Predicting microbial relative growth in a mixed culture from growth curve data

Yoav Ram1\*, Eynat Dellus-Gur1, Maayan Bibi2,

Uri Obolski1, Judith Berman2, and Lilach Hadany1

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1 Dept. Molecular Biology and Ecology of Plants, Tel Aviv University, Tel Aviv 69978, Israel

2 Dept. of Molecular Microbiology and Biotechnology,Tel Aviv University, Tel Aviv 69978, Israel

\*Corresponding author: yoav@yoavram.com

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# Abstract

Fitness is not well estimated from growth curves of individual isolates in monoculture. Rather, competition experiments, which measure relative growth in mixed microbial cultures, must be performed to better infer relative fitness. However, competition experiments require unique genotypic or phenotypic markers, and thus are difficult to perform with isolates derived from a common ancestor or non-model organisms. Here we describe a new theoretical approach for predicting relative growth of microbes in a mixed culture utilizing mono- and mixed culture growth curve data. We validated this new approach using growth curve and competition experiments with bacteria. Our approachprovides a simpler and more cost-effective approach to predict relative growth and infer relative fitness. Furthermore, by integrating several growth phases into the fitness estimation, our approach provides a holistic approach to fitness inference from growth curve data.

Growth curves are commonly used in microbiology, genetics, and evolutionary biology to estimate the fitness of individual microbial isolates. Growth curves describe the density of cell populations in liquid culture over a period of time and are usually acquired by measuring the optical density (OD) of one or more cell populations. The simplest way to infer fitness from growth curves is to estimate the growth rate during the exponential growth phase by inferring the slope of the log of the growth curve1 (see example in Figure 1). Indeed, the growth rate is often used as a proxy of the selection coefficient, *s*, which is the standard measure of relative fitness in population genetics2,3. However, exponential growth rates do not capture the dynamics of other phases of a typical growth curve, such as the length of lag phase and the cell density at stationary phase4 (Figure 1). Thus, it is not surprising that growth rates are often poor estimators of relative fitness5,6.

