Orthogonal synthetic promoter libraries:
To explore design spaces unconstrained by cloning host tolerance

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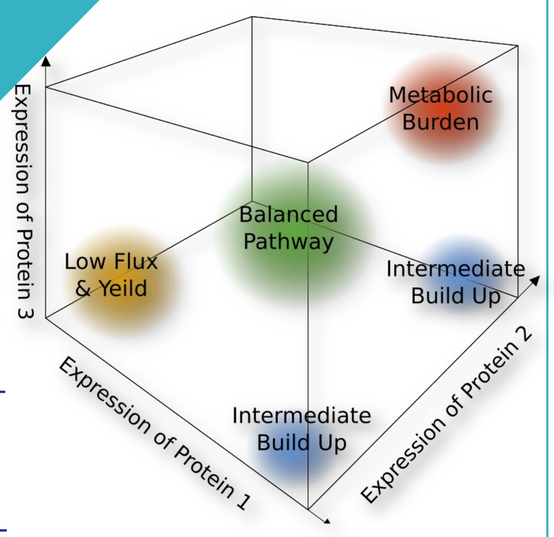
1. Background: Limitations of metabolic pathway engineering in industrially relevant bacteria: the *Clostridium* genus

Introduction

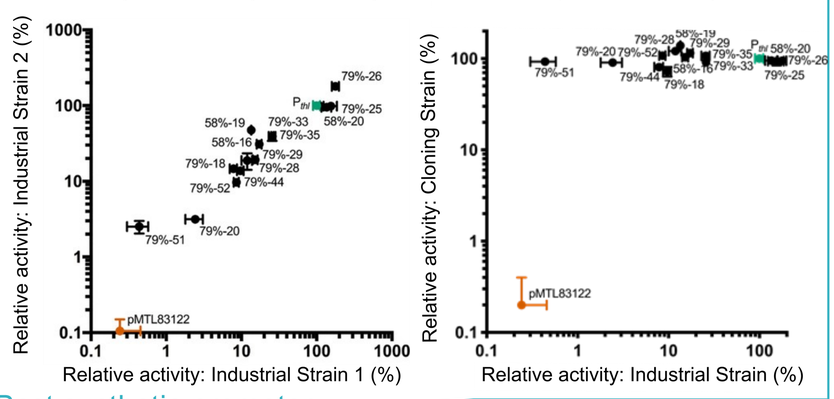
- Many bacteria **naturally** have **properties** and **synthesis pathways** which are attractive to Industrial Biotechnology.
- To improve the product **titres** and **yields**, it is necessary to optimise the pathway **flux**. This often requires advances in the molecular **tools** we have available.
- **Combinatorial assembly** has been demonstrated to rapidly optimise such pathways, and Start-Stop Assembly offers a general purpose platform for combinatorial assembly in a wide range of organisms.
- However, the synthetic biology toolbox for many bacteria are limited to their **native promoters**, which are often incompatible with combinatorial assembly.

[A] Balance protein levels to increase yield and reduce burden.

- Expression of proteins varies from high to low.
- Important to test a range of expression levels.
- Avoid burden (overexpression), bottlenecks (potentially toxic intermediate build-up), or low yield (weak expression).



Activity of Previously Available Synthetic Promoters (Mordaka & Heap, 2018)



[B] Best synthetic promoter library for *Clostridium* toxic in cloning hosts.

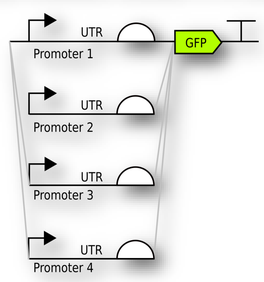
- Synthetic promoter library 2018 (Mordaka & Heap): gave access to range of expression levels.
- Very high expression in cloning hosts = toxicity related cloning issues.
- **Aim of this project: Address issue with a new design.**

2. Are promoters with a 'dampened' TF expression tolerated in cloning hosts?

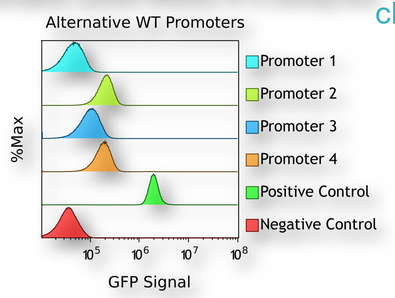
[A] Expression without the TF in cloning hosts

- Background expression levels of the alternative promoters with GFP without TF via flow cytometry.

