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# Construction of synthetic bacterial consortia based on metabolic mutualism

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## Introduction

Within natural ecosystems, microorganisms live in communities and interact with each other<sup>1</sup>. **Division of Labor (DoL)** is an evolutionary strategy widely used in these communities and allows for the distribution of metabolic tasks between individuals for mutual benefit. This strategy reduces the **metabolic burden** of each individual<sup>2</sup> and microbial communities exhibit selective advantages over monocultures in terms of stability, robustness, and functionality<sup>1</sup>. However, piloting population dynamics of cocultures remains challenging as the faster-growing species may dominate over time<sup>3</sup>. One possible strategy to maintain the coexistence of populations is **cross-feeding** of metabolites, a common process in natural consortia<sup>4</sup>.

This project aims to build **mono-species synthetic consortia** composed of *Bacillus subtilis* (*Bsu*) strains exhibiting **obligate mutualism** based on **amino acids cross-feeding** (Fig 1).

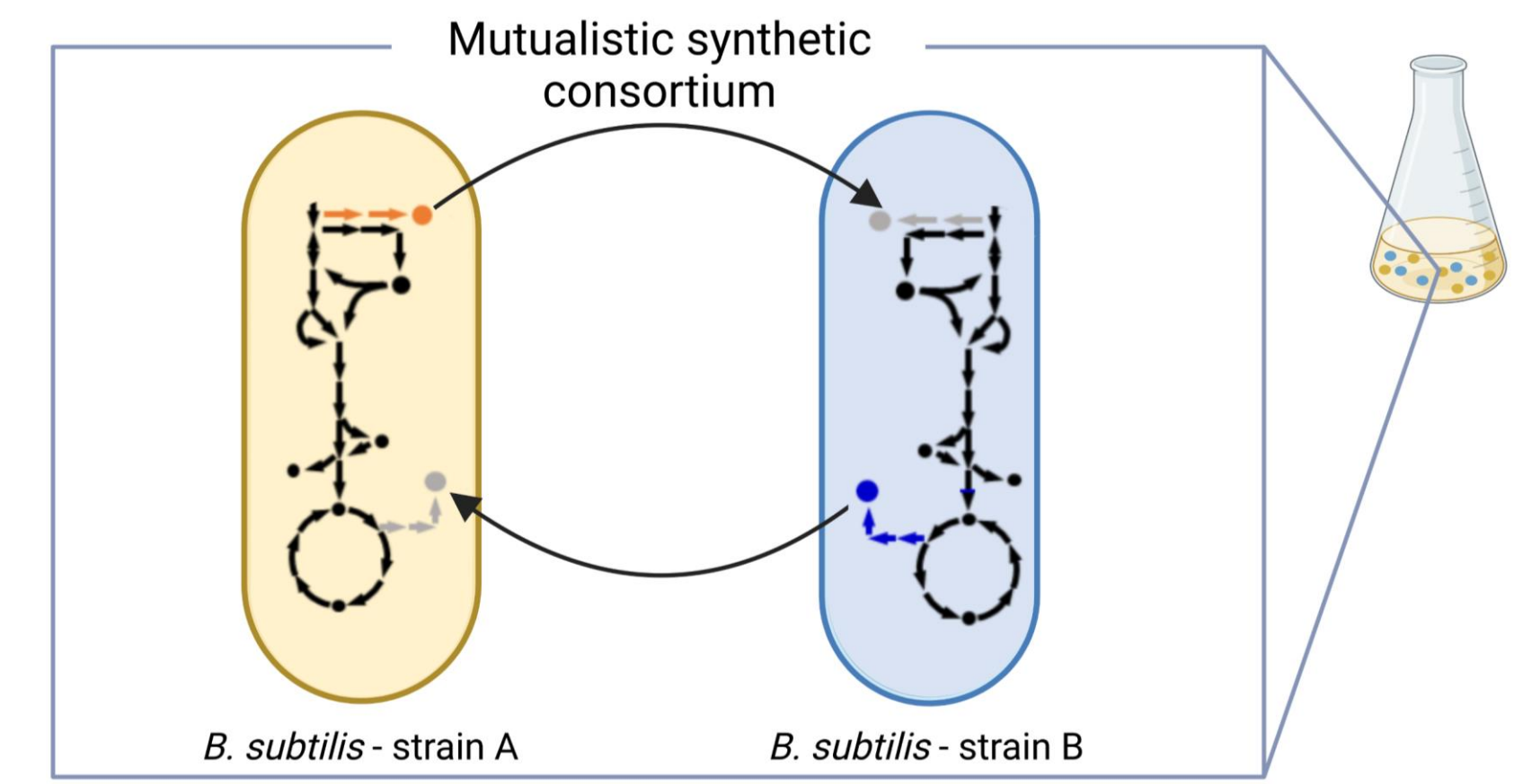


Fig 1 – Synthetic microbial consortium consisting of two *B. subtilis* strains. The exchange of amino acids, i.e., cross-feeding, is required for the growth of both strains.

## 1 - High-throughput screening of mutualistic *B. subtilis* consortia

Thirty-six *B. subtilis* strains auxotrophic for different amino acid were selected from a library of knock-out mutants<sup>5</sup>. Growth of the 36 strains in pairwise cocultures was tested in minimal medium to identify the combination of strains able to complement each other. A liquid-handling robot (Opentron) was used to automatically mix and dispense the 1296 combinations in 384-well plates. The growth of the cocultures was monitored in 384-well plate readers (Fig 2).