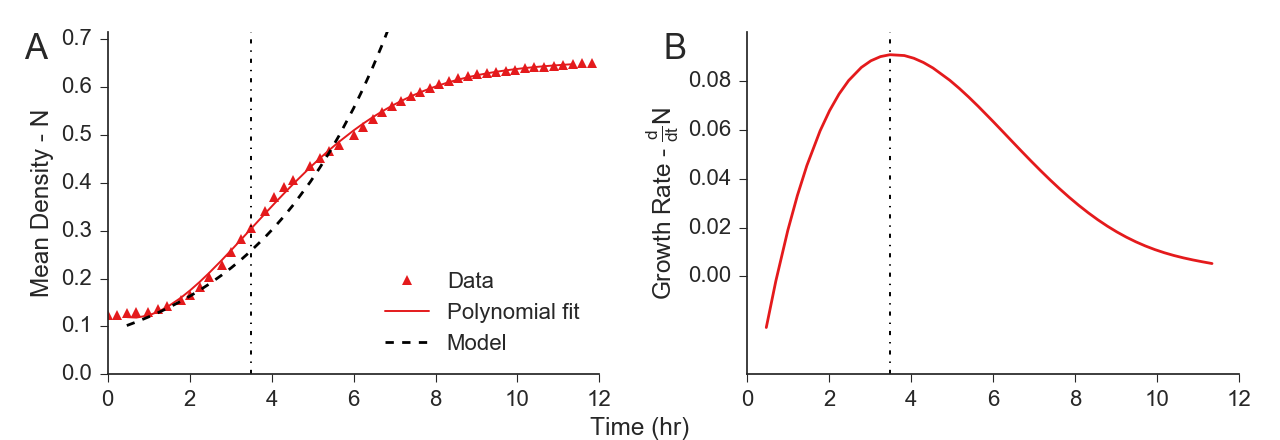


Figure 1. Fitting the exponential model to growth curve data. Growth rates are commonly estimated from growth curves data using this or a similar approach: a polynomial is fitted to the mean of the growth curve data *N(t)*; the time of maximum growth rate *tmax* is found by differentiating the fitted polynomial and finding the maximum; values *a* and *b* are found such that *b+at* describe a tangent line at the point of maximum growth (*tmax, N(tmax)*);the intercept *b* and the slope *a* are interpreted as the initial density *N0=eb* and the growth rate *r=a* in an exponential growth model *N(t)=N0ert* (*N0* is usually disregarded). (A) Red markers represent the mean cell density *N(t)* in 22 growth curves. The solid red line represents the fitted polynomial. The dashed black line represents the exponential model *N0ert* implicitly fitted to the data, with *r=0.27* and *N0=0.058*. The dotted vertical line denotes *tmax*. (B) The red solid curve shows *dN/dt,* the derivative of the mean density (calculated as the derivative of the fitted polynomial). The dotted vertical line denotes *tmax*. Data in this figure corresponds to the red markers in Figure 2A of the red strain in experiment A.

In microbiology, competition experiments are used to infer relative fitness in a manner that accounts for all growth phases7. In pairwise competition experiments, two strains are grown together in a mixed culture: a reference strain and a strain of interest. The frequency of each strain in the mixed culture is measured during the course of the experiment using specific markers, for example, by counting colonies formed by drug resistant or auxotrophic strains7, by monitoring fluorescent markers with flow cytometry8, or counting DNA barcodes reads using deep sequencing9,10. The selection coefficient of the strains of interest can then be estimated from changes in their frequencies during the competition experiments. These methods can infer relative fitness with high precision8, as they directly estimate fitness from changes in strain frequencies over time. However, competition experiments are more laborious and expensive than monoculture growth curve experiments, requiring the development of genetic or phenotypic assays (see Concepción-Acevedo et al.5 and references therein). Moreover, competition experiments are often impractical in non-model organisms. Therefore, many investigators prefer to use proxies of fitness such as growth rates.

Even when competition experiments are a plausible approach (for example, in microbial lineages with established markers7), methods for interpreting and understanding how differences in growth contribute to differences in fitness are lacking. Such differences have a crucial impact on our understanding of microbial fitness and the composition of microbial populations and communities.

Here we present a new theoretical approach that provides a predictive and descriptive framework for predicting relative growth in mixed cultures solely from growth curve data. We implemented this new approach in an open-source software package called (<http://curveball.yoavram.com)> and validated it using experiments in *E. coli*.

# Results

Our new approach consists of three stages: (a) fitting growth models to monoculture growth curve data, (b) fitting competition models to mixed culture growth curve data, and (c) predict relative growth in a mixed culture using the estimated growth and competition parameters to. The predicted relative growth can be used to estimate, for example, relative fitness and population composition. Throughout the manuscript, markers represent experimental data, solid lines represent results of model fitting, and dashed lines represent model predictions.

To test this approach, we performed growth curve and competition experiments with two different sets of *E. coli* strains marked with fluorescent proteins. In experiments A and B we grew DH5α-GFP and TG1-RFP in mono- and mixed culture; in experiment C we grew JM109-GFP and MG1655-Δfnr-RFP (see Figure 2A-C, respectively). In each experiment, 32 replicate monocultures of the GFP strain, 30 replicate monocultures of the RFP strain alone, and 32 replicate mixed cultures containing the GFP and RFP strains together, were grown in a 96-well plate, under the same experimental conditions. The optical density of each culture was measured every 15 minutes using an automatic plate reader for at least 7 hours. Samples were collected from the mixed cultures every hour for the first 7-8 hours, and the relative frequencies of the two strains were measured using flow cytometry. See Materials and Methods for more details.

