction

Diverse biological processes can be reconstituted and studied *in vitro* using purified proteins or lysates. This approach has facilitated fundamental discoveries in molecular biology and biochemistry, such as DNA replication¹ and translation of the genetic code². The cell-free approach allows construction of systems that would be difficult or impossible to develop *in vivo* and to perform measurements and experiments that are difficult to conduct in cells. Apart from basic research on the rules governing biological networks, applications of *in vitro* systems thus far have include biosensors and synthesis of biomolecules³⁻⁵. *E. coli* cell-lysate transcription and translation (TX-TL) systems have become popular to engineer and study genetic networks of increasing complexity⁶⁻¹⁰. Cell-lysate-based TX-TL systems produce high protein yields and allow transcription from native *E. coli* promoters¹¹. However, lysates have the disadvantage that they contain almost all the proteins and macromolecules present in the cytoplasm at the moment of lysis. For bottom-up synthetic biology and reconstitution studies this is not ideal because although simpler than a cell, the lysate still contains many unknown components and uncontrollable variables.

An appealing transcription and translation system, that is commercially available as PURExpress (PURE), is reconstituted from purified components and allows experiments under minimal and defined conditions¹². This reconstituted system contains T7 RNA polymerase (RNAP), purified ribosomes, all necessary translation factors from *E. coli*, tRNAs and enzymes for tRNA aminoacylation and energy regeneration, creatine phosphate as an energy source, and nucleotides and amino acids as precursors. Various genetic networks can be implemented in this system, which to date mostly relied on single subunit phage RNA polymerases for transcription¹³.

Transcription by the multisubunit *E. coli* RNA polymerase (EcRNAP) has been reconstituted in the PURE system and can be implemented by either adding the purified holoenzyme to the reaction mix or by co-expressing its subunits^{14,15}. While transcription rates of the EcRNAP in PURE depend on the concentrations of DNA template and EcRNAP, they are generally considerably lower than for phage polymerases. For example, we observed roughly an order of magnitude lower mRNA concentrations synthesized from a consensus sequence sigma factor 70 ($\sigma 70$) promoter by EcRNAP than by phage T3 RNAP¹³ under similar conditions. *In vivo* elongation rates of EcRNAP range between 28 and 89nt/s¹⁶ and are comparable to the values reported for T7 RNAP *in vitro*^{17,18}. Transcription elongation factors affect EcRNAP transcription elongation rate either by sensitizing or suppressing RNAP pausing. Elongating RNAP frequently backtracks along the DNA forming a transcriptionally

inactive state. Transcription elongation factors GreA and GreB bind to backtracked RNAP and catalyze the endonucleolytic cleavage of nascent RNA within the RNAP active site allowing transcription to continue¹⁹. GreA resolves smaller backtracking events by cleaving 2-3 nt from the 3' end of the RNA, whereas GreB can also rescue longer backtracked complexes²⁰. GreA and GreB are known to increase transcription elongation rate and stimulate promoter escape in a subset of promoters¹⁹⁻²², but GreA and GreB transcription elongation factors are not present in the PURE system.

Here we show that *E. coli* transcription elongation factors GreA and GreB enhance EcRNAP transcription rates in the PURE system up to 6-fold to reach the rate of T7 RNAP transcription in the system. We go on to show that an increase in transcription rates can be observed for several different synthetic $\sigma 70$ *E. coli* promoters. Furthermore, we used the enhanced system to study natural *E. coli* promoters involved in flagella biosynthesis and their activation by two different transcriptional activators *in vitro*, under defined conditions.

Results and discussion

EcRNAP can be added to the PURE system to allow transcription of DNA templates carrying *E. coli* promoters^{14,15} but mRNA synthesis and subsequent protein production is more efficient using phage polymerases such as T7 or T3 RNAP¹³. In bacterial cells multiple proteins can increase RNAP activity, which are not present in the minimal PURE system. The transcription elongation factors GreA and GreB from *E. coli* increase overall transcription elongation rates and stimulate promoter escape in a subset of promoters by re-activating backtracked elongation complexes¹⁹⁻²². We added transcription elongation factors GreA and GreB to a PURE reaction containing EcRNAP with $\sigma 70$ (holoenzyme) and a DNA template expressing EGFP under control of a consensus sequence *E. coli* $\sigma 70$ promoter and found that both proteins increased transcription rates (Figure 1). The transcription rate increase mediated by GreA and GreB followed hyperbolic kinetics and plateaued at concentrations above 5 μM for both GreA and GreB. At the plateau GreA and GreB increased transcription rates about 3-fold and final EGFP protein synthesized about 2-fold for the DNA template concentration tested (Fig. 1B, C). When GreA and GreB were added in combination, we did not observe a significant synergistic effect on mRNA or protein synthesis (Supplementary Fig. 1). We nonetheless used both proteins in an enhanced PURE (ePURE) reaction containing 10 μM of GreA and 10 μM of GreB in addition to the *E. coli* RNAP holoenzyme (0.2 μM EcRNAP, 1 μM $\sigma 70$) for all subsequent experiments.

