­Predicting competition results from growth curves

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# Abstract

Measuring microbial growth in a mixed culture is laborious and expensive as it requires the insertion or identification of unique genotypic or phenotypic markers. We have developed *Curveball* (available at <http://curveball.yoavram.com>), a new method for predicting growth in mixed culture from growth curve data using a revisited population growth model and a newly derived competition model. We validated *Curveball*'s predictions using growth curve and competition experiments with bacteria and yeast. Our method not only result in a simpler and more cost-effective approach for analyzing growth in mixed culture and inferring relative fitness in microbes, but also provide information on the specific growth traits that contribute to differences in fitness and contributes to the integration between population dynamics and population genetics.

# Introduction

A key issue in microbial ecology and evolutionary biology lies in understanding the factors underlying the relationship between growth in monoculture and mixed culture. Such differences have a crucial impact on composition of microbial populations and communities and on microbial fitness. The advent of high-throughput experiments and omics data in microbiology yields information in unprecedented amount and detail. However, development of models for meaningful biological interpretation of this information is ongoing.

## Predicting growth in mixed culture

For over a century, important discoveries have been made by studying growth in monoculture in bacteria (Monod 1949) and yeast (Pearl 1927). However, growth in mixed culture is still poorly understood and often requires the construction of custom models for specific sets of species and strains (Yurtsev et al. 2013).

## From growth to fitness

Models in population dynamics focus on changes in population size and consider a number of growth traits, such as growth rate, maximum population size, and lag duration. Population genetic models focus on changes in frequencies of genotypes within populations and usually assume a single fitness value per genotype. There is statistical correlation between empirical estimates of growth traits and fitness (Bell 2010); however, there is currently no method to predict or interpret fitness from a combination of growth traits.

## Growth curves

Growth curves are commonly used to estimate fitness in microbiology, genetics, and evolutionary biology. Growth curves are acquired by measuring the optical density (OD) of one or more populations of cells growing in liquid culture over a range of time. The simplest way to infer fitness from growth curves is to estimate the growth rate during the exponential growth phase. This is done by taking the log of the mean of the growth curves during the exponential growth phase and using linear regression to estimate the slope of the curve as a measure of the growth rate (Hall et al. 2014). Indeed, growth rates can be proxies of the selection coefficient, *s*, which is a standard approach for representing relative fitness in population genetics (Crow and Kimura 1970; Chevin 2011). However, the selection coefficient can be affected by other phases of growth, such as the lag phase and the stationary phase. Thus, it is not surprising that growth rates can be poor estimates of relative fitness (Concepción-Acevedo et al. 2015).

## Competition experiments

Competition experiments infer relative fitness in a manner that accounts for all growth phases. In competition experiments, two or more strains are grown together in a mixed culture: a reference strain and one or more strains of interest (for example, a wild-type reference strain and a mutant strain of interest). The frequency of each strain in the mixed culture is measured during the course of the experiment. This is done classically by plating assays that distinguish the strains using phenotypic markers (Wiser and Lenski 2015). More recently, flow cytometry has been used with fluorescently marked cells (Gallet et al. 2012b) and deep sequencing read counts have been used to determine the frequencies of different alleles in the population (Bank et al. 2014; Levy et al. 2015). The selection coefficient of the strains of interest can then be estimated from changes in their frequencies during the competition experiments. These methods infer relative fitness with high precision (Gallet et al. 2012b), as they directly estimate fitness from changes in frequencies over time. However, competition experiments are more laborious than growth curve experiments and are typically more expensive, requiring the construction and assaying of genetic or phenotypic markers (Concepción-Acevedo et al. 2015 and references therein). Therefore, many investigators prefer to use proxies of fitness such as growth rates.