## a. Estimate growth parameters

### Growth model

The Baranyi-Roberts model11 can be used to model growth composed of several phases: lag phase, exponential phase, deceleration phase, and stationary phase1. The model implicitly assumes that growth accelerates as cells adjust to new growth conditions, then decelerates as resources become scarce, and finally halts when resources are depleted12. The model is described by the following ordinary differential equation (see eqs. 1c, 3a, and 5a in11; for a derivation of eq. 1 and further details, see Supporting Text 1):

[1]

where is time, is the cell density at time , is the specific growth rate in low density, is the maximum cell density, is a deceleration parameter, and is the adjustment function, which describes the fraction of the population that has adjusted to the new growth conditions by time (). In microbial experiments, an overnight liquid culture of microorganisms that has reached stationary phase is typically diluted into fresh media. Following dilution, cells enter lag phase until they adjust to the new growth conditions. We chose the specific adjustment function suggested by Baranyi and Roberts11, which is both computationally convenient and biologically interpretable: , where characterizes the physiological state of the initial population, and is the rate at which the physiological state adjusts to the new growth conditions.

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

[2]

where is the initial population density. For a derivation of eq. 2 from eq. 1, see Supporting Text 1.

### Model fitting

We estimated the growth model parameters by fitting the model (eq. 2) to the monoculture growth curve data of each strain. The best-fit models (black lines) and experimental data (markers) are shown in Figure 2A-C; see Table S1 for the estimated growth parameters. From these best-fit models we also estimated the maximum specific growth rate , the minimal specific doubling time (minial time required for cell density to double), and the lag duration; see Table 1. The strains differ in their growth parameters: for example, in experiment A (Figure 2A), the red strain grows 41% faster than the green strain, has 23% higher maximum density, and a 60% shorter lag phase.

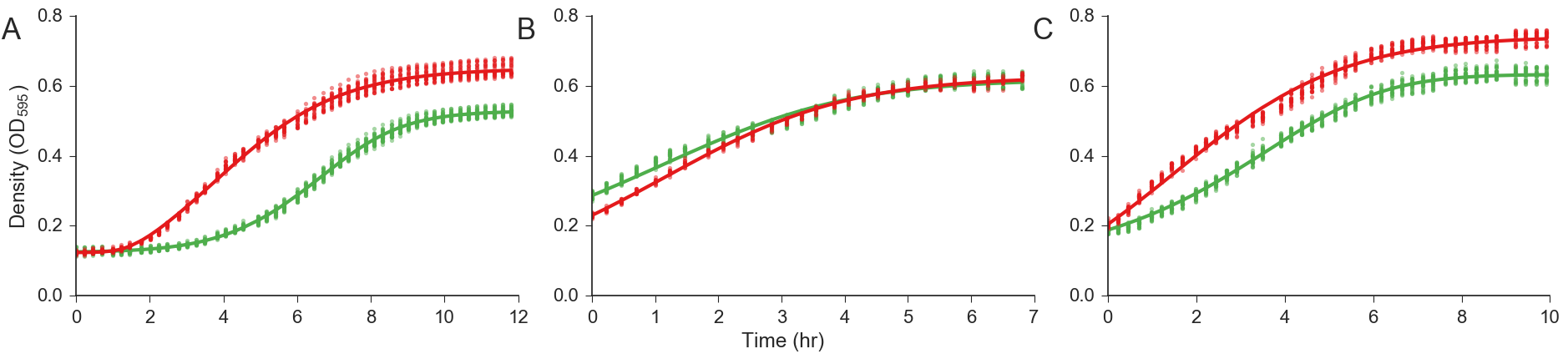


Figure 2. Fitting the growth model to data from three growth curve experiments with *E. coli*. Markers show the optical density (OD) of two strains growing separately in a monoculture in three experiments (green markers for GFP labeled strain; red markers for RFP labeled strains). Lines represent the best-fit model for each strain in each experiment. (A) Strain DH5α labeled with GFP, strain TG1 labeled with RFP. Experiment started by diluting stationary phase bacteria into fresh media, yielding a lag phase culture in which lag phase is longer for the green strain. (B) Strain DH5α labeled with GFP, strain TG1 labeled with RFP. Bacteria were pre-grown in fresh media for 4 hours before the experiment and then diluted into fresh media, such that there is no observable lag phase. (C) Strain JM109 labeled with GFP and, strain K12 MG1655-Δfnr labeled with RFP, experimental conditions as described in (A).

