# Construction and directed evolution of Bacillus subtilis synthetic consortia

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# Construction and directed evolution of *Bacillus subtilis* synthetic consortia

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## Introduction

Over the past two decades, synthetic biologists have harnessed microbes for tasks such as biosensing, and bioproduction, leveraging an ever-expanding array of biological tools. Synthetic circuits can be challenging, especially as they become more complex, leading to a significant burden on the host cell, which can affect their efficiency. One promising strategy to overcome these challenges is to engineer synthetic circuits within microbial consortia. These communities, composed of multiple microbial populations, are naturally occurring in ecosystems and participate in intricate interactions. The division of labor (DoL) is a common evolutionary strategy in these communities, enabling the allocation of metabolic tasks among individuals for mutual benefit (**Figure 1**). Nevertheless, the specific mechanisms that regulate these interactions have remained significantly underinvestigated. One approach to investigate these mechanisms is the rational design of interactions within model organisms.

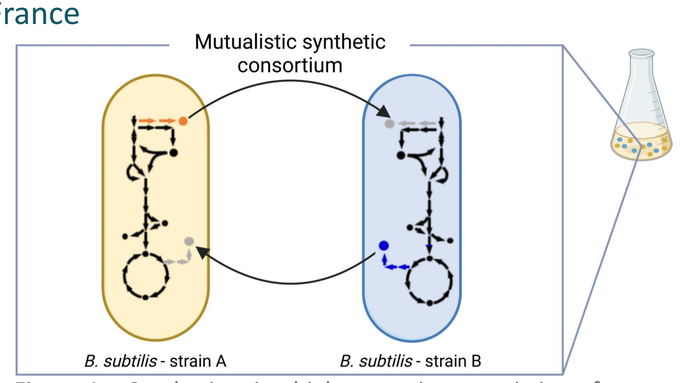



Figure 1 – Synthetic microbial consortium consisting of two *B. subtilis* strains. The exchange of amino acids, i.e., cross-feeding, is required for the growth of both strains.

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

Thirty-six *B. subtilis* strains, each with a specific amino acid auxotrophy, were selected from a knock-out mutant library[5]. They were paired and tested for mutual complementation in minimal medium. Using an Opentron liquid-handling robot, 1296 combinations were dispensed into 384-well plates, and growth was monitored using 384-well plate readers (**Figure 2**).

