“Engineered promoters enable constant gene expression at any copy number in bacteria”

Article Presentation

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Background

- With genetic engineering, it is possible to more efficiently produce products found in nature by engineering specific genes.
- However, the tools to control the expression of these genes have lagged behind in development.
- The balance of gene expression is especially difficult to both achieve and maintain.
- The imbalance in expression of genes encoding the enzymes of a metabolic pathway may cause the accumulation of toxic intermediates or bottlenecks which result in growth inhibition.

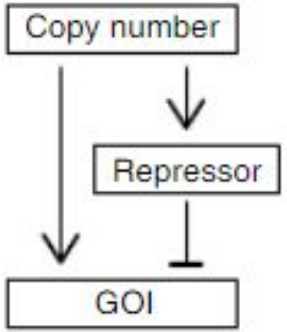
- Ways of balancing gene expression include various promoters or ribosome-binding sites to control each gene.
- However, changes in the host cell are a common cause of disruptions in gene expression, which again necessitates re-tuning of the balance.
- One such factor of uncertainty is the DNA copy number, which, although treated usually as a constant, can vary significantly due to cell growth temperature or rate.
- The stabilization, with respect to DNA copy number, of a genetic system can lead to increased robustness and less chance of disruption after modifications or transference between genetic locations.

Aim & objectives

- To design promoters that maintain constant levels of gene expression at any DNA copy number.
- Stabilization of a promoter requires a regulatory mechanism that counteracts detected changes in the copy number.
- Engineering the stabilization system, an incoherent feedforward loop (iFFL), into *Escherichia coli* promoters using transcription-activator-like effectors (TALEs)
- Application of the stabilized promoters in a metabolic pathway to show that function is maintained after moving the stabilized genes from a plasmid into the genome without re-tuning the system.

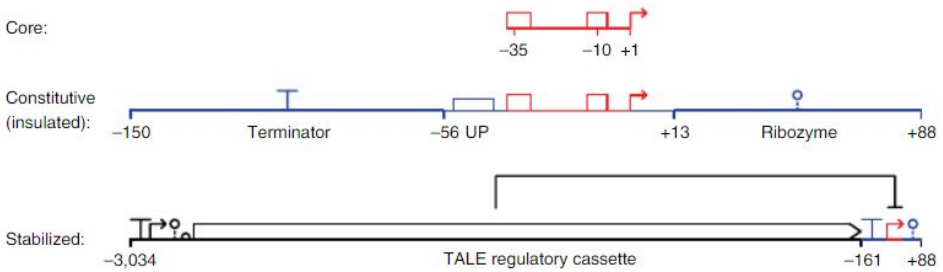
Methods & approaches

Approach



- iFFL based approach
  - Simple
  - Predicted to achieve perfect adaptation
  - Functions in a wide range of contexts
  - In iFFL, the copy number influences both repressor and expression of genes of interest

Design



- Stabilized promoter design
  - The core is the same as the insulated promoter
  - Negative regulation introduced by making promoter responsive to a protein repressor
    - Cancels out the change in expression of genes of interest
  - TALE promoters used to build stabilized promoters
    - Can be programmed to tightly bind arbitrary DNA sequences
    - Have also been shown to achieve 100-fold repression in E. coli
  - Optimal site for operator identified by using a previously characterized TALE and varying the location and orientation of the operator
  - Two optimal TALES were identified: TALEsp1 and TALEsp2
  - The stabilized promoter was created using TALEsp1 and characterizing it with pSC101 plasmid backbones

