Video Article

Monitoring Intraspecies Competition in a Bacterial Cell Population by Cocultivation of Fluorescently Labelled Strains

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Abstract

Many microorganisms such as bacteria proliferate extremely fast and the populations may reach high cell densities. Small fractions of cells in a population always have accumulated mutations that are either detrimental or beneficial for the cell. If the fitness effect of a mutation provides the subpopulation with a strong selective growth advantage, the individuals of this subpopulation may rapidly outcompete and even completely eliminate their immediate fellows. Thus, small genetic changes and selection-driven accumulation of cells that have acquired beneficial mutations may lead to a complete shift of the genotype of a cell population. Here we present a procedure to monitor the rapid clonal expansion and elimination of beneficial and detrimental mutations, respectively, in a bacterial cell population over time by cocultivation of fluorescently labeled individuals of the Gram-positive model bacterium *Bacillus subtilis*. The method is easy to perform and very illustrative to display intraspecies competition among the individuals in a bacterial cell population.

Video Link

The video component of this article can be found at http://www.jove.com/video/51196/

Introduction

Soil bacteria are usually endowed with flexible regulatory networks and broad metabolic capacities. Both features enable the cells to adjust their catabolic and anabolic pathways to compete with their fellows and other microorganisms for the nutrients, which are available in a given ecological niche[1]. However, if the bacteria are unable to adapt to their environment other mechanisms may account for survival of a species. Indeed, as many bacteria proliferate fast and the populations can reach high cell densities subpopulations may have spontaneously accumulated beneficial mutations that provide the cells with a selective growth advantage and therefore increase their fitness. Moreover, mutational hotspots and stress-induced adaptive mutagenesis can facilitate the evolution of a maladapted bacterium[2,3]. Thus, accumulation of mutations and growth under continuous selection is the origin for the enormous microbial diversity, even within the same genus[4,5]. As in nature, shaping of bacterial genomes does also occur in the laboratory due to continuous cultivation under selection. This is exemplified by the domestication of the Gram-positive bacterium *B. subtilis,* which is used worldwide in basic research and in industry. In the 1940s *B. subtilis* was treated with DNA-damaging X-rays followed by cultivation under a specific growth condition[6]. The mutations that have accumulated in the bacteria during their domestication account for the loss of many growth characteristics, *i.e.* the *B. subtilis* laboratory strain 168 lost the ability to form complex colonies[7,8].

Nowadays, for the best-studied model bacteria *Escherichia coli* and *B. subtilis*, a variety of powerful tools is available to genetically manipulate their genomes in order to address specific scientific questions. Sometimes the inactivation of a gene of interest causes a severe growth defect, which is then clearly visible on standard growth medium[9]. By contrast, mutations that cause a weak growth defect and thus only slightly affect fitness of the strain are often ignored. However, in both cases prolonged incubation and passaging of the mutant strains for several generations usually result in the accumulation of suppressor mutants that have restored the phenotype of the parent strain[2,9]. The characterization of suppressor mutants and the identification of the mutations that have restored the growth defect of the parent mutant strain is a very helpful approach that allows elucidation of important and often novel cellular processes[10,11].

We are interested in the control of glutamate homeostasis in *B. subtilis*[12]. Similar to *E. coli*, *B. subtilis* responds to perturbation of glutamate homeostasis (*i.e.* block in glutamate degradation[2]) by the accumulation of suppressor mutants. The genomic alterations in these suppressor mutants that were acquired by spontaneous mutation were shown to rapidly restore glutamate homeostasis[9,13]. Therefore, it is not surprising that adaptation of *B. subtilis* to a specific growth condition during domestication of the bacterium is mirrored in enzyme synthesis and in the evolved enzymatic activities, which are involved in glutamate metabolism[12]. It has been suggested that the lack of exogenous glutamate in the growth medium during the domestication process was the driving force for the emergence and fixation of the cryptic glutamate dehydrogenase (GDH) *gudB*^{CR} gene in the laboratory strain 168[2,14]. This hypothesis is supported by our observation that the reduced amount of GDH activity in the laboratory strain provides the bacteria with a selective growth advantage when exogenous glutamate is scarce[2]. Moreover, cultivation of a *B. subtilis* strain, synthesizing the GDH GudB, in the absence of exogenous glutamate results in the accumulation of suppressor mutants that have inactivated the *gudB* gene[2]. Obviously, the presence of a catabolically active GDH is disadvantageous for the cell because endogenously

produced glutamate that could otherwise be used for anabolism is degraded to ammonium and 2-oxoglutarate (**Figure 1**). By contrast, when glutamate is provided by the medium, a *B. subtilis* strain equipped with high-level GDH activity has a selective growth advantage over a strain that synthesizes only one functional GDH. It is reasonable to assume that high-level GDH activity allows the bacteria to utilize glutamate as a second carbon source in addition to other carbon sources provided by the medium[2] (see **Figure 1**). Thus, GDH activity strongly affects fitness of bacteria, depending on the availability of exogenous glutamate.

