Characterization of plasmid expression level heterogeneity through heterologous eGFP expression in co-expression with isopropanol production in *Cupriavidus necator*

P. Pijpstra[1], A. Boy[1,2], JL. Parrou[2], S. E. Guillouet[1], P. Heindinger[3], R. Kourist[4], N. Gorret[1]

[1] Toulouse Biotechnology Institute (TBI), Université de Toulouse, CNRS, INRAe, INSA, 135 Avenue de Rangueil, Toulouse cedex 04, 31077, France

[2] Plateforme Genome et Transcriptome (GeT-Biopuces), Toulouse Biotechnology Institute (TBI), Université de Toulouse, CNRS, INRAe, INSA, 135 avenue de Rangueil, Toulouse, F-31077, France.

[3] ACIB - Austrian Centre of Industrial Biotechnology, ACIB GmbH, Krenngasse 37, 8010 Graz, Austria

[4] Technische Universität Graz, Institüt für Molekulare Biotechnologie, 8010 Graz, Petersgasse 14

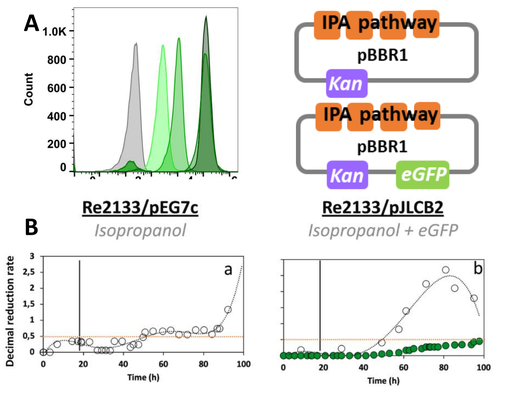
pijpstra@insa-toulouse.fr / ngorret@insa-toulouse.fr

Background

Although axenic cultures in industrial bioprocesses are assumed to be homogenous in phenotype, several studies have proven the opposite, leading to a reduction in production performance and loss of robustness of the bioprocess. Therefore, assuring phenotypic homogeneity during overproduction of biomolecules constitutes a major challenge for synthetic biology, and bioprocess development. A well-established method for strain engineering is through plasmids due to their ease of use, adjustable expression levels and interchangeability between species. However, in the absence of a selective pressure, plasmids are often quickly lost, making plasmid-stability of great interest.

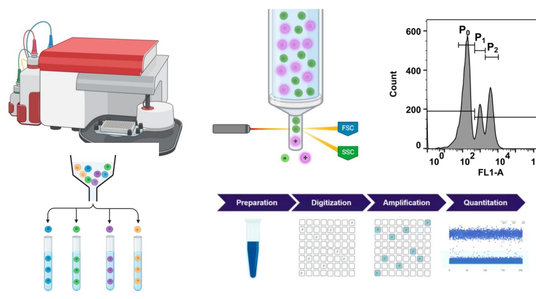
Here we assess strain stability of engineered plasmidic heterologous biomolecule producing *C. necator* through the use of flow cytometry. Additionally, we uncover a major source of population heterogeneity and seek to mitigate this through rethinking the design of the plasmid.

Materials and methods

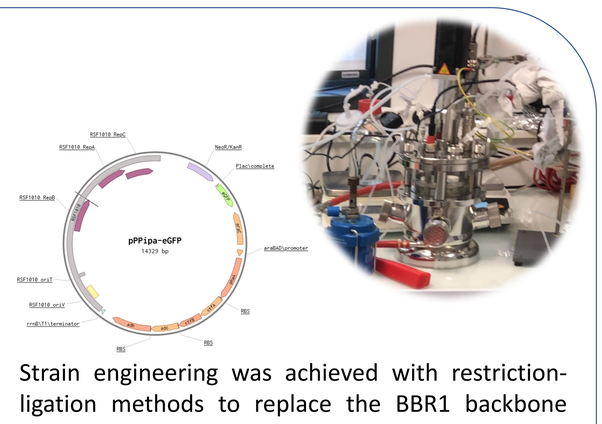


Green fluorescent biosensors were previously developed (Fig A) and applied on heterologous isopropanol producing *C. necator* (Fig B), allowing tracking of subpopulation generation through the course of a well-controlled intensive cultivation[1,2] using flow cytometry and plate counting methods.