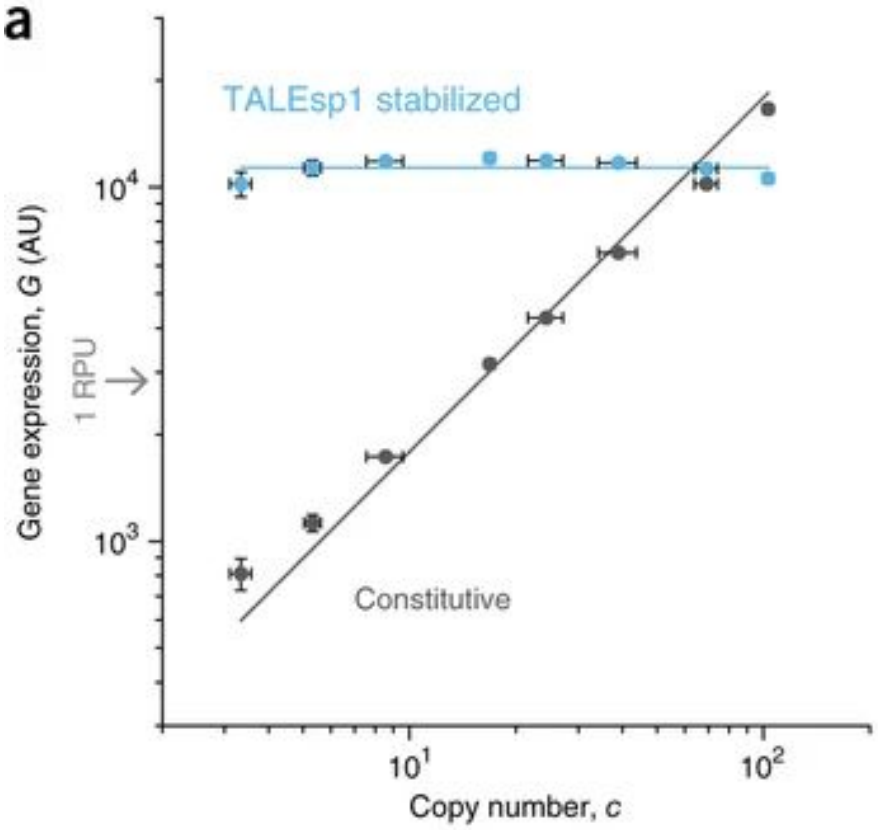
Results

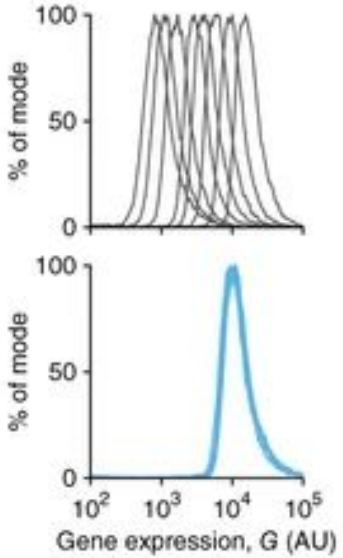
Contents of the results

- Copy number compensation when inserted into plasmid (TALEsp1)
- Effect of different plasmid backbones (TALEsp1)
- Effect of different genomic locations (TALEsp2)
- Effect of perturbations (TALEsp1)
  - Mutations & growth conditions
- Copy number compensation when inserted into genome (TALEsp2)
- Response function (TALEsp1)
- Effects of insertion of a pathway into genome (TALEsp2)

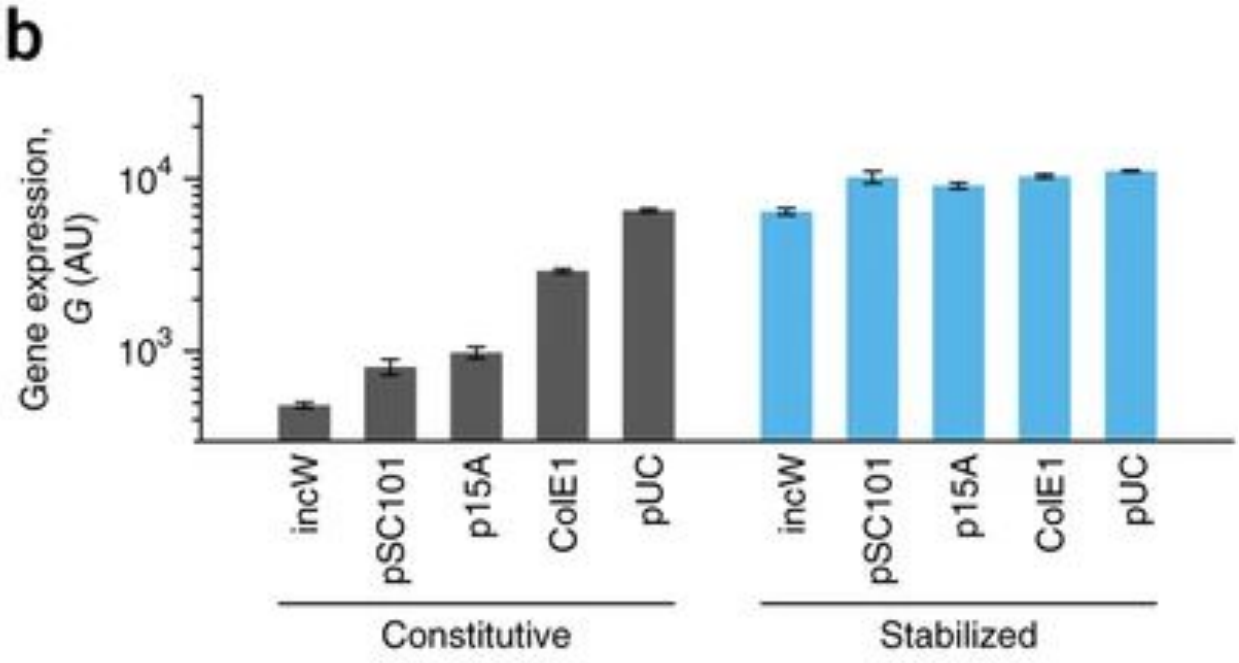
Stabilized promoters compensate the change in copy number