By testing various DNA template concentrations we observed an up to 6-fold increase of transcription rate in ePURE compared to a PURE reaction supplemented with *E. coli* RNAP holoenzyme alone (Fig. 2A). Increased mRNA synthesis led to 3-fold higher final EGFP levels, and the advantage of using the ePURE reaction was strongest for lower DNA template concentrations (Fig. 2B). In the ePURE system we observed comparable mRNA and EGFP synthesis from an *E. coli* $\sigma 70$ promoter and a T7 RNAP promoter (Fig. 2). The ePURE improvement should thus facilitate running genetic networks based on *E. coli* RNAP transcription under defined conditions.

In order to determine if the ePURE system also increases the transcription rate for other promoters we tested the system on synthetic constitutive promoters from the registry of standard biological parts (<http://parts.igem.org>): J23101, J23102, J23106 and J23151, which are well-characterized *in vivo* and *in vitro*²³⁻²⁵, and compared two *E. coli* $\sigma 70$ consensus-sequence repressible promoters¹³ (Fig. 3). In the non-optimized PURE system transcription rates were below the detection limit for the constitutive promoters, whereas in the ePURE system transcription rates increased to measurable levels of up to 0.5 nM/min for J23151, the strongest promoter in the panel (Fig. 3A). The ePURE system significantly enhanced mRNA synthesis for the constitutive promoters except J23106, the weakest promoter we tested, where no measurable increase was observed. Both of the strong repressible promoters showed a ~6-fold increase of transcription rate in the presence of GreA and GreB (Fig. 3B). Final EGFP levels increased significantly for most promoters (Supplementary Fig. S2). Our results on the constitutive promoter panel compare well with relative promoter strengths measured in lysate-based TX-TL reactions^{24,25}.

We next tested whether the ePURE system would allow us to study an endogenous bacterial genetic network and chose to analyze transcriptional regulation of native *E. coli* flagellar promoters. Two main regulators, the FlhDC transcriptional activator and FliA, the flagellar sigma factor, $\sigma 28$, are thought to activate the genes in a tightly controlled temporal order^{26,27}. FlhDC is known as the master regulator and activates $\sigma 70$ -dependent transcription from class 2 promoters. One of the genes FlhDC activates is *fliA* coding for $\sigma 28$, which then activates itself and other genes in a positive autoregulation²⁸. Many of the more than 50 genes in the flagellar regulon, which are divided into at least 17 operons, are transcribed from multiple promoters, and can be activated by both FlhDC and $\sigma 28$ ^{26,29}. Their regulation has been studied extensively *in vivo* using genetics and promoter fusions^{26,27,29}. In a complementary approach, this complex regulatory system can also be studied outside of cells, under reaction conditions that eliminate unknown factors. In this fashion, activation of several

flagellar class 2 and class 3 promoters by FlhDC and $\sigma 28$ was shown by *in vitro* transcription experiments^{28,30,31} and binding of the FlhDC activator to putative promoters was demonstrated by *in vitro* binding assays³².

We analyzed eight flagellar promoters coupled to an EGFP reporter in the ePURE system and show their activation by FlhDC and $\sigma 28$ in the defined TX-TL system (Fig. 4A). We based our expectations on the EcoCyc *E. coli* database³³, which contains information on experimentally known and bioinformatically predicted transcriptional activation. To synthesize our DNA templates we fused 150 to 250 bp long promoter regions that contained the annotated $\sigma 70$ and $\sigma 28$ promoters as well as FlhDC binding sites to identical, strong ribosomal binding sites followed by the EGFP reporter gene. To test their activation, we separately pre-synthesized the FlhDC and the $\sigma 28$ activators and then added these to an ePURE reaction containing a DNA template with the respective flagellar promoters.