Through slow cultivation of the strain in continuous culture conditions (μ=0,03 h^{-1}), subpopulation generation was assessed utilizing flow cytometry and plate counting methods.



We attempted to uncover the origins of the expression heterogeneity through quantitative methods based on cell sorting coupled with digital PCR.

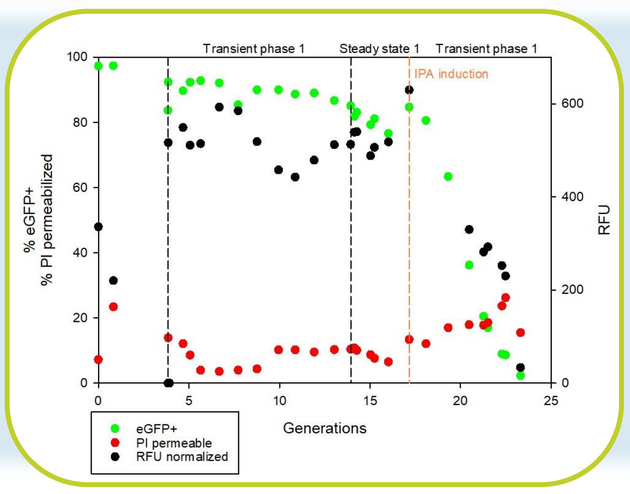


Strain engineering was achieved with restriction-ligation methods to replace the BBR1 backbone with RSF1010 backbone, often described in literature as highly stable backbone.

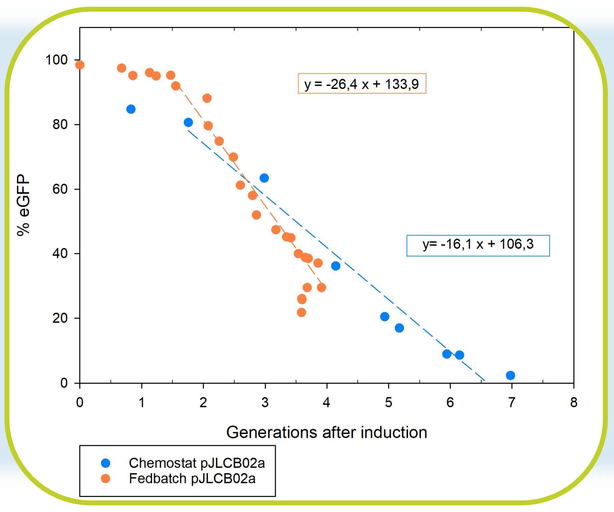
Through slow cultivation of the strain in continuous culture conditions (μ = 0,03 h^{-1}), subpopulation generation was assessed utilizing flow cytometry and plate counting methods.

Results

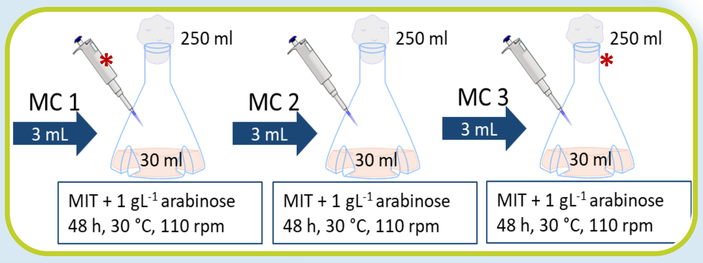
Well-controlled continuous culture with continuously expressed eGFP and inducible heterologous IPA expression (pJLCB02a) indicates a loss of eGFP+ population over generations.