- Copy-number varies due to rapid cell division
- Copy numbers of the plasmid backbones ranged from 3-100 copies per cell
  → 20-fold variance in expression with constitutive promoters
  → Steady expression with stabilized promoters
  → Nearly identical expression levels from all pSC101 variants
- Stable expression in different plasmid backbones

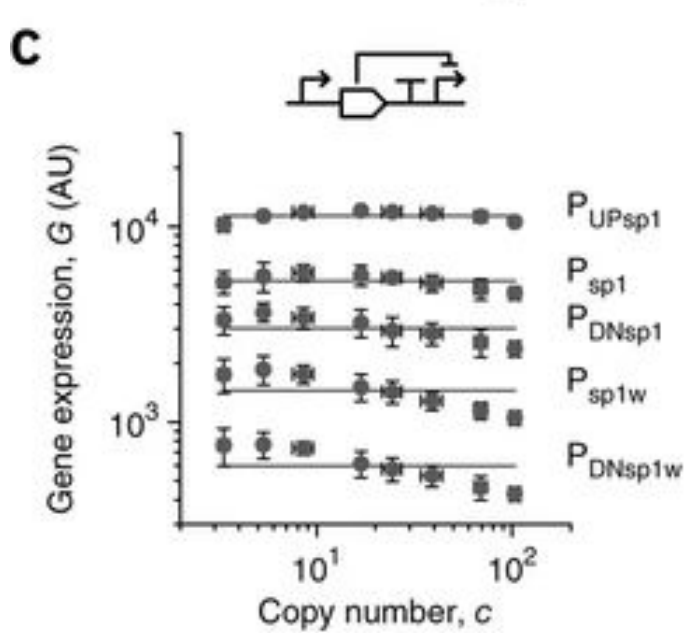




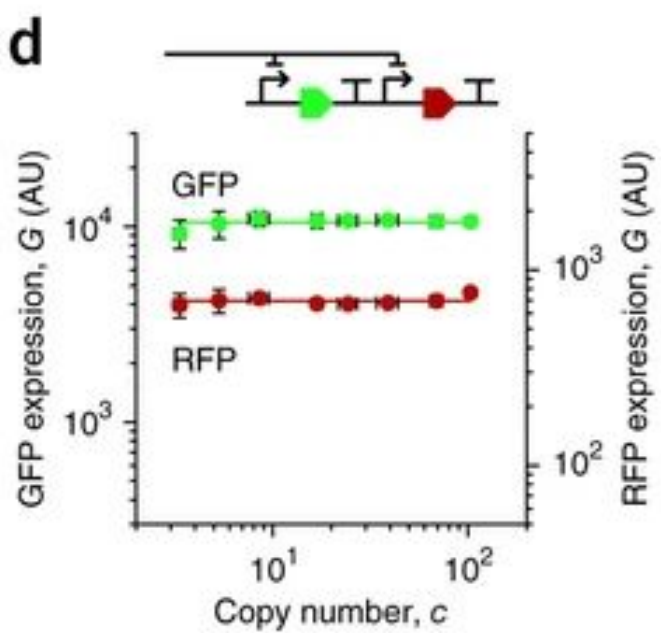
Performances of constitutive and TALEsp1 stabilized promoters in pSC101 plasmid variants with different copy numbers and corresponding cytometry distributions



Performance of the constitutive and the TALEsp1 stabilized promoter in different plasmid backbones



