Incoherent feedforward loop (iFFL) by TALE stabilized promoters

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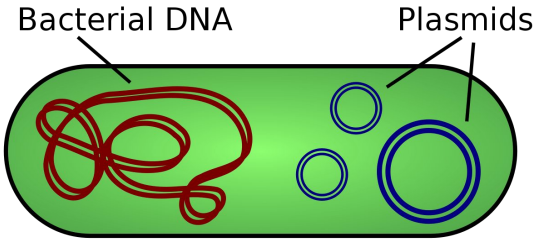

Background

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Background

- **Balancing of gene expression an important goal in genetic engineering, however balance not easily achieved and maintained**
    - Genetic parts to control each gene, directed evolution, computational methods
- **Vast amounts of effort put into improving measurement and reliability of genetic parts**
- **Any change to host cells or change of plasmid backbone may disrupt genetic systems**
    - ‘Re-tuning’ is required to fix the problem

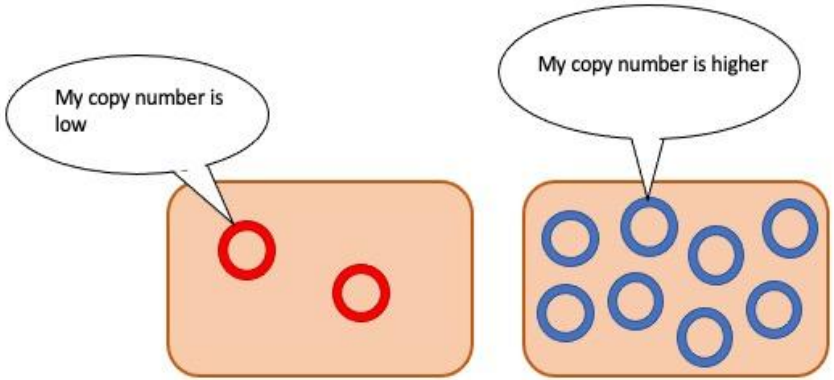
Recap on copy number and promoters

- **Plasmid copy number = expected number of copies per cell**

  High (500-700 copies/cell)

  Medium (20-100 copies/cell)

  Low (15-20 copies/cell)



- **Constitutive vs. regulated promoters**
  - Promoter active always → constitutive
  - Promoter activity only in response to certain stimulus → regulated

Why copy number matters

- **DNA copy number constitutes a huge uncertainty in the design of genetic systems**
- **Copy number of plasmids can vary widely (even though often treated as constant)**
  - Changes to the cell environment (including the host strain that is used)
  - Composition of growth medium
  - Growth temperature
  - Growth rate
  - Changing the size of plasmid or gene(s) being transcribed

Why copy number matters

- **With constitutive promoters, total level of gene expression is dependent on the copy number at which they are present in the cell**
- **Minimal constitutive promoters: σ factor and RNA polymerase binding sequences + transcriptional start site**
  - Performance disturbed if the surrounding sequences are changed
  - Flanking insulators to counteract this
- **Depending on the rate of cell division, copy number of different locations in genome can span up to 8-fold**
- **Stabilization of genetic systems in terms of copy number thought to improve robustness and enable modifications and transfer between genetic locations with less chance of disruption**

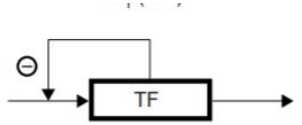
The concept of stabilized promoters

- **Stabilized promoters: additional elements added that decouple gene expression from copy number**
- **Regulatory elements that detect changes in copy number**
  - Promoter activity compensated according to the detected change
- **Aim is to establish a constant level of gene expression regardless of copy number**
- **Several possible approaches to stabilize promoters (next slide)**
  - Feasibility of design needs to be considered

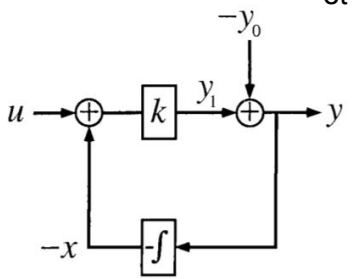
Methods

Regulatory mechanisms

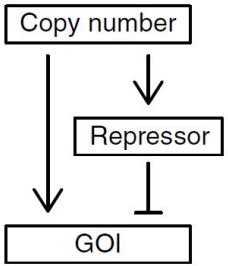
- **Stabilization of promoters can be done by regulatory mechanisms**
  - Autoregulatory feedback
    - straightforward to implement
    - cannot achieve perfect adaptation
    - can cause oscillations
  - Integral feedback
    - can achieve perfect adaptation
    - complicated to implement
  - **Incoherent feedforward loop (iFFL)**
    - simple
    - predicted to achieve perfect adaptation
    - functions in wide range of settings