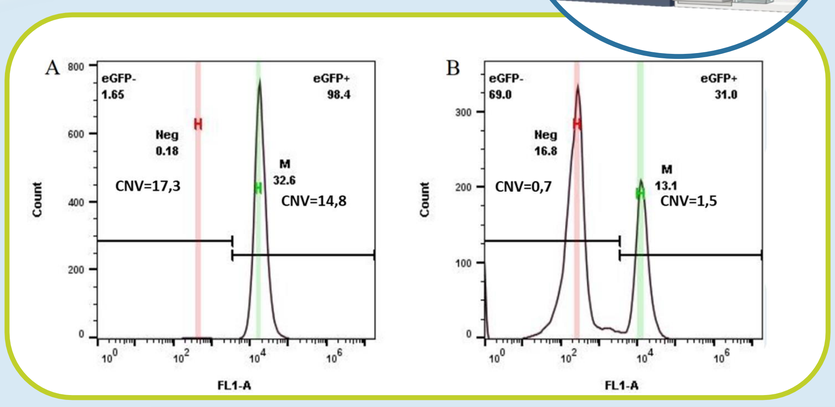


Linear regressions of GFP+ population after IPA induction in well-controlled cultivation of pJLBC02a in intensive and slow cultivation indicate that the mechanism of subpopulation generation is independent of cultivation mechanism and IPA concentration in the bioreactor.



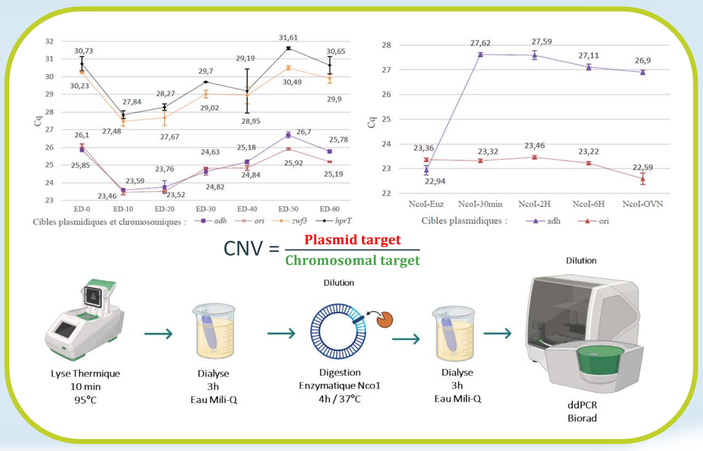
Serial shake flask cultivations on minimal media with IPA induction at the start of every culture were used to explore the mechanisms behind this expression heterogeneity. Analysed samples are indicated by a red asterisk.



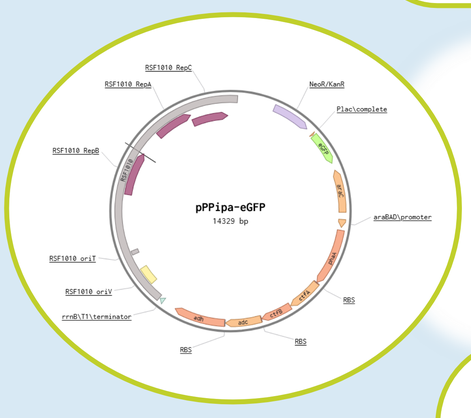


A strain with alternative backbone RSF1010 was constructed and introduced in *C. necator*. This backbone, often described as stable in the literature, was assessed for expression stability.