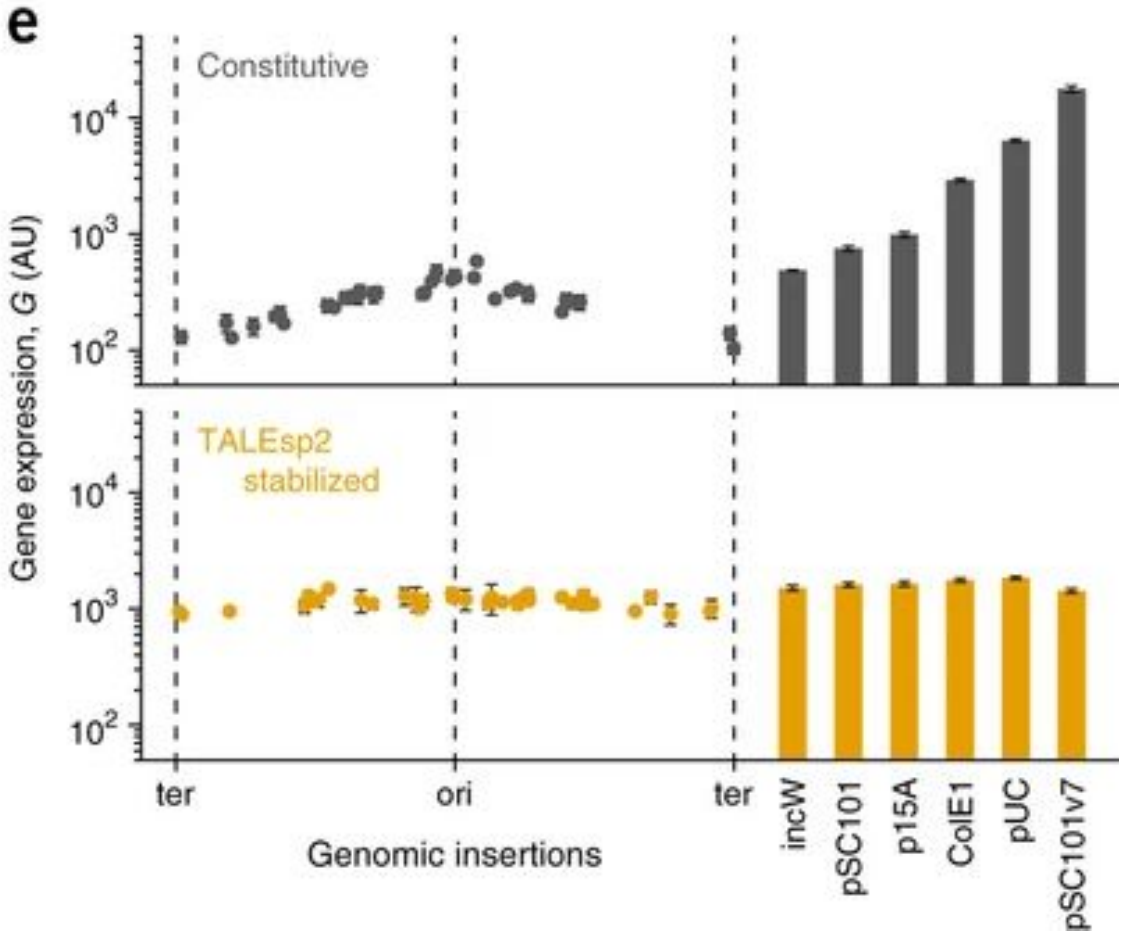
The expression levels of stabilized promoter in pSC101 variants



The expression levels of GFP and RFP operons stabilized by TALEsp1 on the pSC101 plasmid variants

Stabilized promoters reduce the effect of the position of the insertion in gene expression

