CHEM-E8125
Synthetic Biology

Control of nitrogen fixation in bacteria that associate with cereals

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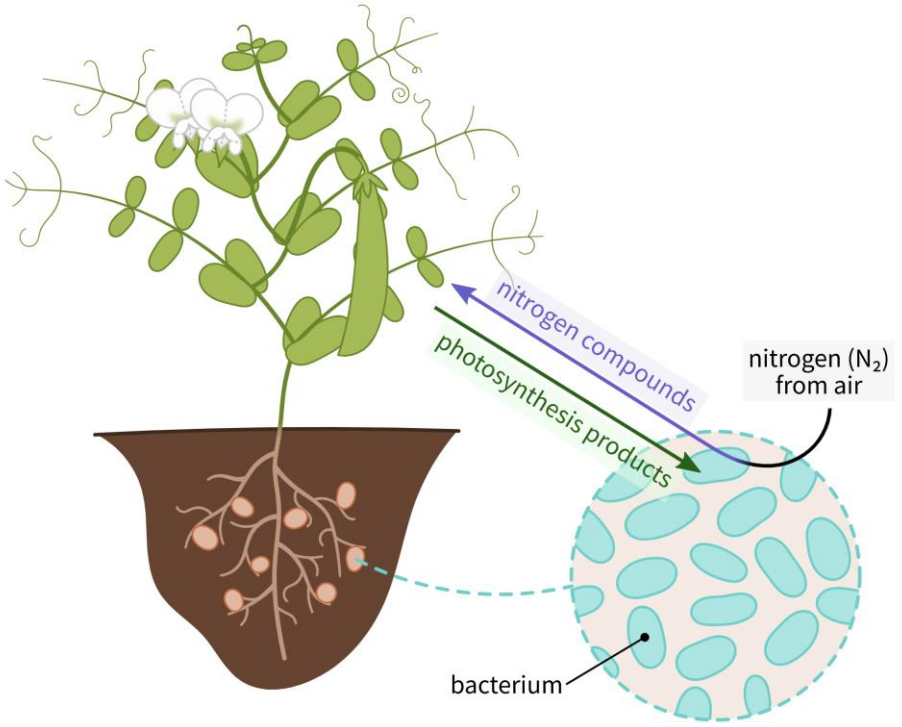
Introduction

- **Nitrogen is a limited nutrient in agriculture**
  - Needs to be added as fertilizer for example in cereals, which can't obtain it from the atmosphere
  - On the other hand, legumes obtain nitrogen through nitrogen-fixing bacteria (rhizobia), residing in root nodules
- **Transferring the nitrogen-fixation ability to cereal crops would reduce need for nitrogenous fertilizer**
- **Possible solution**
  - Engineering of cereal associated bacteria located in soil, root surface (epiphytes) or inside the roots (endophytes) in order to fix nitrogen
- **Nitrogen fixation genes (*nif* genes) are organized in clusters, and conserved genes include those encoding nitrogenase and cofactor biosynthesis**
  - Nitrogenase can make up 20% of cell mass and each ammonium requires about 40 ATP for its production, which strongly represses its transcription under aerobic conditions



"A simplified diagram of the relation between the plant and the symbiotic bacteria (cyan) in the root nodules". In *Wikipedia*.

Main aim

- The main aim was to achieve nitrogenase activity that can be induced in a strain capable of forming an endophytic or epiphytic association with cereals

Methods and approaches

- Ten different native *nif* clusters were tested in three different strains which were
  - *Escherichia coli* MG1655
  - *Pseudomonas protegens* Pf-5
  - *Rhizobium* sp. IRBG74

- In addition to native *nif* clusters, engineered *nif* clusters were made → **refactoring** of *nif* clusters
  - A version of the gene cluster whose DNA sequence has been rewritten, but it encodes the same function
  - Bottom-up process to systematically eliminate the native regulation of a gene cluster and replace it with synthetic genetic parts and circuits

- The induction of *nif* clusters controlled by synthetic sensor so that at undesirable time *nif* transcription is turned off

- The approaches to choose the best version were high nitrogenase activity and reduced oxygen sensitivity and ammonium repression

Performance of native *nif* clusters in *E. coli, P. protegens* Pf-5 and symbiotic rhizobia (1/3)

Goal: To compare native nif clusters' relative performance in different strain backgrounds