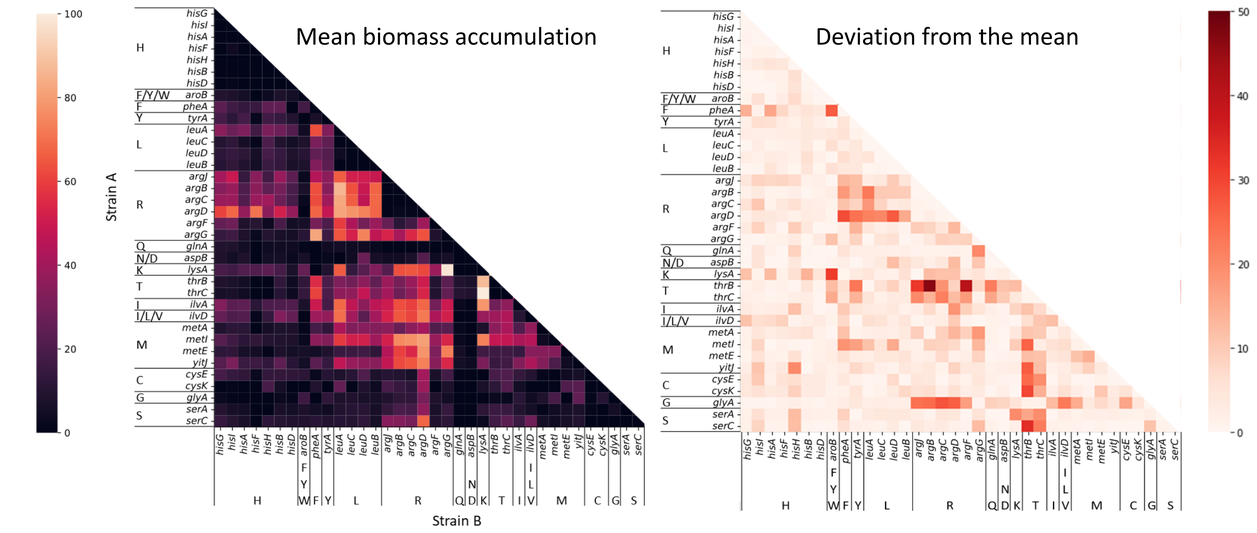



Figure 2: Heatmap of the $OD_{600nm}$ after 19h of culture. Each square is the mean of a duplicate. Color intensity indicates the growth percentage compared to the WT strain in monoculture.

- ❑ Identification of 70 viable mutualistic consortia of *B. subtilis.*
- ❑ The growth of few cocultures with auxotrophic strains deficient in the same amino acid (e.g. Δ*arg*, Δ*met*) showed the exchange of intermediate metabolites.
- ❑ Consortia exchanging **F, L, R, K, T, I, V, M** show high growth.

Based on the precedent screen, 18 auxotrophic strains were selected to assess their growth rate within consortia. The growth rates were monitored using a plate reader in a 96-wells plate, and the calculated using Python scripts (**Figure 3**).

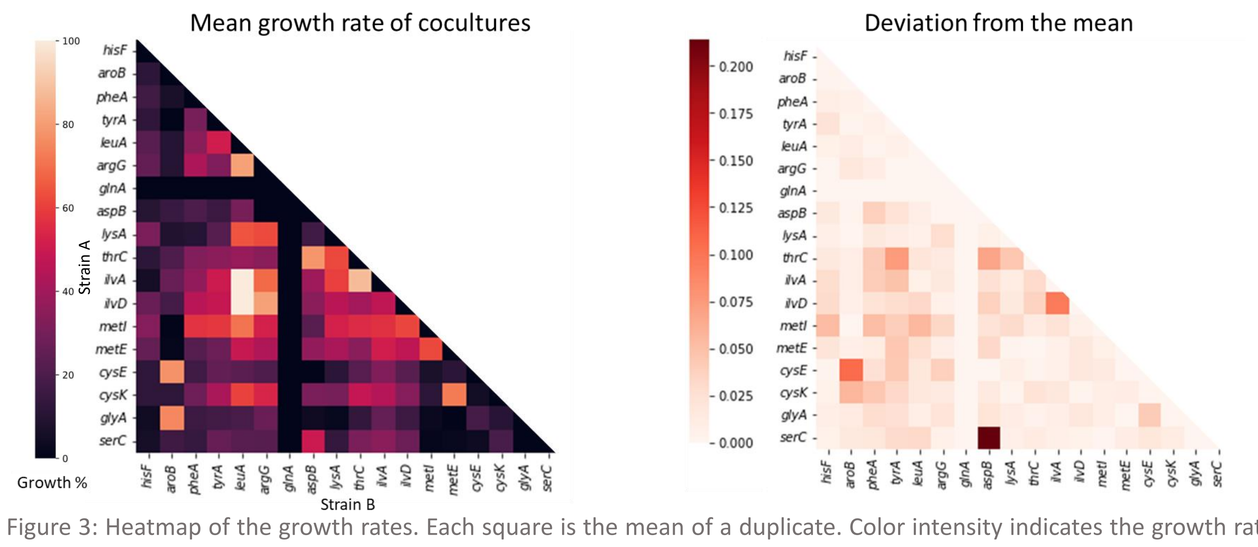



Figure 3: Heatmap of the growth rates. Each square is the mean of a duplicate. Color intensity indicates the growth rate percentage compared to the WT strain in monoculture.