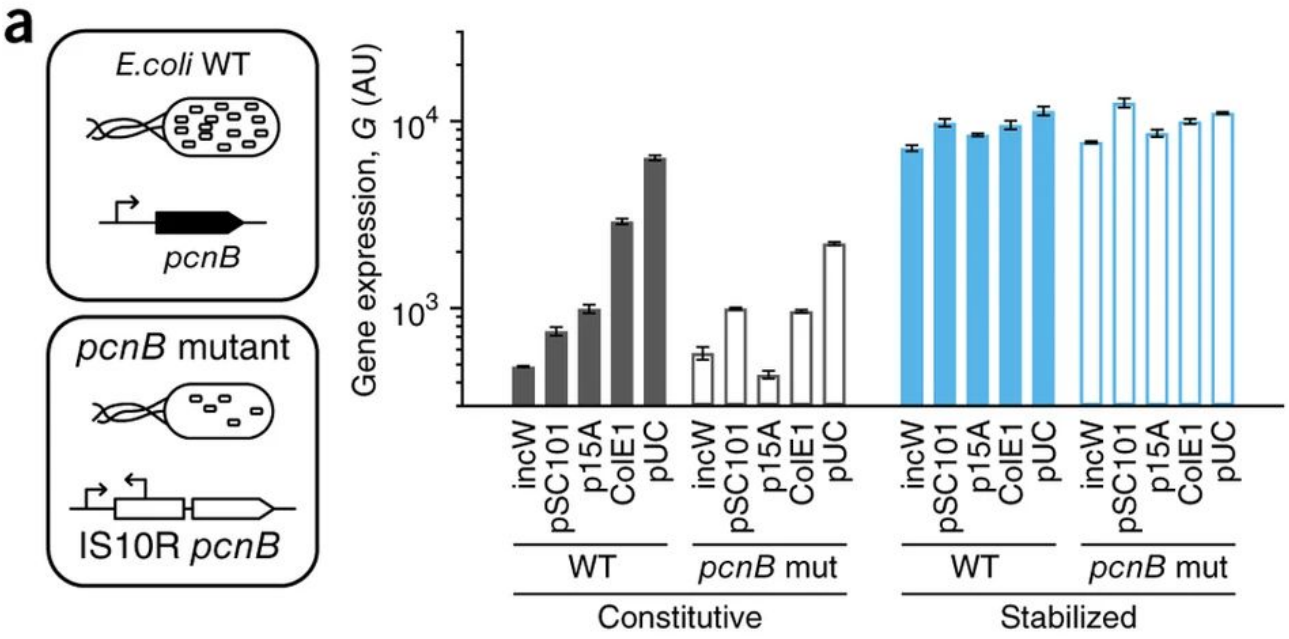
- Constitutive promoter insertions showed maximal expression near the genomic origin of replication
- Nearly no position-dependent differences in gene expression with TALEsp2
- The levels of expression from the stabilized promoter were similar between genomic insertions and promoters carried on plasmids



Performances of constitutive promoter and TALEsp2 stabilized promoters, integrated into the genome and on different plasmid backbones

Stabilized promoters reduce the effect of perturbations on gene expression:

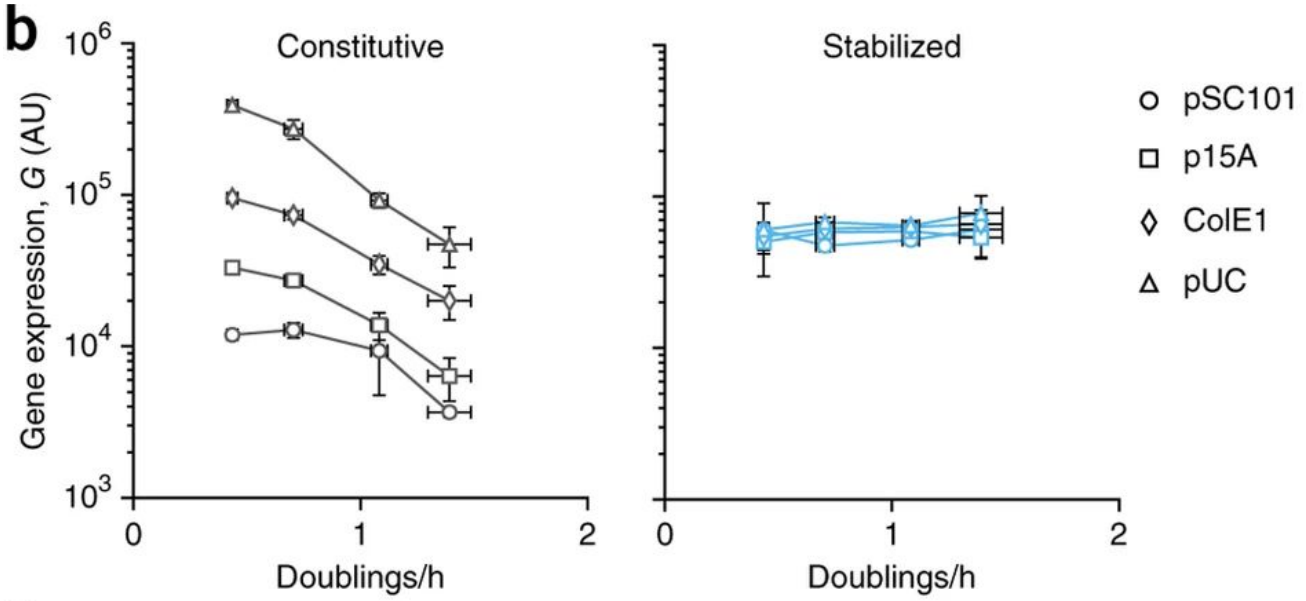
- Mutations in the host genome
  - An interfering mobile genetic element (IS10R) was inserted upstream of *pcnB* gene
    - Encodes a protein that affects the copy number of plasmids that rely on RNA regulation
  - Stabilized promoter was able to enhance the effect of this mutation and similar levels of expression in both wild type and mutant strains were achieved for all of the plasmids