A) Assembly of Constructs for GFP Expression From Orthogonal Promoters Without TF in cloning host



B) Range of GFP Expression Levels Detected For Transcription System Without TF in cloning host

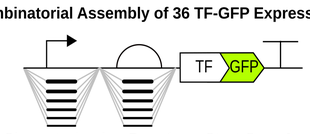


- 3 of 4 promoters had low (yet detectable) levels in cloning hosts without TF.

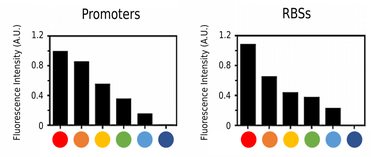
[B] High, medium and low TF expression levels in cloning hosts

- Can we pre-screen to refine libraries in cloning host prior to transfer to *Clostridium*?
- Optimise TF level to 'dampen down' promoter expression.
- Combinatorial assembly = test 36 TF levels to get a high, medium and low.

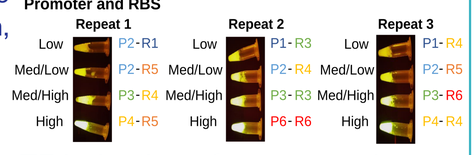
A) Combinatorial Assembly of 36 TF-GFP Expression Levels



B) Part Characterisation Data from Start-Stop Assembly[1]



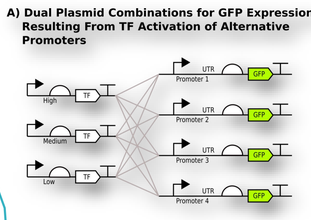
C) A Range of Expression Levels Sequenced To Identify Promoter and RBS



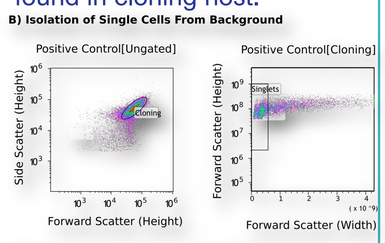
[C] High, medium and low TF induction of promoters in cloning hosts.

- Medium TF level = optimal.
- Low TF level= not sufficient to induce promoters.
- High TF level = toxic.
- BUT later work: medium level too toxic = bottleneck in library of orthogonal promoters found in cloning host.

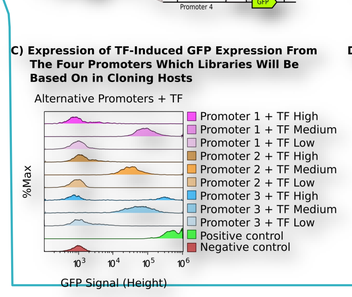
A) Dual Plasmid Combinations for GFP Expression Resulting From TF Activation of Alternative Promoters



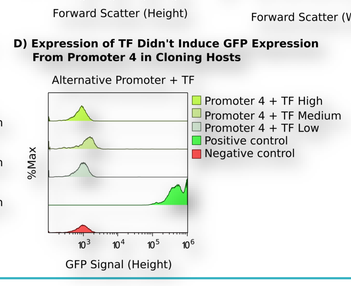
B) Isolation of Single Cells From Background



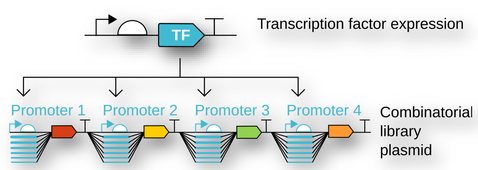
C) Expression of TF-Induced GFP Expression From The Four Promoters Which Libraries Will Be Based On in Cloning Hosts

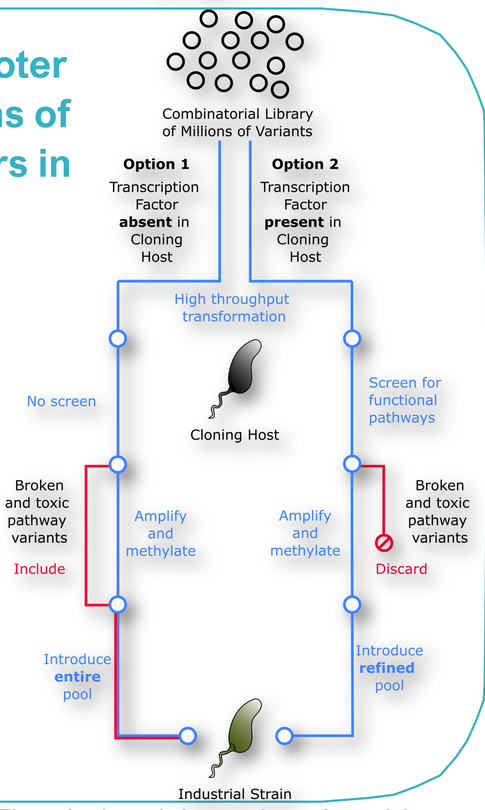


D) Expression of TF Didn't Induce GFP Expression From Promoter 4 in Cloning Hosts