Here we present a very illustrative method to monitor and to visualize intraspecies competition between two *B. subtilis* strains that differ in a single locus on the chromosome (**Figure 2**). The two strains were labeled with the *yfp* and *cfp* genes encoding the fluorophores YFP and CFP, and cocultivated under different nutritional conditions. By sampling over time and by plating appropriate dilutions on agar plates the survivors in each of the cultures could be easily monitored using a common stereo fluorescence microscope. The procedure described in this paper is easy to perform and suitable to visualize the rapid clonal expansion and elimination of beneficial and detrimental mutations, respectively, in a cell population over time.

Protocol

1. Preparation of Agar Plates, Culture Media, Cryostocks, and Precultures

1. Prepare growth media and required reagents (see table of materials and reagents).
2. Streak the *B. subtilis* strains (*e.g.* BP40 (*rocG*$^{+}$ *gudB*^{CR} *amyE::P*$_{gudB}$*-yfp*) and BP52 (*rocG*$^{+}$ *gudB*$^{+}$ *amyE::P*$_{gudB}$*-cfp*) expressing one and two active GDHs, respectively)[2] that will be used in the competition experiment on SP medium agar plates to get single colonies. Incubate the plates overnight at 37 °C.
3. Take single colonies and inoculate sterile culture tubes containing 4 ml LB liquid medium. Grow the bacteria overnight at 28 °C and 220 rpm.
4. Grow overnight cultures at 28 °C to avoid that the cells lyse or sporulate.
5. Measure the optical density of the cultures at a wavelength of 600 nm (OD_{600}) using a standard spectrophotometer.
6. If the cultures have reached an OD_{600} of 2.0, mix 0.75 ml of the cultures with 0.75 ml of a sterile 50% glycerol solution in 1.5 ml reaction tubes. The final OD_{600} should be 1.0 to obtain cryostocks containing about 10^{8} cells/ml.
7. Store the tubes at -80 °C.
8. Make three precultures of each strain labeled with the *cfp* and *yfp* encoding fluorophore genes. Inoculate the precultures (sterile culture tubes containing 4 ml LB liquid medium) with 1 µl cells from -80 °C cryostocks. Incubate the cultures overnight at 28 °C and 220 rpm.

2. Cocultivation of Bacteria, Sample Collection, and Sample Storage

1. Freshly prepare 100 ml C-Glc and CE-Glc minimal medium (see table of reagents and materials), and transfer 20 ml of each medium into sterile 100 ml shake flasks.
2. Take 0.1 ml of the precultures that were grown overnight, dilute them with 0.9 ml LB medium in a 1.5 ml cuvette, and determine the OD_{600}.
3. For the competition experiment, take those precultures of the different strains that have a similar OD_{600} between 1.0-1.5.
4. To obtain mixed cell populations, dilute the cells of the precultures that had the appropriate OD_{600} to an OD_{600} of 0.05 in 20 ml C-Glc and CE-Glc minimal medium supplemented in 100 ml shake flasks. For the competition experiment the two strains should be mixed in a 1:1 ratio.
5. Take 10 ml samples from the flasks, transfer them to 15 ml plastic tubes, harvest the cells by centrifugation for 10 min at 4,000 x g in a standard table top centrifuge, and discard the supernatant.
6. Resuspend the cells in 0.5 ml fresh LB medium, and transfer the cells to a sterile 1.5 ml reaction cup. Add 0.5 ml of 50% sterile glycerol, mix the suspension by rigorous vortexing, and store the samples in an -80 °C freezer until further treatment.
7. Incubate the cells that are left in the shake flasks for up to 24 hr at 37 °C and 220 rpm. Keep the shake flasks in the dark to prevent photo bleaching of the fluorophores.
8. Take further samples of 0.1 ml after 7 hr and 24 hr of growth. Measure and note the OD_{600} of 1:10 dilutions (in C-Glc or CE-Glc minimal medium) of the samples.
9. Take the appropriate amount of cells from each culture to make cryostocks that have an OD_{600} of 1.0. Store the samples at -80 °C until further treatment.