Performance of a constitutive promoter and the TALEsp1 stabilized promoter using different plasmid backbones in WT E. coli DH10B and in a mutant strain with an insertion element interfering with pcnB

Stabilized promoters reduce the effect of perturbations on gene expression:

- Changes in medium and growth conditions
  - Medium composition affects growth rate and plasmid copy number in different ways depending on the origin of replication
  - Average doubling times 40-140 min and 90-fold spread in expression levels with constitutive promoter
  - TALEsp1 eliminated most of these effects

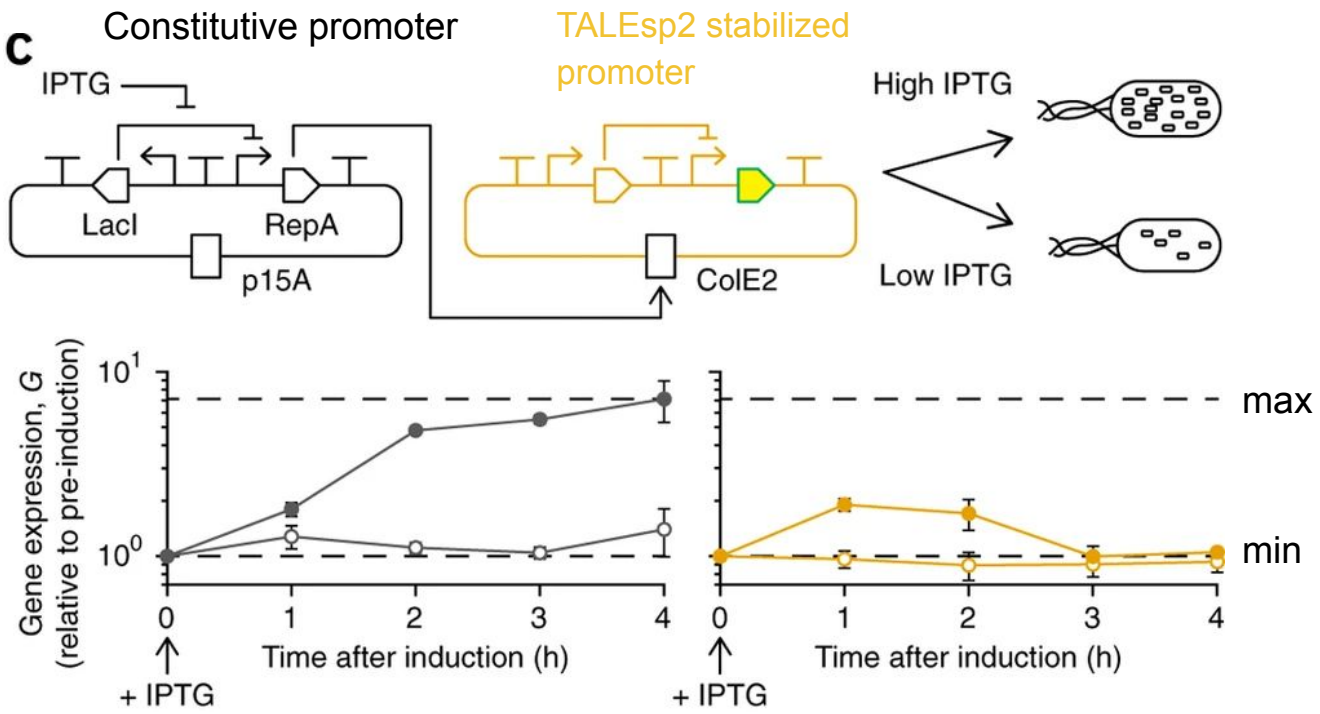


Performance of the constitutive and the TALEsp1 stabilized promoter in different plasmid backbones during exponential growth in E. coli DH10B growing in various media

Used medium from left to right: M9 with 0.4% glycerol and 0.5 mM leucine, M9 with 0.4% glucose and 0.5 mM leucine, M9 with 0.4% glycerol and 0.2% casamino acids and M9 with 0.4% glucose and 0.2% casamino acids

A second stabilized promoter, TALEsp2, was tested for its ability to inhibit copy-number differences caused by rapid cell division by inserting it into the genome

- The number of copies was altered during the experiment with a ColE2 plasmid, in which a trans-acting RepA protein is under isopropyl-β-D-thiogalactoside (IPTG) control from a second plasmid
  → When IPTG concentration was raised from 10 μM to 40 μM, the GFP expression from a constitutive promoter increased by sixfold
  → TALEsp2 was able to reduce the expression after twofold increase and returned the expression to the set point after 3 h



Response to a change in copy number of a plasmid that was controlled by a LacI-repressed, IPTG-inducible trans-factor.