## Outlook

Here we present a new computational method named *Curveball*: a descriptive and predictive framework for estimating growth traits from growth dynamics, predicting competition results, and inferring relative fitness. This integration between growth and fitness is crucial for understanding microbial diversity and adaptation due to the fundamental role of fitness in evolution

# Results

Our method includes four stages: (i) fitting growth models to monoculture growth curves data, (ii) fitting competition models to mixed culture growth curve data, (iii) using the estimated growth and competition parameters to predict the results of competition experiments, and (iv) estimating fitness from the predicted competition results.

## Growth curves

In each experiment we grew two *E. coli* strains, each labeled with a different fluorescent marker – green or red – in monoculture and mixed culture, and measured their changing density over several hours (Figure 1).

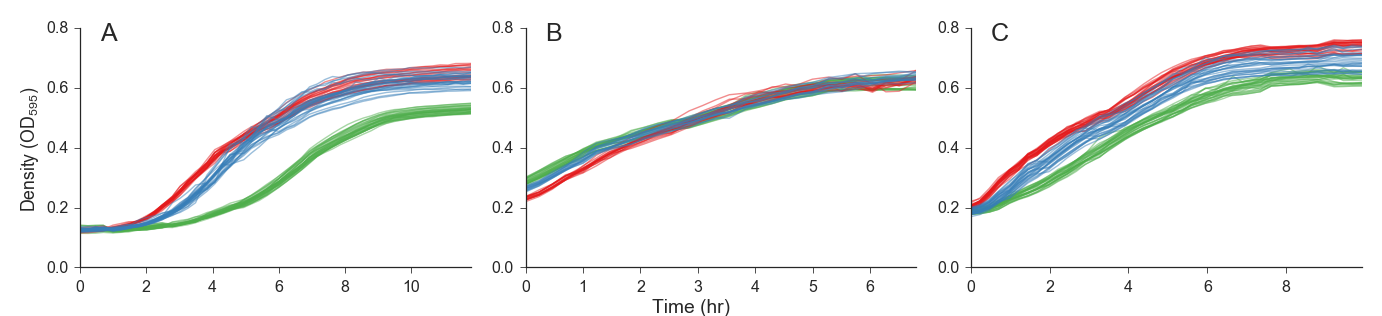


Figure 1. Growth curve data from three experiments with *E. coli*. The optical density (OD) of two strains growing in monoculture (green lines for green labeled strain; red lines for red labeled strains) and mixed culture (blue lines). Each experimental replicate is represented by a separate line. (A) DH5α labeled by green, TG1 labeled by red. Experiment started by diluting bacteria from stationary phase into fresh media. Therefore, there is a clear lag phase, which is longer for the green strain. (B) DH5α labeled by green, TG1 labeled by red. Bacteria were diluted into fresh media before experiment started and allowed to adjust to growth for 4 hours. They were then diluted again into fresh media and the experiment started. Therefore, there is no observable lag phase. (C) JM109 labeled by green, K12 MG1655-Δfnr labeled by red. Experimental conditions similar to A.

## Growth models

Because we are interested in several growth phases – the lag phase, the exponential phase, and the stationary phase – we use an extension of the logistic model, the Baranyi-Roberts model (Baranyi and Roberts 1994).

The Baranyi-Roberts model describes the growth of a cell population under a limiting resource; as the resource becomes scarce, growth decelerates, until it finally halts when the resource is depleted. The model is described by the following ordinary differential equation [see eqs. 1c, 3a, and 5a in (Baranyi and Roberts 1994)]:

(1a)

(1b)

(1c)

where is the population density, is time, is the specific growth rate in low density, is the adjustment function, is the maximum density, and is the cell population surface to mass ratio. For a derivation of eq. 1 and further details, see Appendix A.

The term is used to describe the deceleration in the growth of the population as it approaches the maximum density . When , the deceleration is the same as in the standard logistic model and the density at the time of the maximum growth rate is half the maximum density, . When or , the deceleration is slower or faster, respectively, and the density at the time of the maximum growth rate is (Richards 1959, substituting ).