3. Sample Treatment, Plating, and Incubation for Quantitative Analyses

1. After collecting all samples, thaw the cryostocks, and dilute the cells in a 0.9% saline solution (see table of reagents and materials) up to 10^{-3}.
2. Plate 0.1 ml of the 10^{-3} dilutions on SP medium agar plates and distribute the cells using sterile glass pipettes.
3. Incubate the plates overnight at 37 °C in the dark until single colonies have appeared.

4. Counting the Survivors by Stereo Fluorescence Microscopy for Quantitative Analysis

1. Divide the bottom of the agar plate with a black pen in four parts for a better orientation while counting the survivors under the stereo fluorescence microscope.
2. Place the plate upside down under the microscope and bring the colonies into focus using the cold light source.
3. Once the colonies are in focus, switch to the appropriate filter set for CFP to visualize the surviving cells of the *cfp*-labeled strain. Count the survivors of this strain by labeling the colonies with a pen and note the number.
4. Remove the labels with ethanol and switch to the YFP filter set to visualize the surviving cells of the *yfp*-labeled strain. Count the survivors again by labeling the colonies with a pen and note the number.

5. Sample Treatment and Microscopy for Semiquantitative Analyses

1. For illustrative figures, spot 10 µl of the 10^{-4} dilution (approximately 100 cells) from step 3.1 on a SP agar plate (see **Figure 3**).
2. Incubate the plates overnight at 37 °C in the dark until single colonies have appeared.
3. Place the Petri dish without lid under the microscope and bring the spot into focus using the cold light source.
4. Take pictures of the spots for illustrative figures. Choose an appropriate exposure time for taking the pictures of the colonies with the cold light source.
5. Without moving the plate, change to the CFP filter set, adjust the exposure time and take a picture. Do the same with the YFP filter set and save the pictures for further analyses.

6. Data Analysis

1. Use software such as Excel for the quantitative data analyses. Based on the counted colonies, calculate the percentage of yellow and blue colonies with respect to the whole number of colonies, which are set to 100%.
2. Use the calculated numbers to create a stacked bar diagram (see **Figure 4** and **Figure 5**). Use an image-processing program such as Adobe Photoshop to construct merged pictures of the pictures taken from the different spots. Alternatively, the freely available software ImageJ, downloadable from http://rsbweb.nih.gov/ij/ can be used for image processing.
3. Open the pictures from one spot that were taken with the CFP filter set and the YFP filter set. Optimize the contrast and the brightness to reduce any background fluorescence from the media.
4. Go to one of the pictures and select all. Copy the picture and paste it onto the other picture.
5. Either use the function “color dodge” to merge the pictures or overlay the fluorescent pictures using the channels tab. Merged pictures of the colonies from different media and different time points represent the growth of the different strains within the liquid culture.

7. Specific Tips: Dye Switch Experiment and Cocultivation of Isogenic Strains Labeled with *cfp* and *yfp*

The expression of either of the two fluorophore-encoding genes in *B. subtilis* might influence fitness and thus the growth rate of the bacteria. Therefore, it is recommended to perform the following experiments in order to exclude that the elimination of one competitor strain from the cell population during cultivation is simply due to a negative effect of the fluorophore:

1. Repeat the whole experiment and cocultivate inversely labeled strains (*e.g.* the earlier *cfp*-labeled strain is now labeled with the *yfp* gene and *vice versa*). Although inverse, the obtained results should be comparable to the previous observation that one strain has a selective growth advantage over the other strain.
2. Repeat the whole experiment and cocultivate isogenic strains labeled with *yfp* and *cfp* (*e.g.* BP40 (*rocG*$^{+}$ *gudB*^{CR} *amyE::*P_{gudB}*-yfp*) and BP41 (*rocG*$^{+}$ *gudB*^{CR} *amyE::*P_{gudB}*-cfp*)). Estimate the negative effect of either of the two fluorophores on fitness of the bacteria by following the composition of the cell population over time.