- ❑ Low variability of the duplicates.
- ❑ 28 consortia with a growth rate of at least 50% compared to the wild-type.
- ❑ Consortia with growth rates similar to the WT strain : {Δ*leuA*; Δ*ilvA*} and {Δ*leuA*; Δ*ilvD*}.
- ❑ Three consortia {Δ*leuA*; Δ*pheA*}, {Δ*argG*; Δ*metE*}, {Δ*lysA*; Δ*thrC*} selected for further characterization and directed evolution.

## Characterization of mutualistic *B. subtilis* consortia in batch culture

Genes expressing fluorescent proteins were integrated into the genome to identify and quantify subpopulations within a consortium (**Figure 4A**). The growth rates of the three {Δ*leuA*; Δ*pheA*}, {Δ*argG*; Δ*metE*}, {Δ*lysA*; Δ*thrC*} consortia in M9GM (M9 with glucose and malate) were determined using a multimode microplate reader (**Figure 4B**). Population proportions were analyzed by enumerating fluorescent colonies on plates at different time points (**Figure 4C**) and by flow cytometry to precisely track the dynamics of the subpopulation over time (**Figure 4D**).

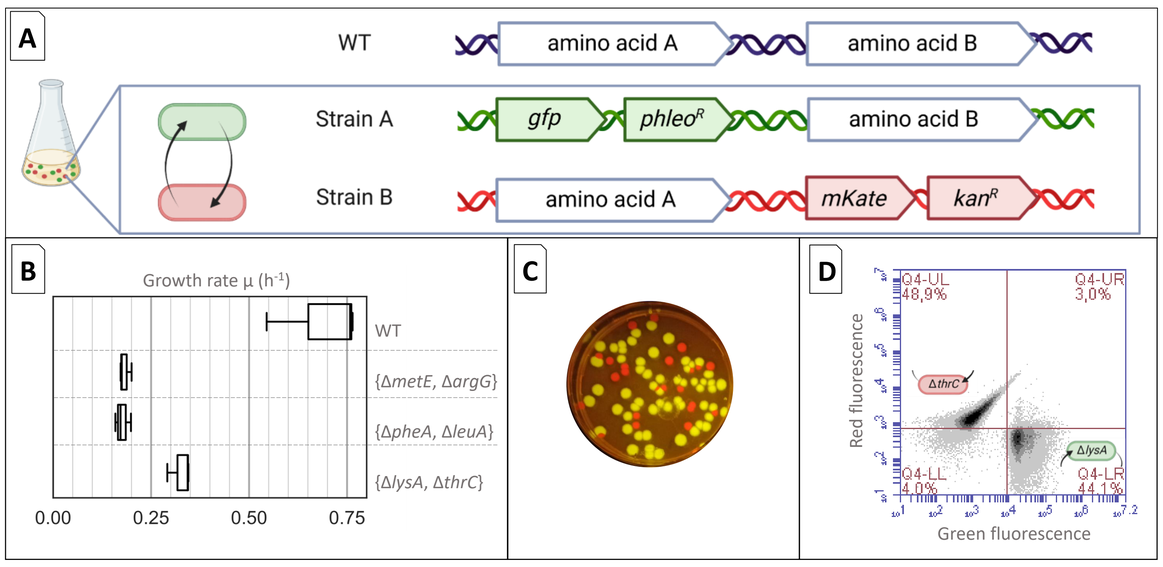
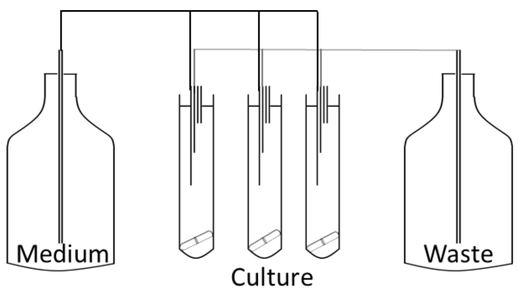



Figure 4: (A) A cassette containing a gene for fluorescent protein and an antibiotic resistance gene were integrated in the genome. (B) Growth rate of the consortia and the WT in monoculture in M9GM. (C) Consortium on LB plate. (D) Flow cytogram of a mix of two strains (*Bsu* Δ*lysA* expressing *gfp* and *Bsu* Δ*thrC* expressing *mKate*).