The adjustment function describes the fraction of the population that is adjusted for growth at time (). Typically, microorganisms are grown in overnight liquid culture, reaching stationary phase, and then diluted into fresh media. Therefore, populations in stationary phase must adjust to growth conditions, and this might take some time. This adjustment phase is called the *lag phase*. The specific adjustment function we use here (eq. 1c) was suggested by Baranyi and Roberts (1994) due to being both computationally convenient and having a biological interpretation: is the initial amount of some molecule (nutrient, enzyme, etc.) that is required for growth; is the rate in which this molecule is accumulated in the cell.

The Baranyi-Roberts differential equation (eq. 1) has a closed form solution:

(2a)

, (2b)

where is the initial population density (usually determined by the dilution chosen by the experimenter). For derivation of eq. 2 from eq. 1, see Appendix A.

We use six forms of the Baranyi-Roberts model (Table 1). The full model is described by eq. 2 and has six parameters. A five parameter form of the model has , as in the standard logistic model. Another five parameter form has both rate parameters set to the same value (), which can make the fitting procedure more stable (Baranyi 1997). A four parameter form has both of the previous reductions, setting and (Baranyi 1997). Another four parameter form of the model has no lag phase, with . This is also known as the Richards model (Richards 1959), the -logistic model (Gilpin and Ayala 1973), or the generalized logistic model (see Wikipedia). This form of the model is useful in cases where there is no observed lag phase: either because the population adjusts very rapidly or because it is already adjusted prior to the growth experiment, usually by priming it in fresh media before the experiment. The last form is the standard logistic model, in which and .

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Name | # Params | Free Parameters | Fixed Parameters | References |
| Baranyi-Roberts | 6 |  | - | (Baranyi and Roberts 1994) |
| - | 5 |  |  | - |
| - | 5 |  |  | - |
| Richards | 4 |  |  | (Richards 1959) |
| Simplified Baranyi-Roberts | 4 |  |  | (Baranyi 1997) |
| Logistic | 3 |  |  | (Verhulst 1838) |

Table 1. Growth models. The table lists the growth models used for fitting growth curve data. All models are defined by eqs. 1 and 2 by setting the respective fixed parameters. is the initial population density; is the maximum population density; is the specific growth rate in low density; is the surface to mass ratio; is the initial physiological state; is the physiological adjustment rate. For detailed discussion of the derivation of these models, see Appendix A.

## Model fitting and selection

We fit all six model forms to the monoculture growth curve data of each strain using the *leastsq* non-linear curve fitting procedure (Jones et al. 2001; Newville et al. 2014). We then calculate the Bayesian Information Criteria (BIC) of each model fit:

,

where is the number model parameters, is the number of data points, are the time points, is the optical density at time point , and is the expected density at time point according to the model. We select the model form with the lowest BIC (Kass and Raftery 1995; Ward 2008).

We repeat the model fitting procedure for the monoculture growth curve data of each strain to produce estimates for all six parameters as well as confidence intervals on these estimates (Fig. SX). The best fit is shown in Figure 2; the estimated growth parameters are given in Table X.