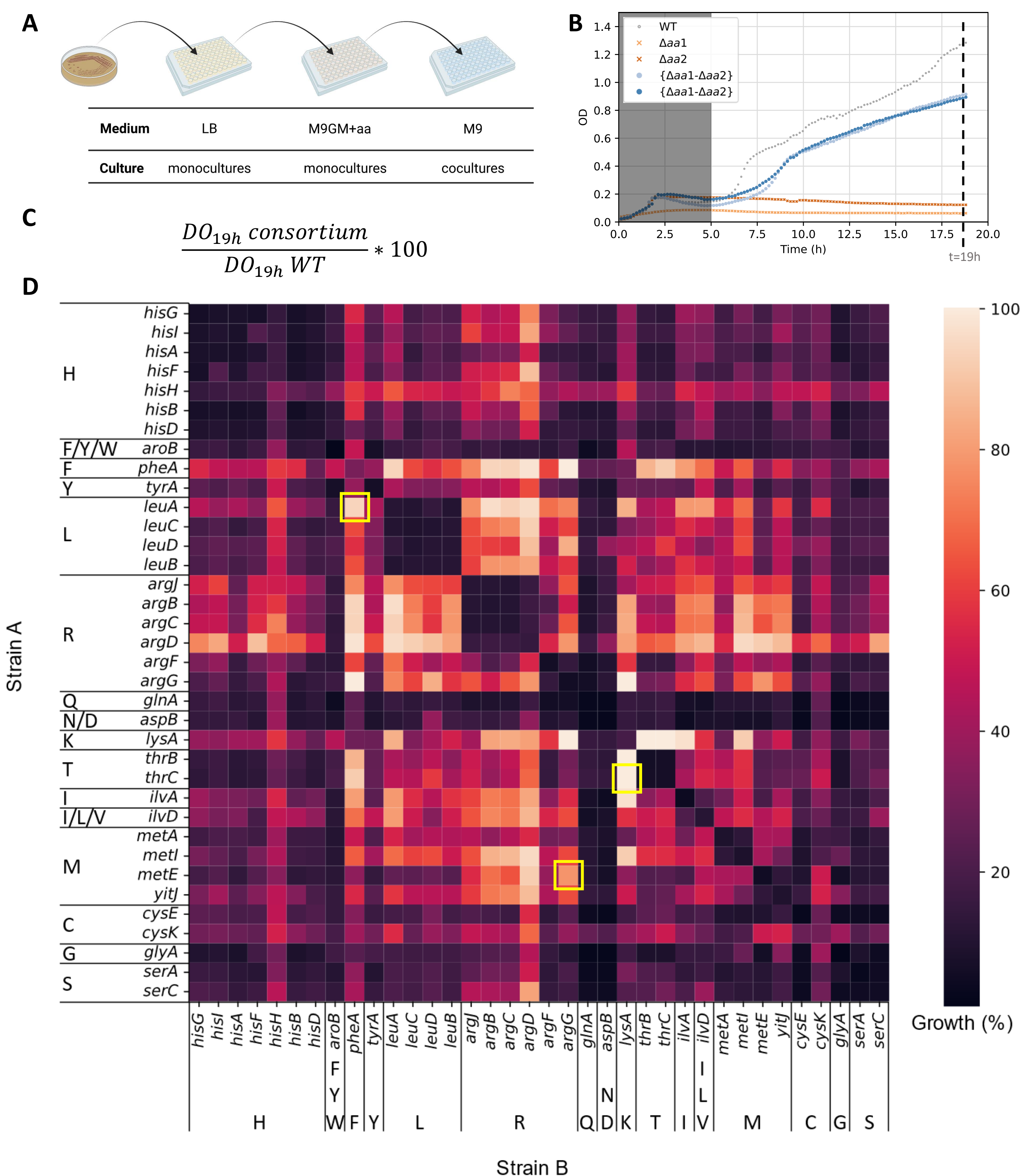


Fig 2 – (A) A first pre-culture is performed in rich medium (LB), a second in minimal medium supplemented with glucose and malate (M9GM) and amino acids. The consortia are then cultivated in 384-well plates in M9GM. The OD<sub>600nm</sub> is measured in a plate reader. (B) Growth curves of a consortium (in blue) and of the corresponding monocultures (in orange), compared to the WT (in grey) in 384-well plates. (C) Criteria for the comparison of all consortia. (D) Heatmap of the OD<sub>600nm</sub> after 19h of culture. Each square is the mean of a duplicate. Color intensity indicates the growth percentage compared to the WT strain in monoculture. Selected consortia for further characterizations are outlined in yellow.

- Identification of viable *B. subtilis* mutualistic consortia.
- Selection of { $\Delta metE$ ,  $\Delta argG$ }, { $\Delta pheA$ ,  $\Delta leuA$ } and { $\Delta lysA$ ,  $\Delta thrC$ } consortia, (80%, 94% and 122 % growth compared to the WT, respectively) for further characterization.

## Conclusion and perspectives

Viable *Bacillus subtilis* co-dependant consortia based on amino acids cross-feeding were identified via the screening of 36 strains in pairwise cocultures. The three consortia selected for further characterization, { $\Delta metE$ ,  $\Delta argG$ }, { $\Delta pheA$ ,  $\Delta leuA$ } and { $\Delta lysA$ ,  $\Delta thrC$ }, are stable and their growth rate reaches 25 to 47% compared to the WT. Proportion of both partners evolve towards a different balance for each consortium.

The fitness of the selected consortia will be improved by **directed evolution** by combining two methodologies. An inducible hypermutator system, developed by Irène Tanneur (SyBER), will be integrated in the consortia. This system increases the mutation rate per generation by a factor of 10 to 1000. Such modified consortia will be cultivated in a continuous culturing device (Fig 5) developed by Cyprien Guérin and Pierre Nicolas (MaIAGE). This chemostat allows to continuously monitor the biomass, and automatically adjust the dilution rate to maintain a desired growth rate. The variants obtained from these experiments will be isolated and characterized in order to find mutations that optimize division of labor.