● = concentration increased from 10 μM IPTG (steady state) to 40 μM

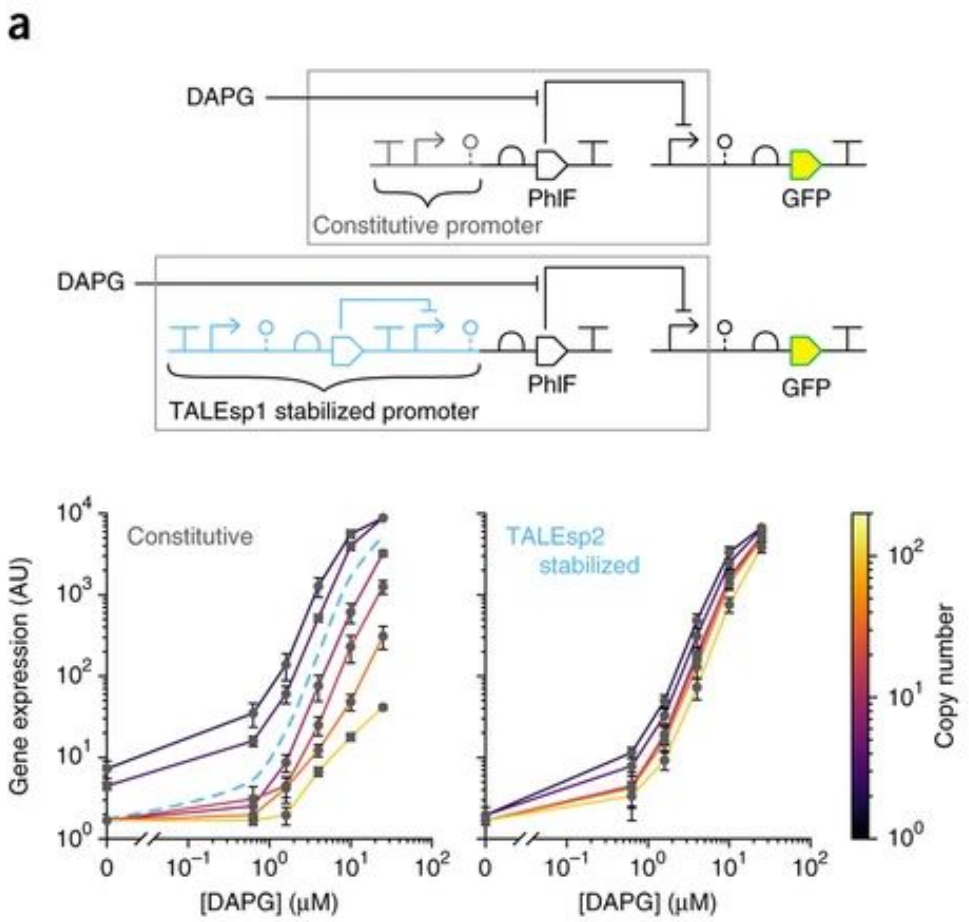
○ = cultures treated with only 10 μM IPTG

The ability of the stabilized promoters to reduce the copy-number dependence of synthetic regulation was also evaluated, i.e., how stabilized promoters could be applied to maintain a constant response function independent of the genetic location of the regulatory protein

- Promoters were applied to a 2,4-diacetylphloroglucinol (DAPG) sensor (based on the PhlF repressor). PhIF represses a PPhIf promoter on a p15A backbone, which is inducible by titrating DAPG.

→ The response function of a constitutive promoter controlled PhlF varied fundamentally depending on the plasmid backbone

→ Plasmids with TALEsp1 stabilized promoter controlled PhIF had similar response functions



The PhlF repressor expressed from different plasmid backbones (low to high copy: incW, pSC101, pSC101v2, ColE1, pUC and pSC101v7), using the constitutive promoter and TALEsp1 stabilized promoter