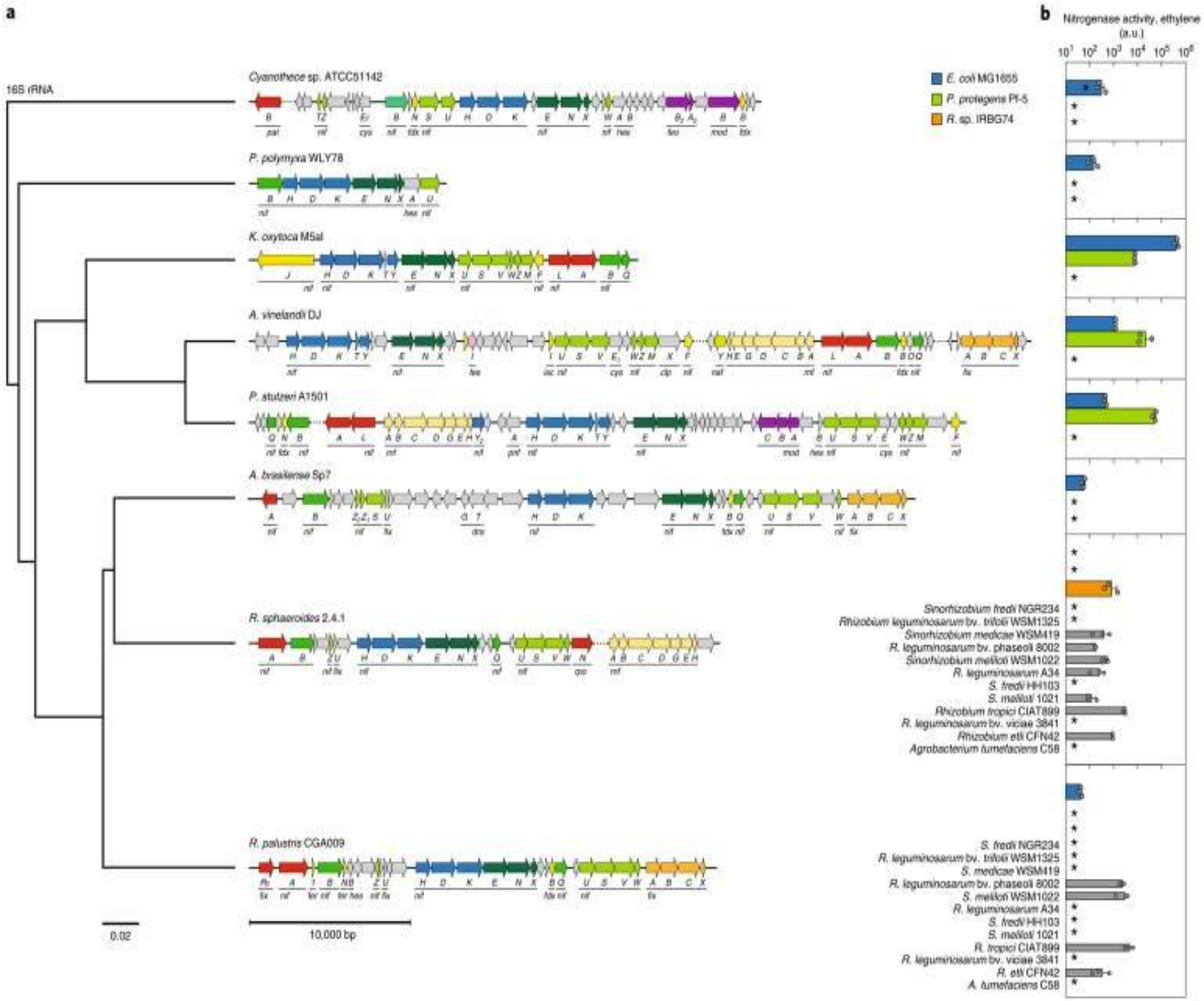
Methods:

- Native nif clusters from different species were cloned
- DNA synthesis and fragment amplification from genomic DNA were used to build the clusters
- Clusters were fused using yeast assembly and cloned into plasmid backbones
- Nitrogenase activity was measured by acetylene reduction assay

Performance of native *nif* clusters in *E. coli, P. protegens* Pf-5 and symbiotic rhizobia (2/3)

Results

- 7/10 cluster were functional in *E. coli, K. oxytoca* the best
- *P. protegens Pf-5* showed high activity with *K. oxytoca, P. stutzeri* and *A. vinelandii*
- *R. sp IRBG74* was active only with the cluster from the *R. sphaeroides.* When native nif cluster was knocked out, introduction of the cluster didn't lead to activity.
- *R. sphaeroides* and *R. palustris* were tested with 12 species isolated from diverse legumes. Activity in 7 strains, but no further experiments.