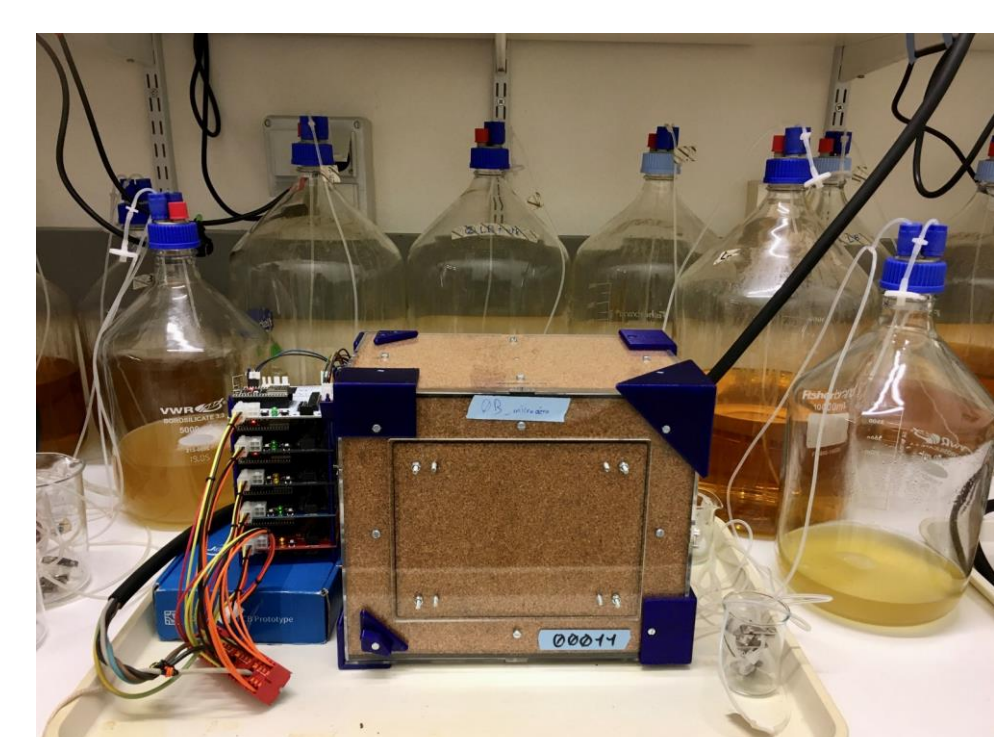


Fig 5 - Continuous culturing device developed by Cyprien Guérin and Pierre Nicolas (MaIAGE)

## 2 - Characterization of mutualistic *B. subtilis* consortia

To discriminate and quantify subpopulations within a consortium, genes expressing fluorescent proteins were integrated in the genome (Fig 3A). The growth of the cocultures in M9GM was monitored in 96-well plates and the growth rate determined (Fig 3B). Proportion of populations were analyzed by counting fluorescent colonies on plates at different time points (Fig 3C). Preliminary results also showed that FACS analysis allow to precisely monitor subpopulation dynamics over time (Fig 3D).