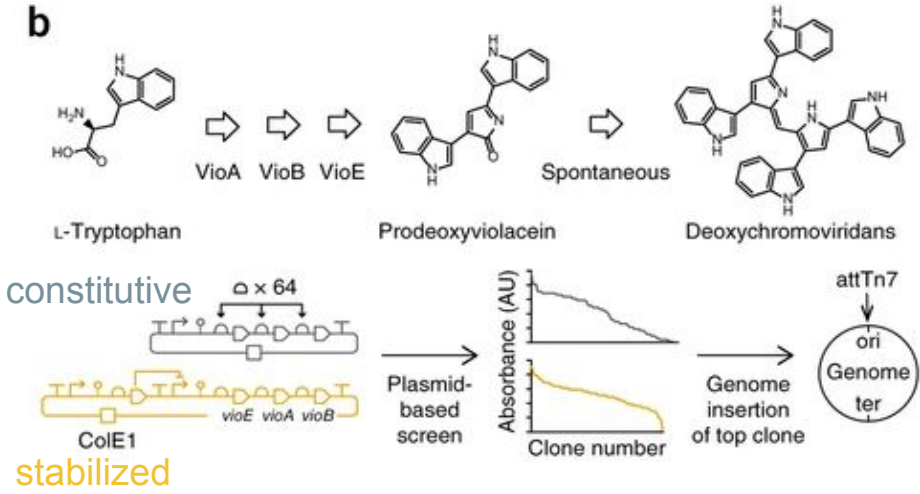
A three-gene (vioA, vioB and vioE) operon that encodes a pathway to synthesize deoxchromoviridans was inserted into a genome together with a promoter to see if the activity is negatively affected

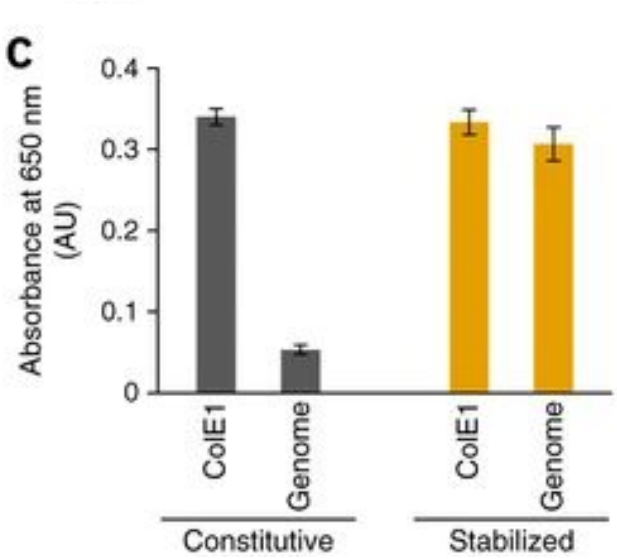
- Libraries were constructed by simultaneously varying three ribosome-binding sites and monitoring deoxychromoviridans production titer by absorbance. The pathways were characterized on a ColE1 plasmid and when integrated into the attTn7 site in the genome.

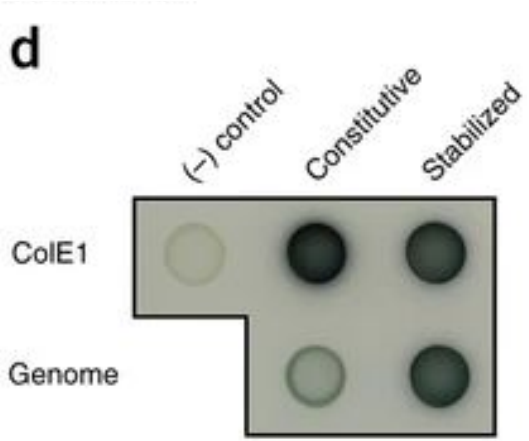
→ With a constitutive promoter the activity declined significantly after insertion into the genome

→ With TALEsp2 stabilized promoter the titer was preserved after genomic insertion

However, titers achieved by RBS libraries with the constitutive and stabilized promoters were nearly identical







Production after 12 h of growth in liquid LB medium

Deoxychromoviridans production of the strains after 24 h of growth on a LB agar plate

Conclusion of the results

- Nearly identical expression in different genome locations and plasmids, independent of the copy number
- Promoter design is durable and robust to genome mutations and medium composition
- Genome insertions of a test operon did not have negative effects on activity

Significance & future

- Plasmids are easy to manipulate and libraries of pathways on plasmids are easier to construct, but library constructs may perform differently when inserted into the genome
  - Stabilized promoters allow for inserting a desired construct into arbitrary locations in genome without negatively affecting the activity
- Stabilization can potentially improve evolutionary robustness
  - Stabilized promoter eliminates the effects of some mutations that could disrupt gene function
- Use of stabilized promoters allows for integrating a variant discovered by directed evolution of a plasmid library into the genome with no need for re-tuning

→ Precision to genetic engineering