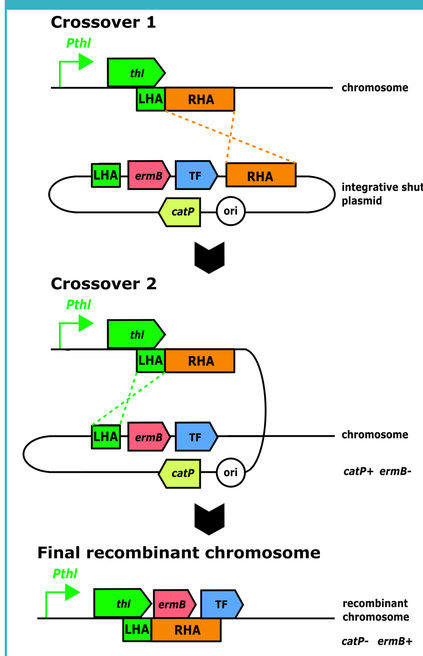
Objectives: orthogonal promoter library to overcome limitations of previously available promoters in cloning hosts.





- Design **alternative promoter libraries** to **overcome toxicity** in cloning hosts; libraries will be **turned on in the presence** of the TF, and **off in the absence** of the TF.
- Investigate the **portability** of these promoters between cloning hosts and *Clostridium*; does **dampened TF expression make promoters less toxic?**
- Build and **optimise pathways** for **green chemical synthesis**.

4. Screening directly in *Clostridium spp.*

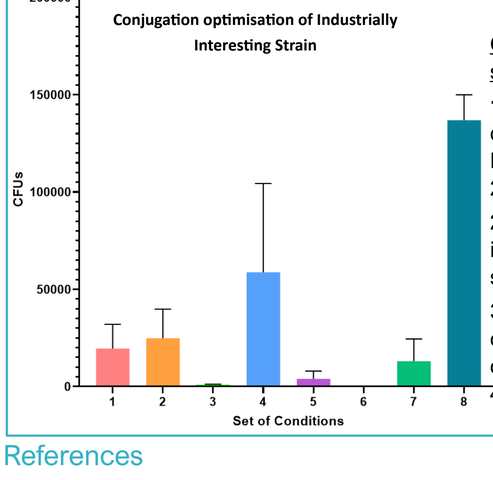


[A] TF inserted downstream of native promoter in genome.

- Built strain: TF downstream of well characterised native promoter.
- Made via Allele Coupled Exchange (ACE) (Heap et al. 2012).
- Decoupled TF regulation from potential native control.

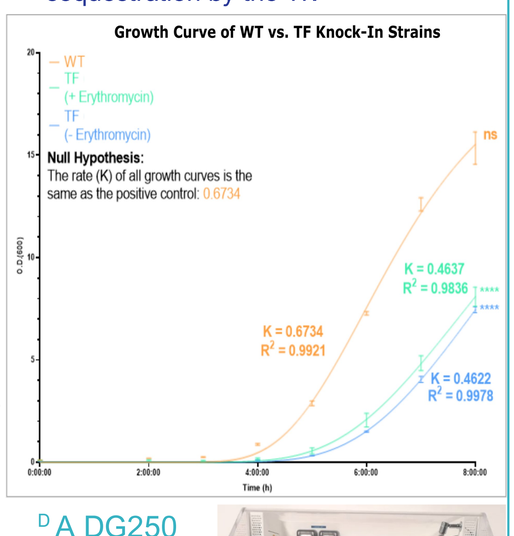
[C] Conjugation optimised for library coverage.

- Require large numbers of variants with good diversity.
- Condition 7 used for maximum diversity despite lower numbers of CFUs per conjugation.
- Replication of transconjugants was minimal prior to selection, reducing duplicates.