All eight promoters tested showed no detectable activity in the absence of FlhDC and $\sigma 28$. When the reaction contained either of the two activators, we observed the expected activation pattern with widely differing promoter strengths (Fig. 4A). Both activators in combination generally did not improve expression compared to only one activator. Most of the time the presence of both activators even led to decreased expression. We attribute this effect to competition between both activators for binding to DNA²⁸ and to the RNAP core enzyme, which binds $\sigma 28$ with a higher affinity than $\sigma 70$ ³¹. Absolute levels of the two activators might be lower in cells than in our assay, which could explain why promoters show additive activation *in vivo*²⁹. Out of eight studied promoters, two promoters, *fliE* and *flgK*, deviated from the annotated regulation pattern in EcoCyc. The *fliE* promoter was predicted to be activated by both $\sigma 28$ ³⁴ and FlhDC³² but we only detected low activation by FlhDC. For the *flgK* promoter only activation by $\sigma 28$ was annotated on EcoCyc and previously shown³¹. We observed strong activation by $\sigma 28$ but also a low but significant activation by FlhDC. A computational search for FlhDC did not identify a FlhDC binding site upstream of the *flgK* gene in *E. coli*³². In *Salmonella typhimurium*, however, transcription of *flgK* is activated by both $\sigma 70$ /FlhDC and $\sigma 28$ ³⁵. Additionally, our study provides experimental evidence for activation of several promoters by FlhDC and $\sigma 28$, which have previously only been predicted computationally, such as activation of *flgB*, *fliE* and *fliD* by FlhDC³² and *flgM* by $\sigma 28$ ³⁶. Thus, our analysis of flagellar promoters in defined conditions demonstrates that native gene activation mechanisms can be obtained using the ePURE system. The finding that FlhDC is a strong transcriptional activator for a number of different promoters should furthermore be useful for the assembly of *in vitro* genetic networks.

Using the genes for FlhDC and $\sigma 28$ and two promoters that showed activation by both activators, we built a synthetic gene network (Fig. 4B), which was implemented and characterized in a microfluidic nano-reactor device¹³. T7 RNAP transcribes the genes coding for FlhD and FlhC. FlhDC then activates $\sigma 70$ *E. coli* RNAP to express the Citrine reporter, which we placed under control of the *flgK* promoter. A control reaction, which did not contain the FlhD and FlhC DNA templates, again demonstrated activation of the *flgK* promoter by the FlhDC activator (Fig. 4C). Transiently we added DNA templates carrying the *fliA* gene ($\sigma 28$) and a Cerulean reporter gene, both under control of the *fliA* promoter, which leads to positive autoregulation of $\sigma 28$. $\sigma 28$ further activates both reporters leading to a fluorescence increase of Citrine and Cerulean in the presence of the *fliA* and Cerulean templates (Fig. 4C). This 5-gene network demonstrates that complex genetic networks dependent on the *E. coli* RNAP and native *E. coli* promoters can be assembled in the ePURE system.

E. coli promoters offer a wide and well-characterized repertoire of promoter-regulator pairs and *E. coli* promoters are also highly modular³⁷ making transcription by the *E. coli* RNAP interesting for *in vitro* synthetic biology¹¹. While lysate-based TX-TL systems can be prepared with high activities of the EcRNAP^{11,38}, we have found activity of the EcRNAP in the reconstituted PURE system to be too low for many applications. PURE is a minimal system that can be rationally improved by a bottom-up approach. Here we enhanced transcription in the PURE system by adding purified transcription elongation factors GreA and GreB. The ePURE mix increased EcRNAP transcription rates from a strong $\sigma 70$ promoter to levels observed with phage T7 RNAP and significantly increased transcription from a number of synthetic *E. coli* promoters of different strengths. We used the ePURE system to characterize activation of eight native *E. coli* flagellar promoters by the transcriptional activators FlhDC and $\sigma 28$, demonstrating that the ePURE system is useful for the characterization of genetic regulation under defined conditions. Inclusion of GreA and GreB proteins should furthermore be useful to increase protein yields in the PURE system when it is desirable to use *E. coli* promoters.