Performance of native *nif* clusters in *E. coli, P. protegens* Pf-5 and symbiotic rhizobia (3/3)

Methods

- RNA-sequencing was used to quantify transcriptional parts.
- *K. oxytoca* nif cluster was transferred to *E.coli, P. protegens Pf.5 and R. sp. IRBG74*
- Ribosome profiling --> translation efficiency

Results of transfer experiments

- *E. coli* the best recipient, but not suitable for agriculture
- *P. protegens Pf5* – moderately high activity, but either constitutively on or sensitive to ammonium
- *Rhizobia* – low activity --> engineering the clusters

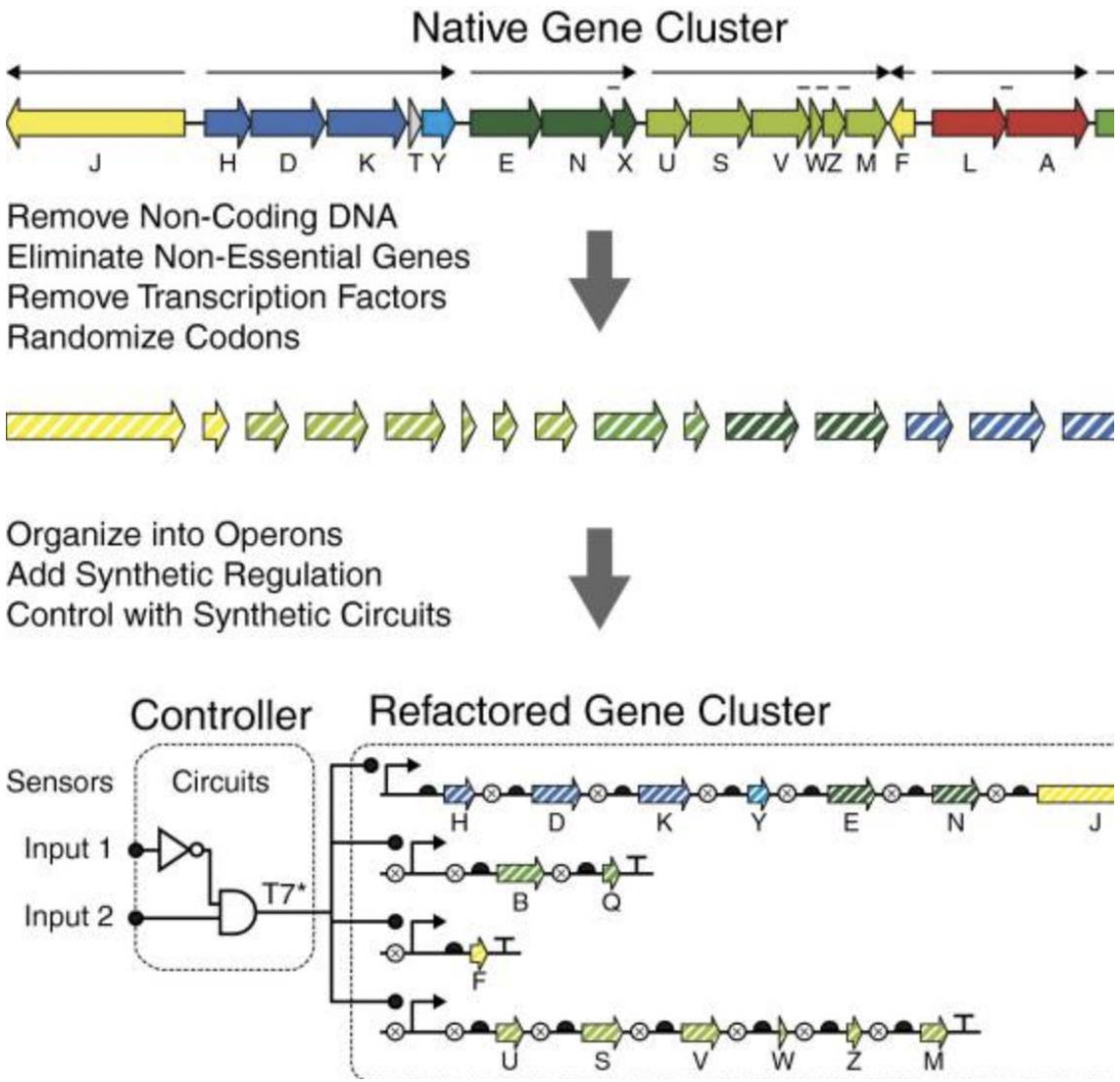
Transfer of refactored *K. oxytoca nif* clusters to *R.* sp. IRBG74