Representative Results

The method described here was successfully applied to visualize intraspecies competition in a cell population consisting of *B. subtilis* strains that were labeled with the *cfp* and *yfp* genes encoding the fluorophores CFP and YFP, respectively. As shown in **Figure 3**, the method can be used to visualize intraspecies competition in a very illustrative manner. By spotting the samples on small areas, the clonal composition of the cell population was made visible at a glance. Although not appropriate for quantitative analyses, this approach is useful for roughly estimating the effect of different growth parameters (*i.e.* nitrogen source) on the development of a cell population that initially contained both strains in equal amounts (**Figure 3**). Moreover, in a small-scale approach the fitness of different *B. subtilis* strains that were cultivated under the same growth condition can be tested using a single agar plate. For quantitative analyses it is recommended to propagate the samples over the whole surface of an agar plate. This will prevent overlay of the colonies and thus allows the distinct identification and count of colonies that emerged from single cells. By plating appropriate dilutions on agar plates the clonal composition of a cell population over time can be precisely determined simply by counting the yellow and blue fluorescent colonies (see **Figure 4**). As we have previously reported, GDH activity strongly affects fitness of *B. subtilis* depending on the availability of exogenous glutamate[2]. Obviously, in the absence of exogenous glutamate high-level GDH activity is disadvantageous for the bacteria as the enzymes RocG and GudB degrade glutamate that is needed in anabolism (see **Figure 1** and **Figure 4A**). By contrast, if provided to the bacteria, glutamate can serve as an amino group donor in transamination reactions. Moreover, glutamate can be fed into carbon metabolism and used as a source of energy due to the presence of the catabolically active GDHs RocG and GudB (**Figure 1** and **Figure 4B**). As shown in **Figures 4C** and **4D**, similar results were obtained in a dye-switch experiment. Again, bacteria equipped with high-level GDH activity were outcompeted by cells with reduced GDH activity in growth media lacking glutamate. By contrast, bacteria synthesizing only one active GDH were eliminated from the culture when the medium was supplemented with glutamate. As shown in **Figures 5A** and **5B**, the initial composition of the mixed cell population remained almost constant over time. Thus, in the competition experiment the elimination of either of the two strains that were equipped with different amounts of GDH activity was not due to a growth defect caused by the fluorophores (see **Figure 4**). Taken together, the usage of fluorophores is a powerful tool for monitoring intraspecies competition in a bacterial cell population.

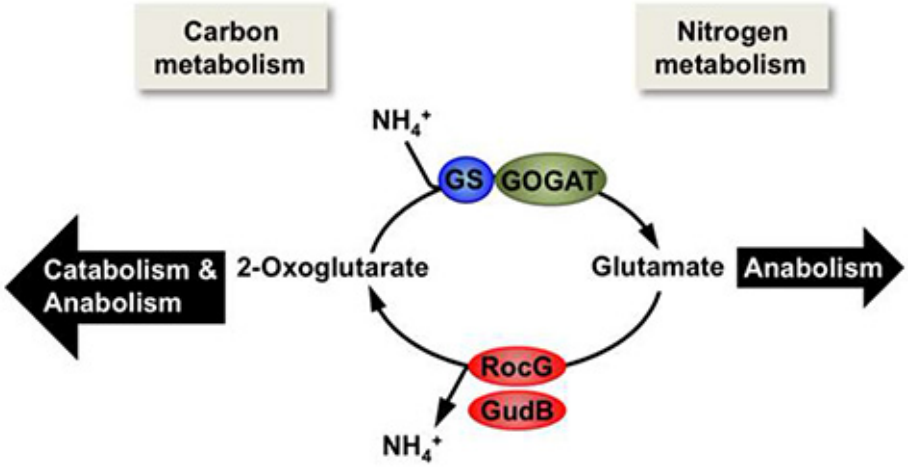

Figure 1. The link between carbon and nitrogen metabolism in *B. subtilis*. When glutamate is not provided by the medium, the major amino donor that is needed for anabolism is synthesized from ammonium and 2-oxoglutarate by the combined action of the glutamine synthetase (GS) and the glutamate synthase (GOGAT). By contrast, in the presence of exogenous glutamate the catabolically active GDHs RocG and/or GudB can degrade glutamate to ammonium and 2-oxoglutarate, which then serves as a carbon source.

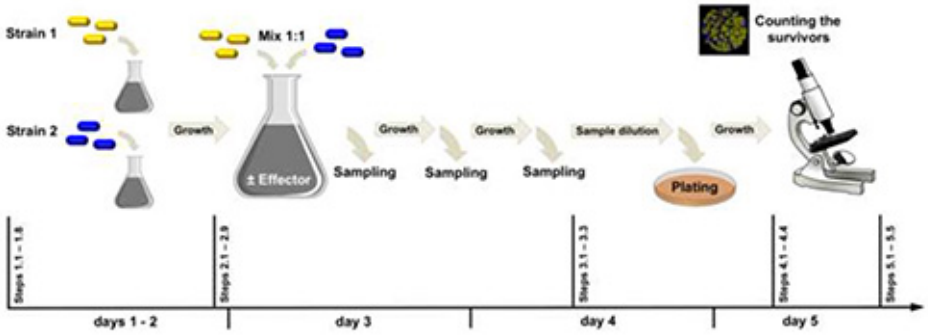

Figure 2. Experimental workflow. Strain 1 (labeled with *yfp*) and strain 2 (labeled with *cfp*) differ in one locus from each other. In the example presented here, we have compared the effect of exogenous glutamate (effector) on the genotypic shift of the cell population that initially contained 50% of *rocG*$^{+}$ *gudB*$^{+}$ (encoding two active GDHs) and 50% of *rocG*$^{+}$ *gudB*^{CR} (encoding one active GDH) cells. Click here to view larger image.