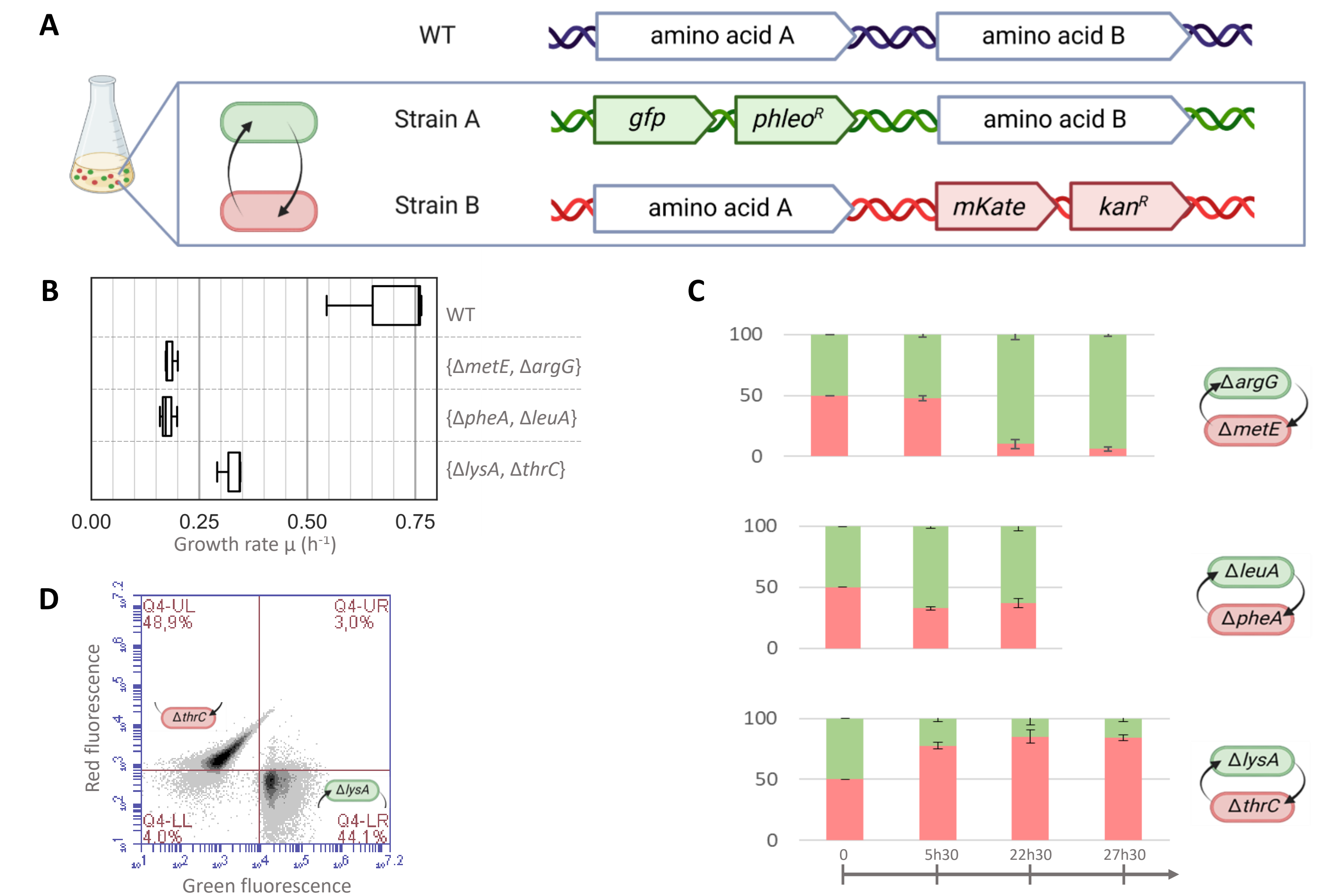


Fig 3 – (A) A cassette composed of an antibiotic resistance gene and a gene coding for a fluorescent protein were inserted in the genome. (B) Growth rate of the consortia and the WT in monoculture in M9GM. (C) Proportion, in %, of each strain in the consortium. The two strains are inoculated at a 50-50 ratio at T=0. (D) Flow cytogram of a mix of two strains (*Bsu*  $\Delta lysA$  expressing *gfp* and *Bsu*  $\Delta thrC$  expressing *mKate*).

- The selected consortia are stable over time.
- The selected consortia reach a growth rate of 0.18 h<sup>-1</sup> to 0.33 h<sup>-1</sup>.
- Proportion of subpopulations reaches an equilibrium, {6%  $\Delta metE$ , 94%  $\Delta argG$ }, {37%  $\Delta pheA$ , 67%  $\Delta leuA$ } and {16%  $\Delta lysA$ , 84%  $\Delta thrC$ }.

## 3 - Estimation of the kinetic parameters of the subpopulations

Growth curve of each subpopulation is inferred from experimental data fitting the following logistic equation:

$$f(x) = \frac{k}{1 + ae^{-\mu x}}$$

$k$  = carrying capacity,  $\mu$  = growth rate,  $x$  = optic density,  $a$  = asymptote

The estimated parameters are  $\mu$ ,  $k_A$  and  $k_B$  (Fig 4). The model assumes that as the growth of each strain is dependent on its partner, the growth rate of each subpopulation is identical. This assumption will be challenged with FACS analysis (on-going experiments).