Condition set:

1. Method described in Purdy et al., 2002
2. Subculture industrial strain (IS) 4h
3. Subculture conjugation donor (CD) 4h
4. Subculture IS 8h
5. Heat shock IS
6. Heat shock IS and conjugate 8h
7. Conjugate 8h
8. Subculture IS 8h & subculture CD 4h

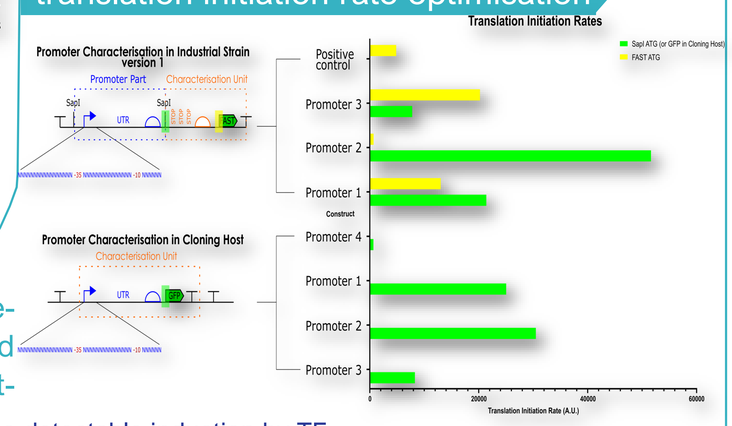


[D] A DG250 Don Whitley workstation was used for anaerobic work using *Clostridium*.

5. Design challenge: *Clostridium* translation initiation rate optimisation

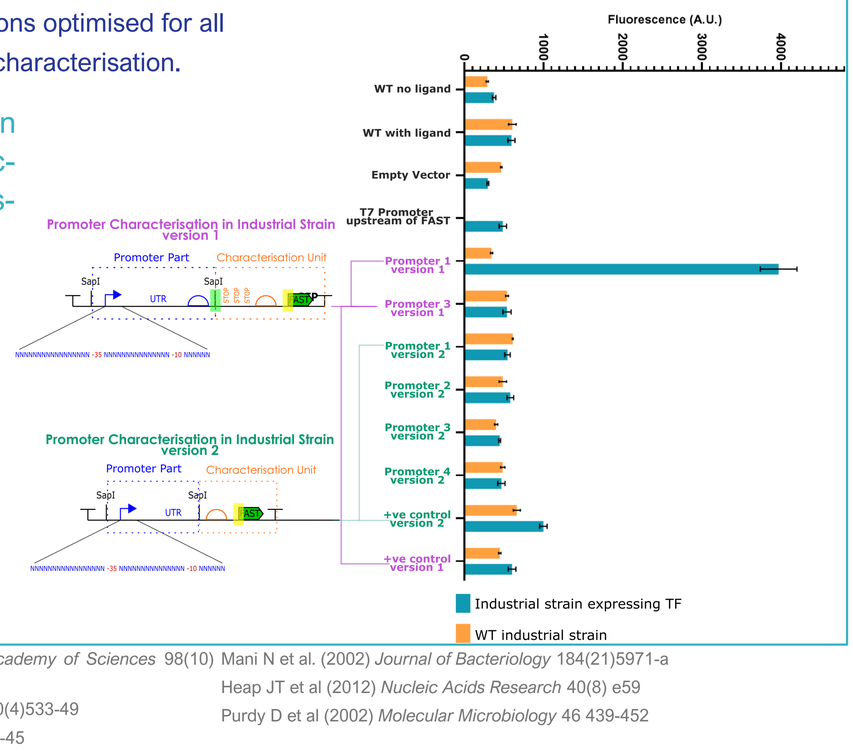
[A] The industrial strain plasmids required an anaerobic reporter, and promoters were cloneable via Start-

- In industrial strain, promoters 2 and 3 = no detectable induction by TF.
- Promoter 1 = very good induction by TF (see figure below).
- Investigated region surrounding the start codon: context changed with new fluorescent reporter.
- RBS calculator found low translation initiation rate (TIR) for promoter 2. Also found low TIR for promoter 4 (previously appeared inactive in cloning host (section 2.C, figure section D)).
- Crucially = highlighted variable TIRs between promoters, leading to miscalculation of relative promoter library strengths.
- DNA context around start codons optimised for all ~ 10,000 TIR, for comparable characterisation.



[B] Only Promoter 1 (version 1) showed detectable induction by the TF in the Industrial Strain.

- 'Improved' promoters (version 2) = no detectable expression from TF induction following TIR optimisation.
- Highlights need for empirical characterisation in non-model organisms over *in silico* prediction.
- Promoter 1 version 1 will be taken forth for library..



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