Negative autoregulatory feedback loop (Becksei, A. et al. 2000)



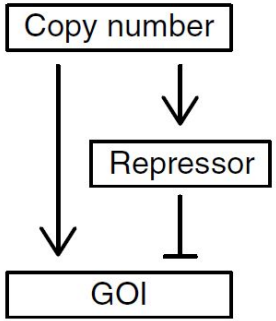
Integral feedback loop (Rahimi, N. et al. 2016)



Incoherent feedforward loop (Segall-Shapiro, T. et al. 2016)

Incoherent feedforward loop (iFFL)

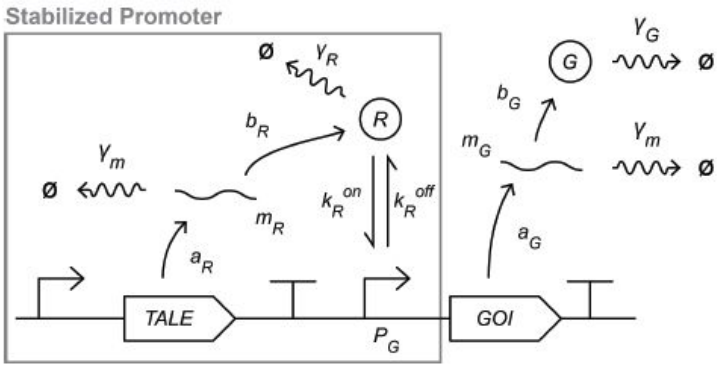
- **Incoherency rises from input signal both positively and negatively controlling the output**
- **Input-signal = copy number**
  - On its own, promoter is expected to correlate positively with copy number
- **The system in this study centered on a stabilized promoter**
- **The promoter is made responsive to the protein repressor**
  - Increased copy number ⇨ increased expression of the repressor ⇨ cancel out the change in the expression gene of interest



Incoherent feedforward loop (iFFL)

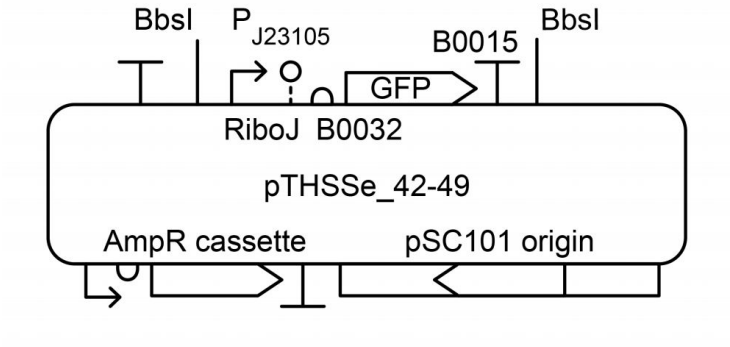
- **Transcription activator-like effectors TALEs were chosen to build stabilized promoters**
- **They bind to the DNA to TALE protein specific binding site** ⇨ repressor
- **TALE can be programmed to tightly bind chosen DNA sequences**
- **Transcriptional repression in bacteria at steady-state follows Hill equation**
  - G=concentration of GOI protein, c=copy number, R=concentration of repressor, n=cooperativity of repression

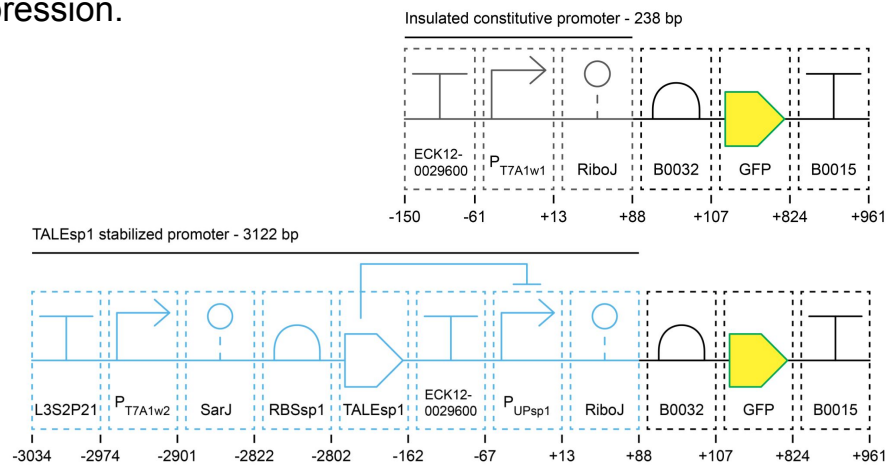
$$G \propto \frac{c}{R^n} \Rightarrow G \propto c^{(1-n)}$$