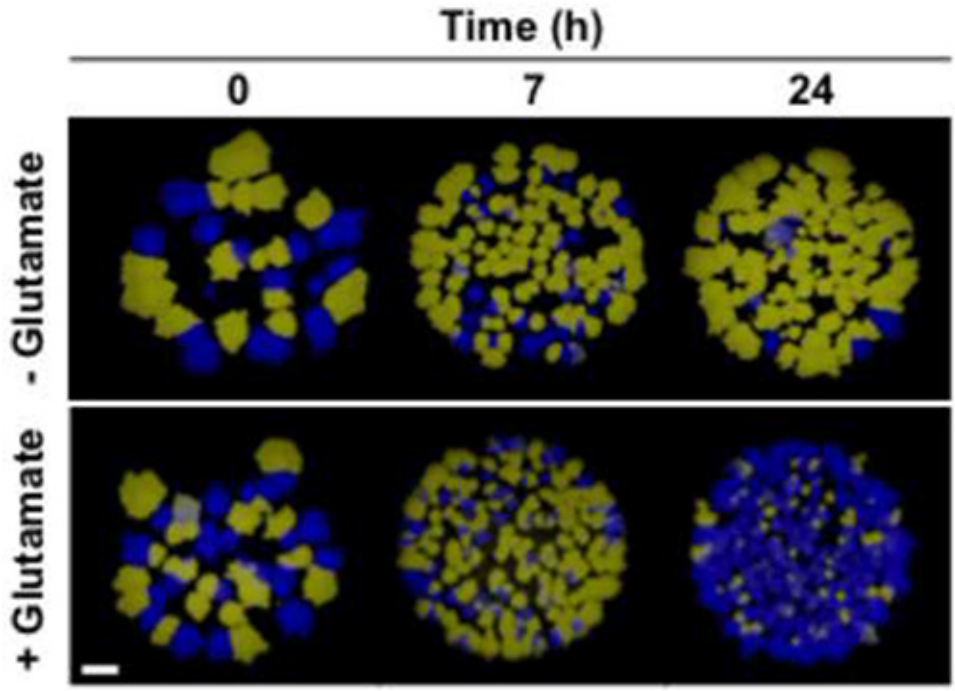

Figure 3. Semiquantitative approach to visualize intraspecies competition in a descriptive way (see section 5). Prior to cocultivation (0 hr), and after 7 hr and 24 hr of growth dilutions (10^{-4}) of cells were spotted on SP agar plates. The surviving cells that have formed colonies after 12 hr of incubation at 37 °C were identified by stereo fluorescence microscopy. Exposure time, 0.6 sec; scale bar, 1 mm. This figure was modified from Gunka *et al*. 2013[2].