- ❑ Consortia stable for up to 40 generations
- ❑ Growth rate of 0.2 $h^{-1}$ for {Δ*lysA* ; Δ*thrC*}, and 0.15 $h^{-1}$ for {Δ*leuA* ; Δ*pheA*} and {Δ*argG* ; Δ*metE*}.
- ❑ Proportions of populations within the consortia are : 20%-80% for {Δ*lysA* ; Δ*thrC*}, 80%-20% for {Δ*leuA* ; Δ*pheA*} and 90%-10% for {Δ*argG* ; Δ*metE*}.

## Short-term evolution of a consortium



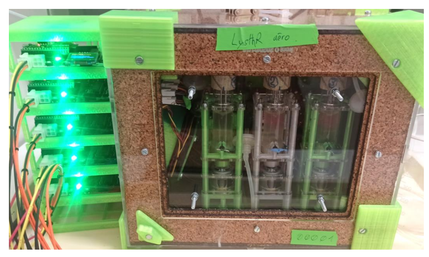



Figure 5: Continuous culturing device developed by Cyprien Guérin and Pierre Nicolas (MaIAGE)

In a recent collaboration with Cyprien Guérin and Pierre Nicolas (MaIAGE, INRAE), a state-of-the-art modular system of computer-controlled mini-bioreactors has been successfully implemented in the SyBER team **(Figure 5)**. This advanced system served as a pivotal tool for executing directed evolution experiments, precisely tailored to enhance the fitness of the {Δ*lysA*; Δ*thrC*} consortium.

Experiments were performed in triplicate, using 14 mL mini-bioreactors, with continuous monitoring of bacterial density and precise flux control. Notably, the {Δ*lysA*; Δ*thrC*} consortium grew continuously in a chemostat culture for 380 generations.

## Characterization of evolved consortia

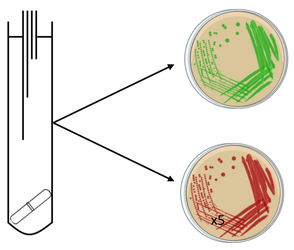



Figure 6: Isolation of variants via LB plating

Clones were isolated from the chemostat via plating on LB agar plates (**Figure 6**). The two auxotrophic subpopulations were distinguished based on their distinct fluorescence. Combinatorial co-culture of the isolated clones led to a **1.4-fold increase** in growth rate compared to the non-evolved consortium **(Figure 7)**. Subsequently, 10 clones were selected for whole genome sequencing to identify the mutations responsible for the improved performance, and are currently being characterized.

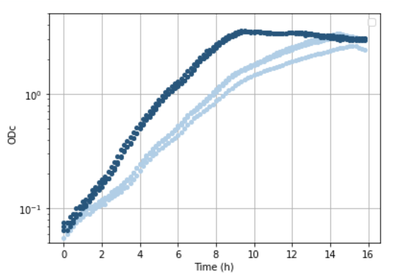



Figure 7: Growth curves of non evolved (light bluedark ) and evolved (blue) {Δ*lysA*; Δ*thrC*} consortia.

## Conclusion

We identified viable consortia of *B. subtilis* strains that co-depend on amino acids cross-feeding through screening 36 strains in pairwise cocultures. Three specific consortia, namely {Δ*metE*, Δ*argG*}, {Δ*pheA*, Δ*leuA*}, and {Δ*lysA*, Δ*thrC*}, were chosen for further characterization. These consortia are stable and exhibit a growth rate ranging from 25% to 47% compared to the wild type. Notably, the proportion of both partner strains in each consortium evolves towards a unique equilibrium. One of the selected consortia, {Δ*lysA*, Δ*thrC*}, was subjected to directed evolution to enhance its fitness. The experiment yielded variants, which were subsequently isolated and characterized to identify the mutations that would improve the division of labor within the consortium.

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