Stabilized promoters

- Characterized the effect of copy number on an insulated constitutive promoter → moved onto a set of pSC101 plasmid backbones with a range of different copy numbers.
- The stabilized promoter was tested with different back bones that had different origins of replication, and variation in copy number, size, gene expression and, possibly, cellular localization.
- The promoter and RBS controlling TALE expression were chosen to express the repressor to a sufficient level for good adaptation at copy numbers and for maintaining high gene of interest expression.





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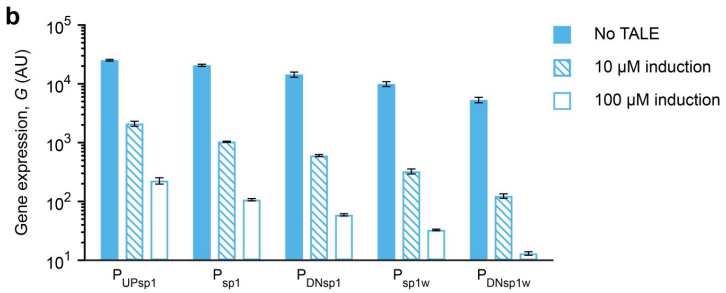
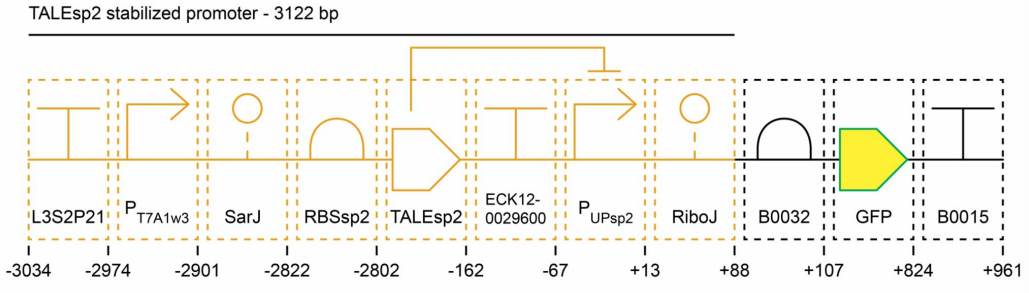
Stabilized promoters

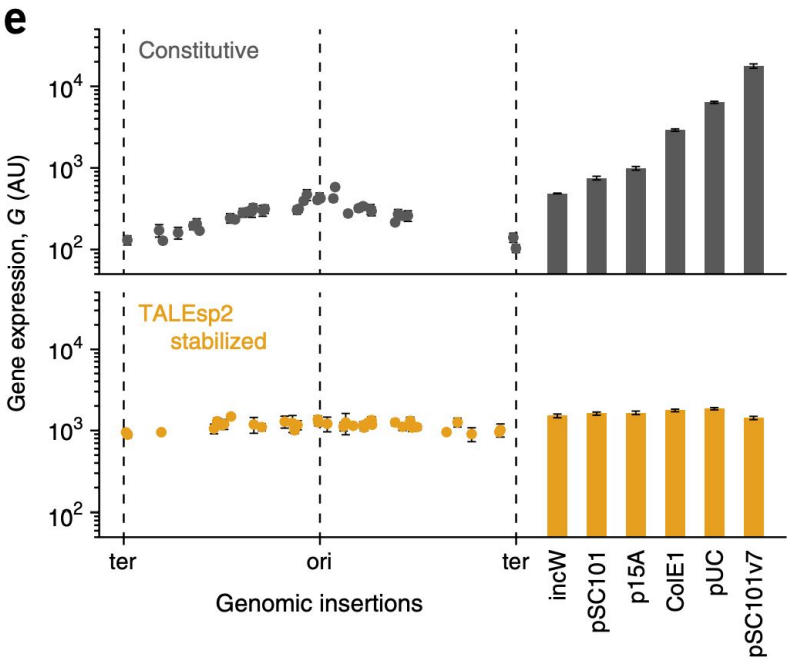
- A series of stabilized promoters were built that had variable expression levels and were controlled by the same TALE.
- Mutation were made to the TALEsp1-repressible promoter sequence → generated four promoters with a range of strengths that were repressed by the repressor.
- The promoters were combined with TALEsp1 expression cassette → a set of four stabilized promoters were created that represented a range of strengths.
- A second stabilized promoter that used TALEsp2 repressor was built to use stabilized promoters at copy numbers below the copy number of pSC101. For the promoter the expression of TALE was increased to achieve good stabilization at copy numbers down to ~ 1/cell.