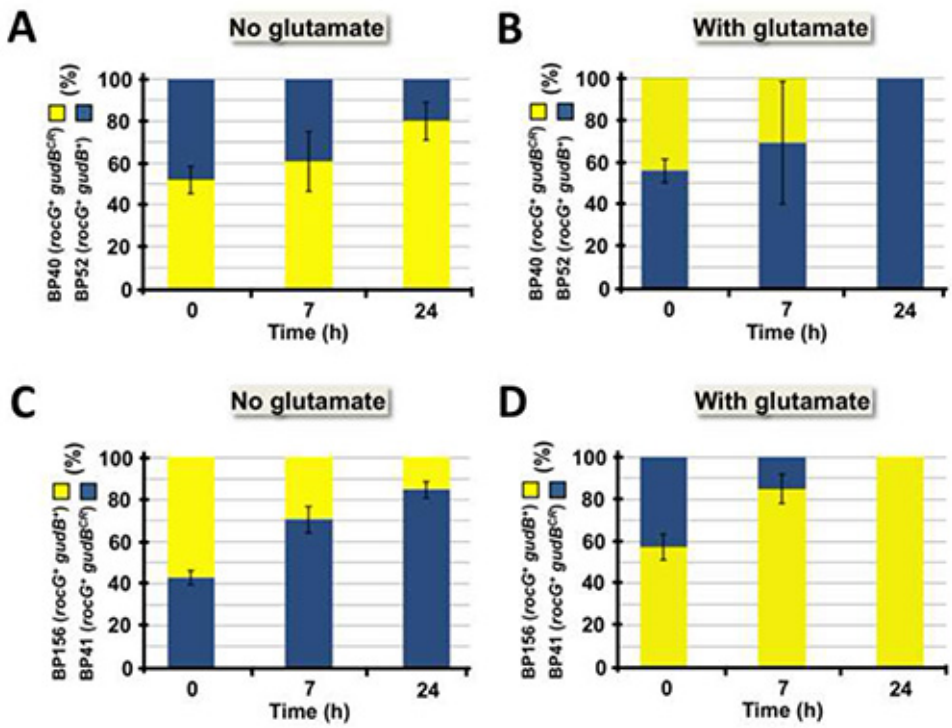

Figure 4. Quantification of intraspecies competition. After sample dilution and propagating the cells (see steps 3.1-3.3) on SP medium the plates were incubated overnight at 37 °C. Yellow and blue colonies were quantified as described in Protocols 4 and 6. The black error bars represent standard deviations for at least four independently repeated experiments. Each agar plate contained at least 100 countable colonies. (**A**) In the absence of exogenous glutamate the *B. subtilis* strain BP40 (yellow) equipped with only one functional GDH outcompetes strain BP54 (blue), which synthesizes both glutamate-degrading enzymes, RocG and GudB. (**B**) By contrast, synthesis of two functional GDHs is advantageous for the bacteria when exogenous glutamate is available because in addition to glucose, glutamate is used as a carbon source. As shown in (**C**) and (**D**), comparable results were obtained in a dye switch experiment. This figure was modified from Gunka *et al.* 2013[2]. Click here to view larger image.

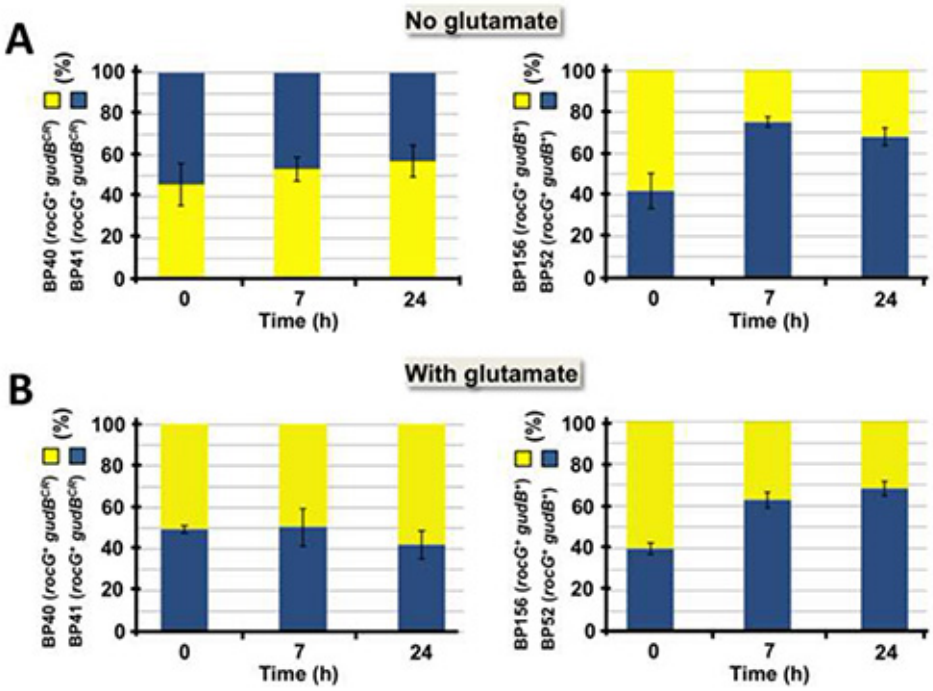

Figure 5. Control experiment to evaluate the effect of the fluorophore-encoding *cfp* and *yfp* genes on fitness of the bacteria. Mixed populations of the isogenic strains BP40 (*rocG*$^{+}$ *gudB*^{CR} *amyE::yfp*) and BP41 (*rocG*$^{+}$ *gudB*^{CR} *amyE::cfp*) or BP52 (*rocG*$^{+}$ *gudB*$^{+}$ *amyE::cfp*) and BP156 (*rocG*$^{+}$ *gudB*$^{+}$ *amyE::yfp*) were grown in the absence (**A**) and in the presence (**B**) of exogenous glutamate. The surviving cells were counted as described in Protocols 1-4 and 6, respectively. The bars represent standard deviations for at least four independently repeated experiments. This figure was modified from Gunka *et al*. 2013[2]. Click here to view larger image.