- Due to difficulty transferring activity with a native *nif* cluster into *R.* sp. IRBG74 they tried to transfer the refactored v2.1 cluster

  → this failed because promotors were less active and terminators misfunctional. Almost no nitrogenase activity in *R.* sp. IRBG74 and *P. protegens* Pf-5. More activity in *E. coli* MG1655.

- They designed new refactored cluster v3.2

  → *Nif* cluster active both in *R.* sp. IRBG74 and *P. protegens* Pf-5, though it was less active in *E. coli* MG1655 than v2.1. However, the activity was not very high and increasing inducer concentration too much activity declined rapidly.



***Nif* cluster v2.1**. Temme, K., Zhao, D., & C.A. Voigt (2012). Refactoring the nitrogen fixation gene cluster from Klebsiella oxytoca . Proc. Natl. Acad. Sci., 109(18): 7085 7090

Replacement of *A. caulinodans nif* regulation with synthetic control

- Engineering of *A. caulinodans,* in order to eliminate ammonium repression
- Goals:
  - Moving clusters of *A. caulinodans* to R. sp. IRBG74
  - Modification of *nif* regulatory control system by using synthetic sensors
- Regulatory control was achieved by replacing the regulatory control of clusters with synthetic regulation, engineered to reduce ammonium repression
- IPTG inducible system was used and it worked well
- Observations:
  - Complete recovery of activity when controller co-expressing *NifA* and *RpoN* was induced
  - WT strain activity strongly repressed (95%) by ammonium
  - 50% of activity was recovered when *NifA* double mutant with *RpoN* were co-expressed, when ammonium was present
  - Oxygen tolerance was nearly identical in native and inducible gene clusters (both had a broad tolerance)

Controllable *nif* activity in *P. protegens* Pf-5

- *K. oxytoca, P. stutzeri and A. vinelandii* nif clusters were functional in *P. protegens PF-5.* However, either nif was strongly repressed by ammonium or it was constitutively on
- To obtain regulatory control, NifA master regulators were removed and expressed from a controller
- A controller that was able to induce the clusters from all 3 species was build - (nifA of P. stutzeri, IPTG-sensor)

Results

- The native *P. stutzeri* and *A. vinelandii* clusters were strongly repressed by ammonium, the inducible clusters were not
- Oxygen sensitivity was tested – gave information at which oxygen % optimal nitrogenases activity occurs

Why and how is this important and path forward

Importance

- Finding alternative ways to introduce nitrogen to the corps

  --> Reducing the need for fertilizers as a source of nitrogen

  --> Optimizing the nitrogen flux for maximal effect

- Environmental and economic impact

Path forward

- Increasing the flux of nitrogen delivery by redirecting metabolism and using transporters

- Engineering a plant to produce orthogonal carbon sources --> synthetic symbiosis

Thank you!

Questions?

References

1. Ryu et al. **Control of nitrogen fixation in bacteria that associate with cereals** Nature Microbiology volume 5, pages 314–330 (2020).
2. Temme, K., Zhao, D., and C.A. Voigt, Refactoring the nitrogen fixation gene cluster from Klebsiella oxytoca . *Proc. Natl. Acad. Sci*., **109(18)** (2012) 7085 7090. doi: 10.1073/pnas.1120788109.
3. "Nitrogen fixation Fabaceae" *Wikimedia Commons,* https://commons.wikimedia.org/w/index.php?title=File:Nitrogen_fixation_Fabaceae_en.svg&oldid=600017523 (accessed May 5, 2023).