Evaluation of the stabilized promoter

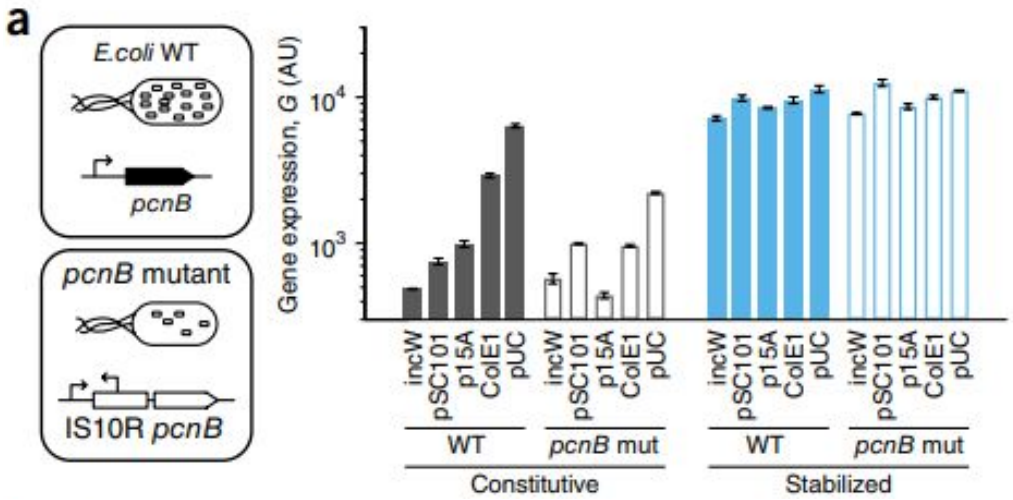
- Stabilized promoter TALEsp2 was inserted randomly in the genome with Tn5 transposon system to study its ability to buffer against copy-number differences caused by rapid cell division.
- Positions of single-insertion events were determined with arbitrary PCR → yielded strains with 35 insertions of the stabilized promoter across the genome.
- The process was repeated with insulated constitutive promoter to get 35 additional strains with distributed insertions.



Results

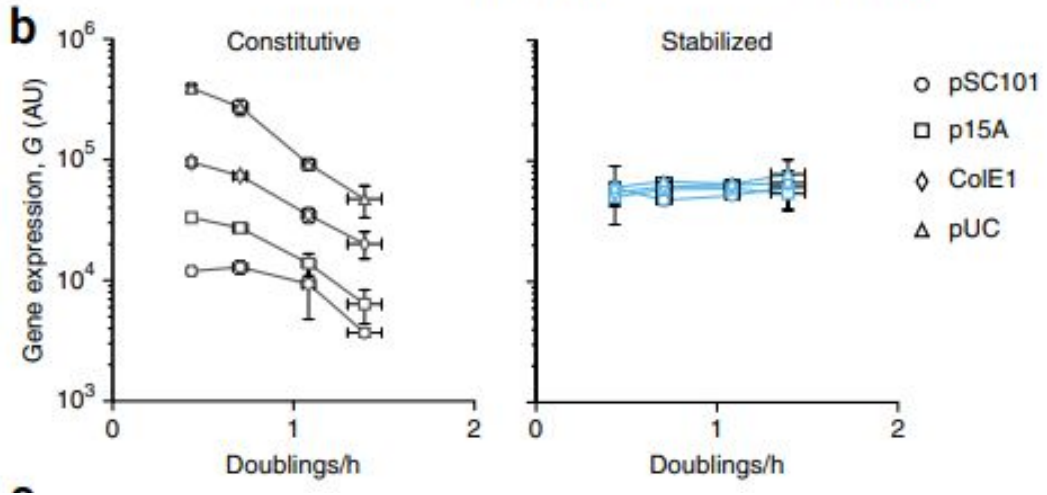
Stabilized promoters reduce the effect of perturbations that affect copy number

- Many perturbations can affect gene expression through DNA copy number as an intermediate=> stabilized promoters would buffer against these changes;
- pcnB encodes a protein that affects the copy number of plasmids that rely on RNA regulation (e.g., p15A, ColE1 and pUC);
- The stabilized promoter was able to ameliorate the effect of this mutation and achieve similar levels of expression across both strains for all of the plasmid backbones.