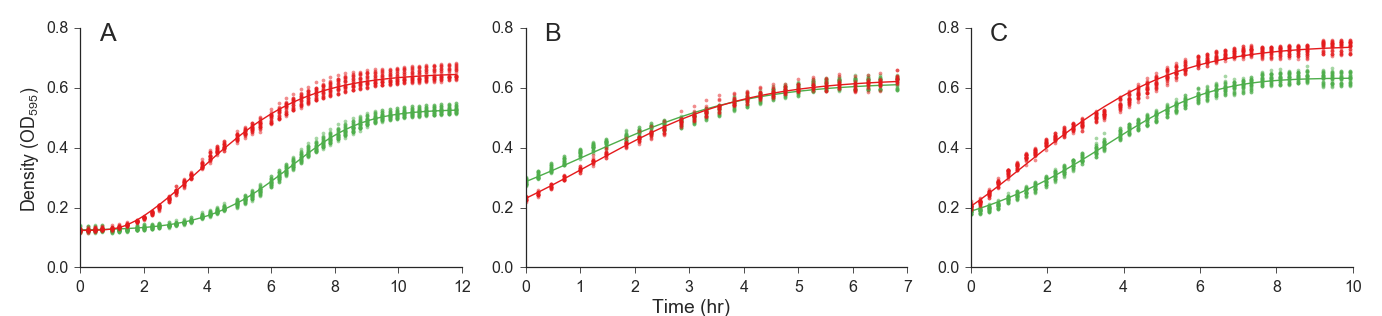


Figure 2. Fitting growth model to growth curves data. The figure shows the best model fit (solid lines) to the growth curves data (markers) of two strains (green and red) growing in monoculture. Experiments are the same as in Figure 1.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Parameter | Exp. A/Green | Exp. A/Red | Exp. B/Green | Exp. B/Green |  |  |
|  | 0.125 | 0.124 | 0.286 | 0.23 |  |  |
|  | 0.528 | 0.65 | 0.619 | 0.631 |  |  |
|  | 0.265±0 | 0.587±0.003 | 0.304±0.012 | 0.483±0.017 |  |  |
|  | 4.417±0 | 1\* | 2.484±0.164 | 1.512±0.083 |  |  |
|  | 0.0001±0 | 0.008±0.006 | **-\*** | **-\*** |  |  |
|  | 3.316±0 | 3.738±0.64 | **-\*** | **-\*** |  |  |
| max. specific growth rate | 0.26 | 0.376 | 0.256 | 0.37 |  |  |
| lag duration | 3.796 | 1.578 | 0 | 0 |  |  |

Table 2. Estimated parameters from growth model fitting. Where applicable, the estimation standard deviation is given after the ± sign. \* denotes cases where the best model had a parameter fixed. – denotes cases where the selected model had no lag phase. is the initial population density; is the maximum population density; is the specific growth rate in low density; is the surface to mass ratio; is the initial physiological state; is the physiological adjustment rate. For detailed discussion of the derivation of these models, see Appendix A.

## Competition model

For modeling growth in mixed culture, we assume that interactions between the strains or species are solely due to resource competition. The competition model for two strains is given by this differential equation system (see Appendix B for more details):

|  |  |  |
| --- | --- | --- |
|  |  | *[3a]*  *[3b]*  *[3c]*  *[3d]* |

is the density of strain and and are the values of the corresponding parameters for strain which we get from the fitting the monoculture growth curve data. is a competition coefficient, the ratio between inter- and intra-strain competitions, or the ratio between strain *i* competitiveeffect on cells of strain *j* and its effect on cells from its own strain. See Appendix B for a definition based on a resource consumption approach.

The competition model explicitly assumes that interactions between the strains are solely due to resource competition. Therefore, all interactions are described by the deceleration of the growth rate of each strain in response to growth of the other strain. We do not however assume the same limiting resource or resource efficiency for both strains, as we use different maximum densities for each strain and competition coefficients (see Appendix B for a formal description).

Eq. 3 is fitted to the growth curve of a mixed culture that includes both strains, in which the combined OD of the strains is recorded over time. This fitting provides estimates for the competition coefficients . The fitting is performed to minimize the squared differences between and the observed OD from the mixed culture (Figure 3A,C).

## Competition prediction

Once all growth and competition parameters have been estimated, eq. 3 is solved by numerical integration, providing a prediction for and . This predicts the *frequency* of each strain over time during growth in a mixed culture, such as in a competition experiment.

## Prediction verification

To verify our method, we performed growth curve and competition experiments with two different sets of *Escherichia coli* strains marked with fluorescent proteins. In set 1, we competed DH5α-GFP vs. TG1-RFP; in set 2 we competed JM109-GFP with MG1655-Δfnr-RFP.