Discussion

Several methods have been developed to analyze competitive fitness of bacteria[16]. In many cases the bacteria were labeled with different antibiotic resistance cassettes[17]. Similar to our approach, labeling of the cells with antibiotic resistance cassettes allows the evaluation of competitive fitness of the bacteria during cocultivation under defined growth conditions. Moreover, this method can be used to determine competitive fitness of cells that differ from each other in a specific locus on the chromosome[17]. However, there are some disadvantages using antibiotic resistance cassettes to monitor competitive fitness. As the expression of the resistance genes is mostly driven by promoters having unequal strength the enzymes conferring resistance to the antibiotics are probably produced at different levels. Therefore, weak fitness effects may be not detectable with this approach. In our approach, both fluorophore genes were integrated with the same selection marker and their expression is driven by the same promoters[2]. Another disadvantage to monitor competitive fitness with antibiotic resistance cassettes might be that the approach is more laborious as two types of agar plates supplemented with the appropriate antibiotics are needed for the colony counting. Alternatively, fitness of bacteria can be determined by simply monitoring the growth rate and by the calculation of a so-called fitness index[16]. Obviously, this is the most precise approach because the bacteria are cultivated individually and toxic compounds that might be produced by a strain with a certain genotype will not affect growth of the competing strain. Moreover, there is no need to use antibiotics that might affect growth of the cells. However, both approaches are not very illustrative as the numbers describing competitive fitness can only be presented in a rather neutral way.

The use of fluorophore encoding genes to monitor and quantify intraspecies competition has several advantages over other methods. If both strains have integrated the fluorophore encoding genes by double homologous recombination into the chromosome, there is no need to use antibiotics in any of the cultivation steps. Therefore, samples that are taken from the culture during cultivation can be analyzed on the same growth medium and both strains are capable of growing. This approach allows visualization of intraspecies competition in a very illustrative way. Moreover, using this semiquantitative approach several growth conditions can be tested at the same time and many different strains can be compared in parallel. Finally, there is no need for fixation of the samples on microscope slides because the samples that were taken from the bacterial culture during cocultivation can be stored in a freezer[18]. Thus, all samples and replicates can be analyzed at the same time.

Two critical steps in our protocol have to be mentioned. It is important to note that the cryostocks should contain equal amounts of cells in the same growth phase of each competitor strain. An initial disproportion of the strains in the cryostocks and consequently in the shake flasks before starting the experiment will have a strong impact on the outcome of the competition experiment. Therefore, it is wise to check the composition of the cryostocks prior to the experiment. Moreover, an appropriate amount of cells should be evenly propagated over the plate. Otherwise the emerged colonies are too close to each other and a precise determination of the surviving cells will become difficult.

There are also some limitations and drawbacks of the fluorophore-based approach. Cocultivation in a multi-well plate reader and simultaneous detection of the CFP and YFP signals is not possible as the excitation and emission spectra of the fluorophores are too close to each other. However, this technical problem might be circumvented using different fluorescent proteins, such as those emitting green and red light, optimally

based upon the same protein scaffold. Another drawback of the fluorophore-based approach could be that some mutations cause only a weak growth defect of the bacteria. Thus, if the growth defect that might be caused by either of the fluorophore-encoding genes is stronger than the growth defect caused by a certain mutation, the fluorophore-based approach is not appropriate to analyze intraspecies competition. Therefore, before creating a whole set of strains it is recommended to first label only the parent strain with both, *cfp* and *yfp,* and to cocultivate the strains. The growth experiments will reveal how strong the fluorophores affect fitness of the bacteria (see **Figures 5A** and **5B**).

In the future it will be interesting to test whether the fluorophore-based approach to monitor intraspecies competition will be more accurate and less laborious if the surviving cells are counted using flow cytometry. Recently, flow cytometry has been shown to be a powerful tool to analyze the composition of *B. subtilis* biofilms[19]. Moreover, it might be more appropriate to analyze weak fitness effects that affect competitive fitness of bacteria by continuous cocultivation of the fluorophore-labeled bacteria in a fermenter. In contrast to shake flasks, this approach allows to keep the growth conditions constant and thus to monitor intraspecies competition over a long period of time.