Materials and Methods

DNA template preparation

Linear DNA templates were produced by two-step PCR as described previously^{13,39} using the primers listed in Table S1. Flagella promoters were PCR amplified from *E. coli* BL21(DE3) genomic DNA and replaced the 5' extension primer during two-step PCR. All linear DNA

templates prepared for this study are listed in Table S2.

Preparation of GreA, GreB and EcRNAP holoenzyme

EcRNAP subunits (expression plasmid pVS10) were co-expressed in *E. coli* Xjb(DE3) cells (Zymo Research, Irvine, CA, USA) and the $\alpha_2\beta\beta'\omega$ assembly (β' subunit contained C-terminal His₆-tag) was purified by a combination of immobilized-metal affinity, heparin and anion exchange chromatographies as described⁴⁰. *E. coli* σ 70 protein containing N-terminal His₆-tag (expression plasmid pET28- σ 70⁴¹) was expressed in *E. coli* Xjb(DE3) cells and purified by a combination of immobilized-metal affinity, heparin and anion exchange chromatographies as described for EcRNAP except that the lysis and the wash buffers during immobilized-metal affinity chromatography contained 1 M NaCl. *E. coli* GreA and GreB proteins containing C-terminal His₆-tags (expression plasmids pIA578 and pIA577, respectively) were purified by immobilized-metal affinity chromatography followed by gel filtration as described⁴². All proteins were dialyzed against the storage buffer (20 mM Tris-HCl pH 7.9, 150 mM NaCl (1M NaCl for GreA and GreB), 0.1 mM EDTA, 50% glycerol, 0.1 mM DTT) and stored at -20°C. A stock solution of 50x holoenzyme (1:5 *E. coli* core RNAP : σ 70 factor at 10 μ M and 50 μ M) was prepared by incubating the proteins in storage buffer at 30°C for 20 min, then the stock was stored at -20°C until use.

Batch reaction setup and measurements

TX-TL reactions were performed in the PURExpress *In Vitro* Protein Synthesis kit (New England Biolabs) supplemented with Protector RNase Inhibitor (Roche), 1 μ M Cy3 and Cy5 binary probes³⁹, 200 nM *E. coli* core RNAP and 1 μ M σ 70 factor. The ePURE system additionally contained 10 μ M of each GreA and GreB transcription elongation factors. Platereader batch TX-TL reactions, and measurement of the mRNA concentration were performed as previously described³⁹. The initial mRNA synthesis, or transcription rate (TX), was determined by fitting the mRNA concentration (m) of the first 40 min of the reaction to:

$$m(t) = \frac{TX}{deg} * (1 + e^{-deg*t}),$$

where t is time and deg signifies the mRNA degradation rate (fixed at 0.0085 min⁻¹)³⁹. The final EGFP concentration was determined at the plateau of the protein synthesis reaction.

To test activation of flagellar promoters by FlhDC and σ 28, we pre-synthesized the activators from T7 RNAP templates in a standard PURE reaction without EcRNAP and Gre proteins. FlhDC was produced by combining *flhD* and *flhC* templates at 10nM each, and σ 28 was produced from 10 nM *fliA* template for 100 min at 37°C. The activators then were stored in

aliquots at -80°C until use. These were prepared by combining the FlhDC and $\sigma 28$ pre-synthesis reactions 1:1 for testing activation by both activators and by diluting 1:1 with Tris buffer for testing of each activator separately.

Flagellar gene network in a nano-reactor device

We assembled the genetic network from 5 individual DNA templates. Final concentrations were 1 nM for P_{T7} -*flhD*, 2.5 nM for P_{flgK} -Citrine, and P_{T7} -*flhC*, and 2 nM for P_{fliA} -*fliA* and P_{fliA} -Cerulean. The templates P_{fliA} -*fliA* and P_{fliA} -Cerulean were only added transiently. The microfluidic chip was prepared and used as described¹³ using a dilution time, t_d , of 39.6 ± 0.4 min. Citrine and Cerulean concentrations were determined from a calibration with purified proteins¹⁰.

Supporting Information

Supporting figures S1 and S1. Supporting tables S1 and S2.