In each competition experiment, we grew 32 populations of the green strain alone, 30 populations of the red strain alone, and 32 populations of both of the strains together, all in the same experimental conditions. The optical density of each population was measured every 15 minutes using a plate reader. A sample was taken from the mixed populations every hour for the first 7-8 hours, and the frequencies of the two strains was measured using flow cytometry (see Materials and Methods).

We compare our model prediction with the exponential model which is most commonly used in the literature to estimate relative fitness from growth curve (Hall et al. 2014). Briefly, the log of population density () is drawn vs. time, the inflection point – point of maximum growth rate - is found, the growth rate is estimated from the slope of the tangent line to the inflection point, and fitness is inferred from the ratio of the growth rates (Chevin 2011).

Figure 3shows the comparison of our method prediction (in green and red solid lines) with data from the competition experiment (colored error bars) and the exponential model (dashed black lines) for different sets of bacterial strain and experiments. Our method is clearly better for predicting competition in mixed culture, as can be seen from the figure as well as from statistical analysis (XXXX).

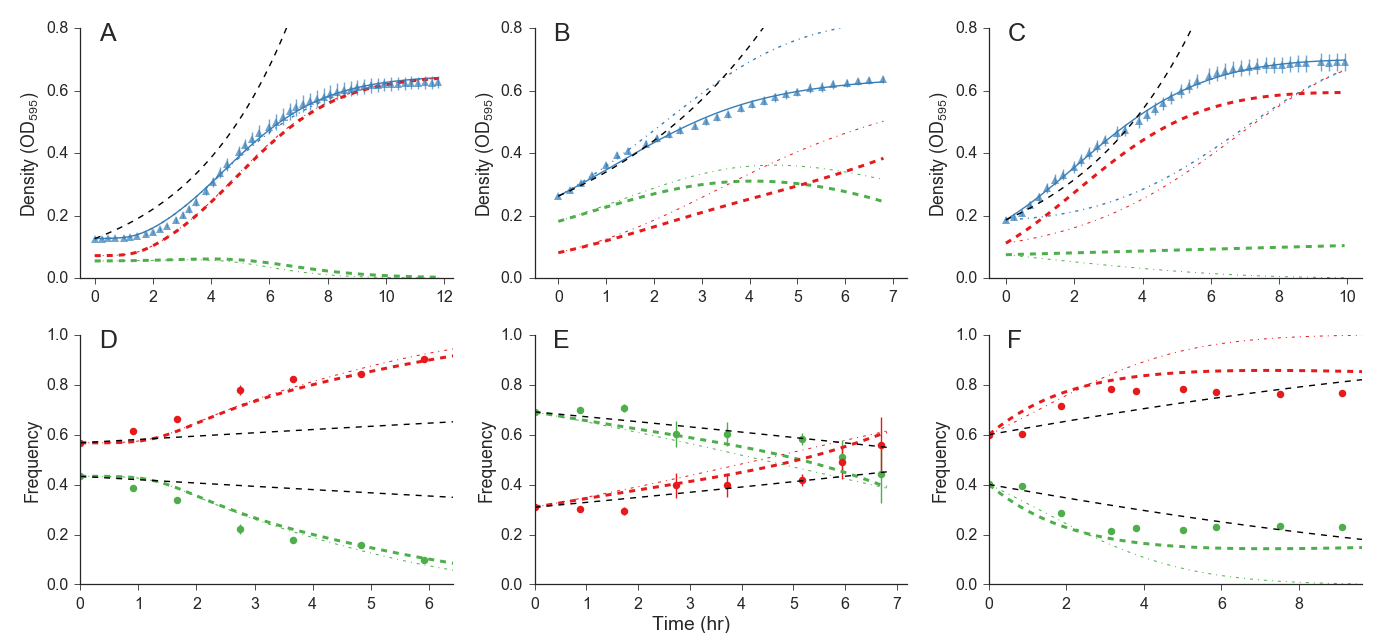


Figure 3. Predicting results of competition experiments. Growth of two *E. coli* in mixed culture, competing for resources. (A-C) Blue error bars denote measured total optical density in mixed culture, the solid blue line is the competition model (eq. X) fitted to the data. The black line represents the exponential model prediction; the green and red dashed lines represent the competition model prediction for each strain. (D-F) Green and red error bars are the measured frequencies of the two strains. The dashed green and red lines are the competition model prediction. The dashed black lines are the exponential model prediction. Error bars show mean values ± standard deviation. Mean residual squared errors: A, 0.00012; C, 0.00011. Estimated competition coefficients: A, a1=10,a2=0.27; C, a1=3.7, a2=2.

## Fitness inference

The best way to estimate relative fitness of two strains is by performing pairwise competition assays: growing them in mixed culture and measuring the change in their frequencies over time. Our method allows doing this by simply measuring their densities in mono- and mixed culture, without requiring direct measurement of strain frequencies, thus allowing the estimation of fitness in a simpler, more cost-effective, way.

A common method for estimating relative fitness or selection coefficients from pairwise competition results is (Wiser and Lenski 2015):