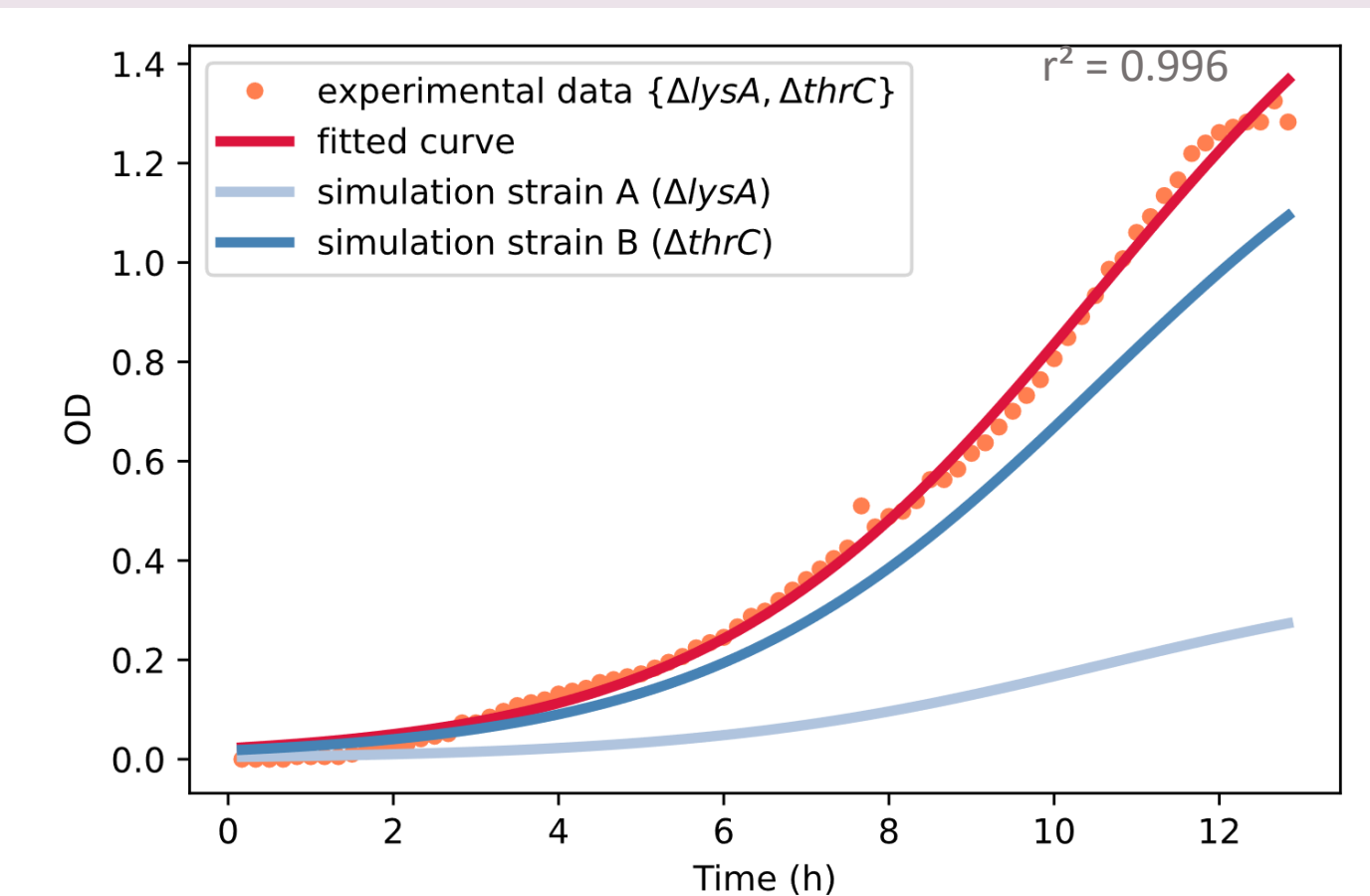


Fig 4 – A logistic function (in red) is fitted to the experimental data (in orange). The end-point proportion of subpopulations allowed to determine  $k_{\Delta lysA}=0,16*k$  and  $k_{\Delta thrC}=0,84*k$ , as well as to simulate growth of each partner (in blue).

<sup>1</sup> Rafieenia et al. 2022 - Division of Labor for Substrate Utilization in Natural and Synthetic Microbial Communities. *Current Opinion in Biotechnology* 75 (June): 102706.

<sup>2</sup> Giri et al. 2019 - Defining Division of Labor in Microbial Communities. *Journal of Molecular Biology* 431 (23): 4712-31.

<sup>3</sup> Roell et al. 2019 - Engineering Microbial Consortia by Division of Labor. *Microbial Cell Factories* 18 (1): 35.

<sup>4</sup> Pande et al. 2017 - Bacterial Unculturable and the Formation of Intercellular Metabolic Networks. *Trends in Microbiology* 25 (5): 349-61.

<sup>5</sup> Koo et al. 2017 - Construction and Analysis of Two Genome-Scale Deletion Libraries for *Bacillus Subtilis*. *Cell Systems* 4 (3): 291-305.e7.