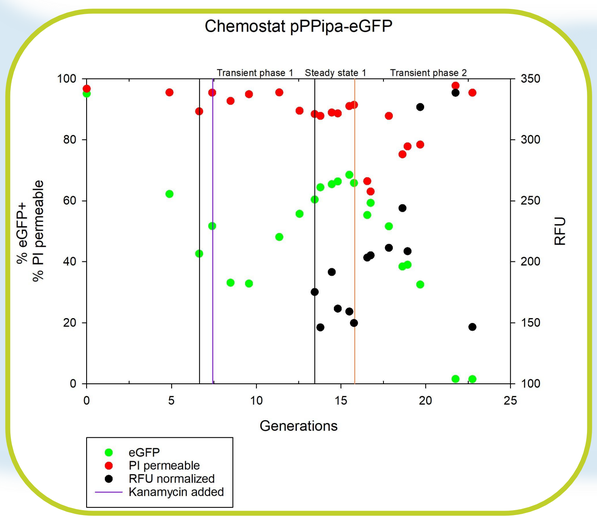
Fluorescent assisted cell sorting achieved separation of the two samples, one at the start (Fig A) and at the end (Fig B) of the shake flask cultivation (previously indicated by the red asterisk), into their respective subpopulations. Two intervals were selected, representing both eGFP- and eGFP+ subpopulation (represented in red and green, resp.). This revealed that IPA production leads to an overall decrease in CNV (eGFP+$_{noIPA}$= 14.8; eGFP$_{noIPA}$ = 17.3; eGFP+$_{noIPA}$= 1.5; eGFP-$_{noIPA}$ = 0.7).



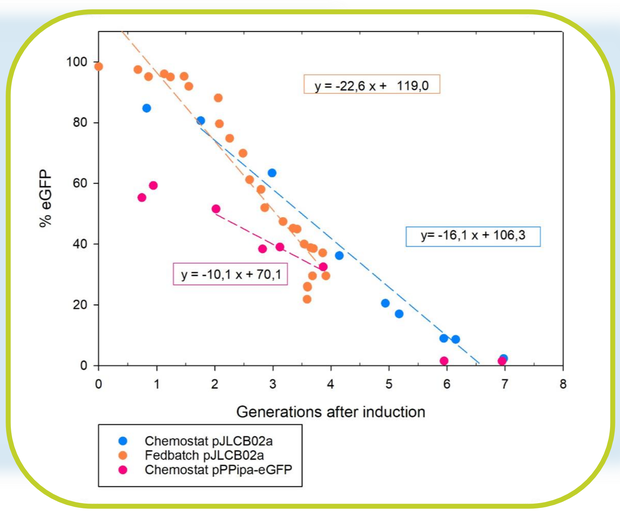
Copy Number Variation (CNV) determination was optimized to include thermal lysis of the cell and enzymatic digestion. Through selection of reference genes on the genome and genes on the plasmid, quantification of plasmid copy number is achieved.



A well-controlled continuous culture with continuously expressed eGFP and inducible IPA expression with pPPipa-eGFP suggests that after induction, eGFP- population grows.



Comparison of linear regression of eGFP+ population after IPA induction suggest increased stability of the new backbone.



Conclusions and outlook

Flow cytometry is a useful tool for strain evaluation and bioprocess development. The development of non-invasive biosensors with a good compromise between accurate sensing and minimal metabolic load remains relevant towards understanding bottlenecks in bioprocesses. By gaining in-depth understanding the mechanisms behind subpopulation generation, engineering efforts can be directed towards the creation of industrially robust strains.

References

[1] Boy, C., Lesage, J., Alfenore, S., Guillouet, S. E., & Gorret, N. (2022). Study of plasmid-based expression level heterogeneity under plasmid-curing like conditions in Cupriavidus necator. *Journal of Biotechnology*, *345*(December 2021), 17–29. https://doi.org/10.1016/j.jbiotec.2021.12.015

[2] Boy, C., Lesage, J., Alfenore, S., Guillouet, S. E., & Gorret, N. (2022). Co-expression of an isopropanol synthetic operon and eGFP to monitor the robustness of Cupriavidus necator during isopropanol production. *Enzyme and Microbial Technology*, *161*(August). https://doi.org/10.1016/j.enzmictec.2022.110114

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 955740. This poster reflects only the author's view, the Agency is not responsible for any use that may be made of the information it contains.