(4)

where and are the densities of the strains and is time, usually chosen to be 24 hours. Eq. 4 can be applied to the predicted competition results to infer the relative fitness of the strain of interest (Fig. 1D).

# Discussion

We present a new computational method to predict the growth in a mixed culture from growth curves of mono- and mixed cultures, without measuring frequencies of strains in the mixed culture. We tested and verified our method, which performed well and better than the model commonly used in the literature.

We have released an open-source software package called *Curveball* which implements our method (<http://curveball.yoavram.com> and <https://github.com/yoavram/curveball>). The software includes a command line interface (CLI) that can be used by non-programmers, as well as an application programming interface (API) that can be used by Python programmers. It can be freely used and extended by the community.

Growth curve experiments, in which only optical density is measured, require much less effort and resources than pairwise competition experiments, in which the cell frequency or count of each strain in measured (Concepción-Acevedo et al. 2015; Wiser and Lenski 2015; Hegreness et al. 2006; Gallet et al. 2012b). As automatic 96-well microplate readers become more and more common in microbiology labs, growth curve experiments can be set up in less than 30 minutes, after which measurements are automatically collected by the plate reader (Hall et al. 2014; Concepción-Acevedo et al. 2015). Importantly, our method does not only result in a simpler and more cost-effective approach to high-throughput fitness inference, but also provides information on the specific growth traits that contribute to differences in fitness. By providing researchers with a simple yet powerful method to estimate and interpret fitness, we hope our research will help to standardize the way fitness is measured and reported and improve communication between empirical and theoretical evolutionary biologists and ecologists.

Current methods for estimation of fitness from growth curves use the growth rate as a proxy of fitness. The growth rate and other proxies of fitness have several disadvantages: (i) they can't capture the full scope of effects contributing to differences in fitness; (ii) they depend on specific experimental conditions that differ for different organisms and from lab to lab; and (iii) they can't be used as parameters in standard population genetics models that test hypotheses and predict evolutionary dynamics. In contrast, our method integrates several growth phases into the fitness estimation, and our growth and competition model can be extended to include other phases and factors of growth, such as biphasic growth and cell death.