Disclosures

The authors declare that they have no competing financial interests.

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References

1. Buescher, F.I., *et al*. Global network reorganization during dynamic adaptations of *Bacillus subtilis* metabolism. *Science*. **335**, 1099-1103 (2012).
2. Gunka, K., Stannek, L., Care, R.A. & Commichau, F.M. Selection-driven accumulation of suppressor mutants in *Bacillus subtilis*: the apparent high mutation frequency of the cryptic *gudB* gene and the rapid clonal expansion of *gudB*$^+$ suppressors are due to growth under selection. *PLoS One*. **8**, e66120 (2013).
3. Al Mamum, A.A.M., *et al.* Identity and function of a large gene network underlying mutagenic repair of DNA breaks. *Science*. **338**, 1344-1348 (2012).
4. Koeppel, A.F., Wertheim, J.O., Barone, L., Gentile, N., Krizanc, D. & Cohan, F.M. Speedy speciation in a bacterial microcosm: new species can arise as frequently as adaptations within a species. *ISME J.* **7**, 1080-1091 (2013).
5. Maughan, H. & Nicholson, W.L. Increased fitness and alteration of metabolic pathways during *Bacillus subtilis* evolution in the laboratory. *Appl. Environ. Microbiol*. **77**, 4105-4118 (2011).
6. Burkholder, P.R. & Giles, N.H. Induced biochemical mutations in *Bacillus subtilis*. *Am. J. Bot.* **34**, 345-348 (1947).
7. McLoon, A.L., Guttenplan, S.B., Kearns, D.B., Kolter, R. & Losick, R. Tracing the domestication of a biofilm-forming bacterium. *J. Bacteriol*. **193**, 2027-2034 (2011).
8. Zeigler, D.R., *et al*. The origins of 168, W23, and other *Bacillus subtilis* legacy strains. *J. Bacteriol*. **190**, 6983-6995 (2008).
9. Gunka, K., Tholen, S., Gerwig, J., Herzberg, C., Stülke, J. & Commichau, F.M. A high-frequency mutation in *Bacillus subtilis*: requirements for the decryptification of the *gudB* glutamate dehydrogenase gene. *J. Bacteriol*. **194**, 1036-1044 (2012).
10. Commichau, F.M., *et al*. Characterization of *Bacillus subtilis* mutants with carbon source-independent glutamate biosynthesis. *J. Mol. Microbiol. Biotechnol*. **12**, 106-113 (2007).
11. Beckwith, J. Genetic suppressors and recovery of repressed biochemical memory. *J. Biol. Chem*. **284**, 12585-12592 (2009).
12. Gunka, K. & Commichau, F.M. Control of glutamate homeostasis in *Bacillus subtilis*: a complex interplay between ammonium assimilation, glutamate biosynthesis and degradation. *Mol. Microbiol*. **85**, 213-224 (2012).
13. Yan, D. Protection of the glutamate pool concentrations in enteric bacteria. *Proc. Natl. Acad. Sci. U.S.A*. **104**, 9475-9480 (2007).
14. Belitsky, B.R. & Sonenshein, A.L. Role and regulation of *Bacillus subtilis* glutamate dehydrogenase genes. *J. Bacteriol*. **180**, 6298-6305 (1998).
15. Commichau, F.M., Herzberg, C., Tripal, P., Valerius, O. & Stülke, J. A regulatory protein-protein interaction governs glutamate biosynthesis in *Bacillus subtilis*: the glutamate dehydrogenase RocG moonlights in controlling the transcription factor GltC. *Mol. Microbiol*. **65**, 642-654 (2007).
16. Gordo, I., Perfeito, L. & Sousa, A. Fitness effects of mutations in bacteria. *J. Mol. Microbiol. Biotechnol*. **21**, 20-35 (2011).
17. Rabatinová, A., *et al*. The δ subunit of RNA polymerase is required for rapid changes in gene expression and competitive fitness of the cell. *J. Bacteriol*. **195**, 2603-2611 (2013).
18. Capra, E.J., Perchuk, B.S., Skerker, J.M. & Laub, M.T. Adaptive mutations that prevent crosstalk enable the expansion of paralogous signalling protein families. *Cell*. **150**, 222-232 (2012).
19. García-Betancur, J.C., Yepes, A., Schneider, J. & Lopez, D. Single-cell analysis of *Bacillus subtilis* biofilms using fluorescence microscopy and flow cytometry. *J. Vis. Exp*. **15**, doi:pii: 3796. 10.3791/3796 (2012).