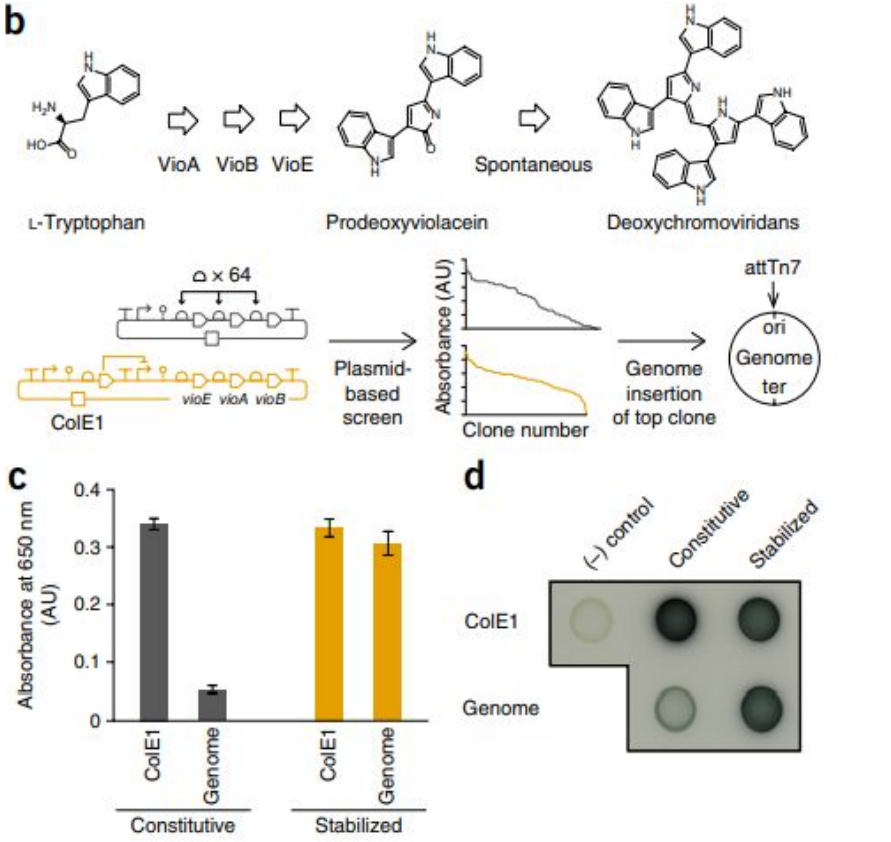
Stabilized promoters in different media eliminate fold-spread

- Changes in medium and growth conditions also can change copy number and break genetic circuits and metabolic pathways;
- Four variants of M9 medium were made with different carbon sources (glucose or glycerol) or amino acids (casamino acids or leucine);
- When the performance of the **constitutive** promoter was compared between all of the different media and the origins of replication, there was a 90-fold spread in expression levels and the TALEsp1 stabilized promoter eliminated most of this effect.



Stabilized promoters enable the insertion of library constructs into the genome without negative effects

- Three-gene operon (vioA, vioB and vioE) that encoded a pathway to synthesize deoxchromoviridans was used as a test;
- For product to be synthesized most effectively, specific expression levels of these three genes are required. Libraries by simultaneously varying all three RBSs and screening for deoxychromoviridans production titer were constructed;
- When a tuned pathway was controlled by a constitutive promoter, the activity declined considerably after insertion into the genome;
- When a tuned pathway was controlled by the TALEsp2 stabilized promoter, the titer was preserved after genomic insertion



Conclusion

- **The aim of the project was to design promoter which would produce the proteins independently at copy number perturbed by genomic mutations or changes in growth medium composition. Using IFFLs, they were able to:**
  - improve the effect of pcnB protein which affects the copy number;
  - eliminate fold spread in different media;
  - achieve the same level of gene expression irrespective of the plasmid backbone or it's location in the genome.
- **All experiments show that using of stabilized promoters is more effective in gene expression comparing to constitutive promoters.**

References

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Segall-Shapiro, T. H., Sontag, E. D., & Voigt, C. A. 2018. Engineered promoter enable constant gene expression at any copy number in bacteria. Nature biotechnology. 36.4. pp. 352.

http://parts.igem.org/Part:BBa_K2918008

Time for questions

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