The growth model that we use - the Baranyi-Roberts model - has a differential equation form (eq. 1) and a closed form analytical solution (eq. 2). We have also shown here that it can be derived from a resource consumption perspective (Appendix A) and that it can be extended to competition model with multiple strains under the assumption of resource competition (Appendix B). Hence, this model is very useful for our method: the closed form of the monoculture model is used to fit to the growth curve data, whereas the differential equation of the mixed culture model is used to predict the competition dynamics

Our method assumes that the two strains interact solely via resource competition. If the investigators know or suspect that additional interactions exist (*i.e.*, frequency-dependent interactions such as social or sexual selection, mutualism, and interference), our model can serve as a null hypothesis: the results of competition experiments can be compared to model predictions and a goodness of fit test can be used to decide if additional interactions are significant. This can be of tremendous values, as frequency-dependent interactions are hard to detect in the lab despite having a significant effect on microbial evolution (Hibbing et al. 2010). Moreover, frequency-dependent interactions could be measured, either in terms of the difference in selection coefficients (between the coefficient calculated from the empirical results and coefficient calculated from the model prediction) or by fitting the empirical results to an extended model that includes density-dependent interactions (Masel 2014). Developing a statistical framework for detecting and measuring frequency-dependent interactions will be the subject of future work.

## Conclusions

We developed and tested a new method to analyze growth curves, predict growth and competition in mixed culture, and infer relative fitness. Our method improves fitness estimation from growth curves, has a clear biological interpretation, and can be used to interpret growth in mixed culture and competition experiments.

# Materials and Methods

**Strains and plasmids.** *Escherichia coli* strains DH5α, TG1, JM109, and K12 MG1655 Δfnr were used as a host in all the experiments. Plasmids containing a GFP or RFP gene, and genes conferring resistance to kanamycin (KanR) and chloramphenicol (CapR) were used as vectors. These plasmids have roughly the same effect on growth, as shown previously (Zelcbuch et al. 2013) and in Fig. SX. All experiments were performed in LB media with 30 μg/mL kanamycin and 34 μg/mL chloramphenicol. Fluorescence was confirmed using fluorescent microscopy (Nikon Eclipe Ti, Fig. SX).

**Growth and competition experiment.** Strains were inoculated into 3 ml LB and grown overnight at 30°C with shaking. Saturated overnight cultures were diluted into fresh media so that the OD will be above the OD of blank media (1:1-1:20 dilution rate). In experiments without lag phase, cultures were grown for 3-5 hours after dilution, in 30°C, their OD monitored until the exponential growth phase was reached. These cells were then inoculated into 100 μL LB in a 96-wells flat-bottom microplate (Costar) as following:

* 36-48 wells containing a monoculture of a GFP-labeled strain
* 34-46 wells containing a monoculture of a RFP-labeled strain
* 48-72 wells containing a mixed culture of both GFP- and RFP-labeled strains
* 2 wells with blank media

The cultures were grown at 30°C in an automatic microplate reader (Tecan infinite F200 Pro). OD595 readings were taken every 10 minutes with continuous shaking between readings.

Samples were taken from the incubated microplate at the beginning of the experiment and once an hour for 6-8 hours: 1-10 µL were removed from 4 wells (different wells for each sample), and diluted into cold PBS+ buffer. These samples were analyzed with a fluorescent cell sorter (Miltenyi Biotec MACSQuant VYB) to count the number of GFP- and RFP-labeled cells (GFP was detected with the 488nm/520(50)nm FITC laser; RFP was detected with the 561nm/615(20)nm dsRed laser). Samples were further diluted to allow precise detection in the cell sorter (Gallet et al. 2012a), see Table SX for details.

Fluorescent cell sorter output data was analyzed using R (R Development Core Team 2012) with the *flowPeaks* package that implements an unsupervised flow cytometry clustering algorithm (Ge and Sealfon 2012). The analysis scripts are included in supporting files XXX; data will be deposited in Dryad.

**Data analysis.** Growth curve data was analyzed using *Curveball*, an new open-source Python (Van Rossum and others 2007) package that implements the method presented in this manuscript. *Curveball* includes both programmatic interface (API) and a command line interface, and therefore doesn't require programming skills. See <http://curveball.yoavram.com> or <https://github.com/yoavram/curveball> for an installation guide and a basic tutorial.