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Figure 1:

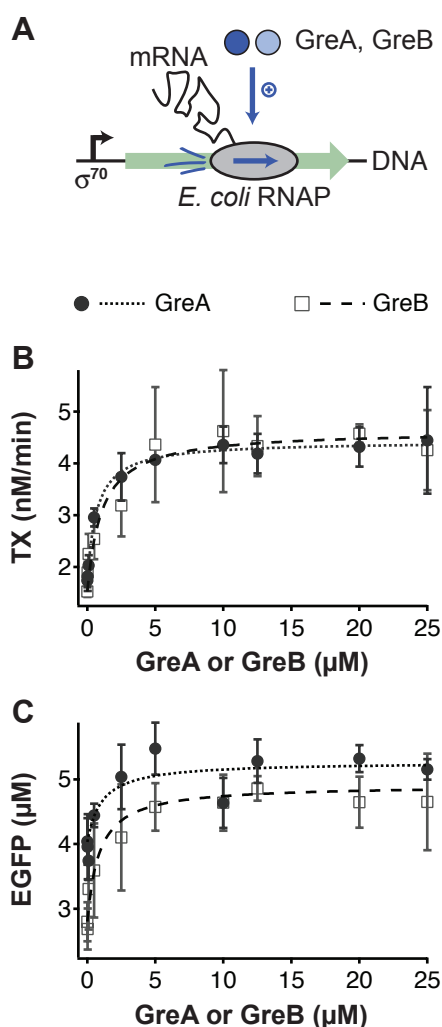


Fig. 1: GreA and GreB transcription elongation factors increase EcRNAP transcription rates in a PURE TX-TL reaction.

(A) GreA and GreB proteins enhance transcription of a DNA template encoding EGFP under control of a consensus sequence σ^{70} EcRNAP promoter in a PURE TX-TL reaction. Transcription rates (B) and final EGFP concentrations (C) increase with increasing GreA and GreB concentrations following hyperbolic kinetics. Lines represent fits to the Michaelis-Menten equation. The DNA template carried the σ^{70}_{tet} promoter and was used at a concentration of 8 nM. All GreA and GreB concentrations were tested at least in duplicate.

Figure 2:

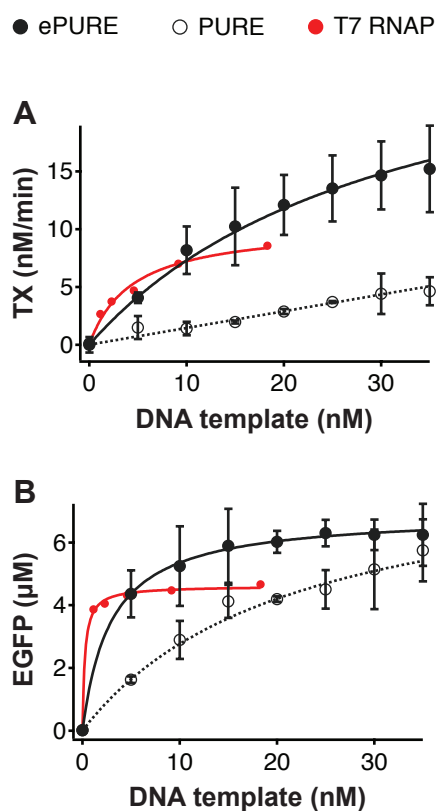


Fig. 2: Enhanced transcription in the ePURE system as a function of DNA template concentration.

Effect of varying DNA template concentrations on transcription rates (A) and final EGFP concentration (B). The DNA template carried the σ^{70} tet promoter and DNA concentrations were tested in two independent experiments. Data for comparison to the T7 RNAP was previously collected in a PURE reaction without EcRNAP and GreA and GreB proteins³⁹. Lines represent fits to the Michaelis-Menten equation.

Figure 3:

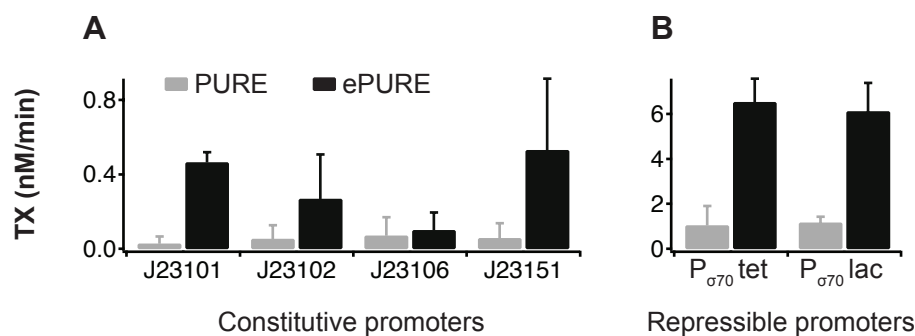


Fig. 3: Enhanced transcription from different synthetic $\sigma70$ EcrNAP promoters in the ePURE system.

Comparison of transcriptions rates from different synthetic $\sigma70$ EcrNAP promoters in the PURE and the ePURE system. The ePURE system improved transcription of a panel of constitutive promoters from the registry of standard biological parts (A) and of TetR and LacI repressible consensus sequence promoters (B). DNA templates were at a concentration of 8 nM and encoded EGFP. Values are averages of two independent experiments with error bars showing the standard deviation.

Figure 4:

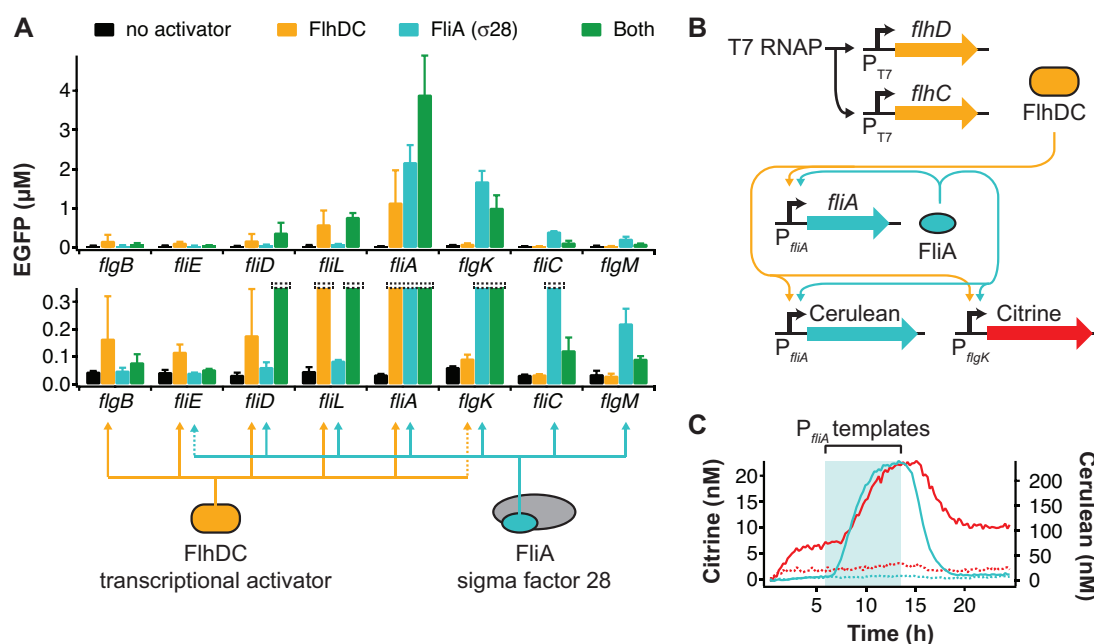


Fig. 4: *E. coli* flagellar promoter activation by FlhDC and $\sigma 28$ factor in ePURE.

(A) Addition of pre-synthesized FlhDC and $\sigma 28$ to an ePURE reaction activated EGFP expression from eight native *E. coli* promoters involved in flagella synthesis. The activators were added separately or in combination. The activation pattern expected from annotations on EcoCyc is shown beneath the experimental results. Dotted lines represent deviations of our results from the expectation and are discussed in the text. All promoter-EGFP templates were used at 6.5nM. Values are averages of two independent experiments with error bars showing the standard deviation. (B) A 5-gene genetic network built from FlhDC and $\sigma 28$ activators and two native flagellar promoters control expression of Citrine and Cerulean reporters. (C) The flagella gene network was characterized in a microfluidic nano-reactor device in a continuous ePURE reaction. The P_{fliA} -*fliA* and P_{fliA} -Cerulean templates were added transiently during the shaded region of the plot. Solid lines are Cerulean (cyan) and Citrine (red) concentrations for the full network, dotted lines represent a control experiment omitting the P_{T7} -*flhD* and P_{T7} -*flhC* templates. The figure shows a representative result of two experiments.