*Curveball* makes use of several python packages: NumPy (van der Walt, Colbert, and Varoquaux 2011), SciPy (Jones et al. 2001), Matplotlib (Hunter 2007), Pandas (McKinney 2010), Seaborn (Waskom et al. 2015), LMFIT (Newville et al. 2014), Scikit-learn (Pedregosa et al. 2011), and SymPy (SymPy Development Team 2014).

**Figure reproduction**. Data was analysed and figures were produced using a Jupyter notebook (Perez and Granger 2007) available as a supporting file XXX or at <https://github.com/yoavram/curveball_ms/blob/master/supp.ipynb>.

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# Appendices

## Appendix A: Monoculture model

We derive our growth models from a resource consumption perspective (Otto and Day 2007, 365; Gopalsamy 1986). Denote by the density of a limiting resource and by the density of the population cells, both in total mass per unit of volume. Because cells intake resources via their surface, we denote to be the total surface of the cells, in total area per unit of volume. The parameter can be considered the surface:mass ratio for all cells; for a single, spheroid cell, this would be 2/3 [?].

We assume that the culture is well-mixed and homogeneous and that the resource is depleted by the growing population without being replenished. Therefore, the intake of resources occurs when cells meet resource via mass action law with resource intake rate . Once inside the cell, resources are converted to cell material at a rate . The differential equations describing this process are:

|  |  |  |
| --- | --- | --- |
|  |  | [A1a]  [A1b] |

However, we are interested in cell mass, rather than cell surface:

,

which gives us the equivalent differential equations:

|  |  |  |
| --- | --- | --- |
|  | , | [A2a]  [A2b] |

with . To solve this system, we use a conservation law approach by setting (Dilao and Domingos 1999). We find that *M* is constant:

,

and so we can substitute in eq. A1b:

|  |  |  |
| --- | --- | --- |
|  | . | [A3] |

Substituting again , and defining , we get

|  |  |  |
| --- | --- | --- |
|  | , | [A4] |

which is the Richards differential equation (Richards 1959), with the maximum population density *K* and the specific growth rate in low density.

Eq. A4 via eq. A3, which is a logistic equation and therefore has a known solution. Setting the initial cell density :

.

Eq. A4 is an autonomous differential equation ( doesn't depend on ). To include a lag phase, Baranyi and Roberts (1994) suggested to add an adjustment function , which makes the equation non-autonomous (dependent on ):

|  |  |  |
| --- | --- | --- |
|  | . | [A5] |

Baranyi and Roberts suggested a Michaelis-Menten type of function (Baranyi 1997), which has two parameters: *q0* is the initial fraction of the population adjusted to growth, and *m* is the adjustment rate, or the rate at which additional cells adjust to growth. Integrating eq. A5 produces eq. 2. Baranyi and Roberts suggested to fix to avoid instability of the fitting procedure (Baranyi 1997; Clark et al. 2010).

## Appendix B: Mixed culture model

We now consider the case in which two species or strains grow in the same culture, competing for a single limiting resource, similarly to eq. A2:

|  |  |  |
| --- | --- | --- |
|  |  | *[B1a]*  *[B1b]*  *[B1c]* |

We define , and (where *j* is 1 when *i* is 2 and vice versa) to find that and is constant. We then substitute into the differential equations for . Denoting and , we get:

|  |  |  |
| --- | --- | --- |
|  |  | *[B2a]*  *[B2b]* |

where .

We get a similar result if each strain is limited by a different resource that both strains consume, and the intake rates depend only on the strain:

|  |  |  |
| --- | --- | --- |
|  |  | *[B3a]*  *[B3b]*  *[B3c]*  *[B3d]* |

Here, we notice first that and therefore is a constant. We then substitute in eqs. B3a-d and continue as above. This only changes the definition of .

If the intake rates depend only on the resource:

|  |  |  |
| --- | --- | --- |
|  |  | *[B4a]*  *[B4b]* |

Then we define and and again continue as above.

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