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | Experiment A | | Experiment B | | Experiment C | |
| *Strain*  *Parameter* | **GFP** | **RFP** | **GFP** | **RFP** | **GFP** | **RFP** |
| Initial density | 0.125 | 0.124 | 0.286 | 0.23 | 0.188 | 0.204 |
| Max density | 0.528 (0.525, 0.532) | 0.650 (0.643, 0.658) | 0.619 (0.612, 0.625) | 0.628 (0.624, 0.632) | 0.633 (0.627, 0.638) | 0.741 (0.735, 0.746) |
| Max specific growth rate | 0.268 (0.262, 0.275) | 0.376 (0.371, 0.382) | 0.256 (0.251, 0.261) | 0.369 (0.355, 0.384) | 0.228 (0.226, 0.231) | 0.42 (0.391, 0.426) |
| Min doubling time | 2.695 (2.636, 2.77) | 1.844 (1.809, 1.88) | 4.372 (4.269, 4.481) | 2.451 (2.397, 2.506) | 3.117 (3.087, 3.147) | 2.075 (2.035, 2.124) |
| Lag duration | 3.93 (3.82, 4.028) | 1.578 (1.513, 1.64) | 0.004 (0.002, 0.013) | 0.014 (0.002, 0.029) | 0.711 (0.684, 0.749) | 0.039 (0.033, 0.081) |

Table 1. Estimated growth parameters. 95% confidence intervals, calculated using bootstrap (1000 samples), are given in parentheses. Min doubling time is the minimal time required to double the population density. Densities are in OD595; growth rate in hours-1, doubling time and lag duration in hours. See Table S2 for additional parameter estimates.

## b. Estimate competition coefficients

### Competition model

To model growth in a mixed culture, we assume that interactions between the strains are solely due to resource competition. We derived a new two-strain Lotka-Volterra competition model13 based on resource consumption (see Supporting Text 2):

|  |  |  |
| --- | --- | --- |
|  |  | [3a]  [3b] |

where is the density of strain , and are the values of the corresponding parameters for strain (obtained from fitting the growth model (eq. 2) to monoculture growth curve data), and are competition coefficients, the ratios between inter- and intra-strain competitive effects. Note that each strain can have a different limiting resource and resource efficiency, based on the maximum densities and competition coefficients determined for each strain.

### Model fitting

The competition model (eq. 3) was fitted to data from the growth of the mixed culture that includes both strains, in which the combined OD of the strains was recorded over time (but not the frequency or density of individual strains). The fitting provides estimates for the competition coefficients and was performed by minimizing the squared differences between (the sum of the integrals of the system in eq. 3) and the total OD from the mixed culture (Figure 3A-C).

## c. Prediction and validation of relative growth

### Model prediction

With estimates of all the competition model parameters, we solved the competition model (eq. 3) using numerical integration, thus providing a prediction for the cell densities and of the two strains growing in a mixed culture. From these predicted densities, the frequencies of each strain over time were estimated:

.

### Experimental validation

The green and red error bars in Figure 3D-F show the results of the competition experiment, that is, the frequency of each strain growing together in a mixed culture. These experimental results are compared to the model predictions in green and red dashed lines and to the exponential model predictions in black dashed lines (see Figure 1 for details on the exponential model). Our model performs well and clearly improves upon the exponential model for predicting competition dynamics in a mixed culture: the colored dashed lines match the data much better than the black dashed lines.

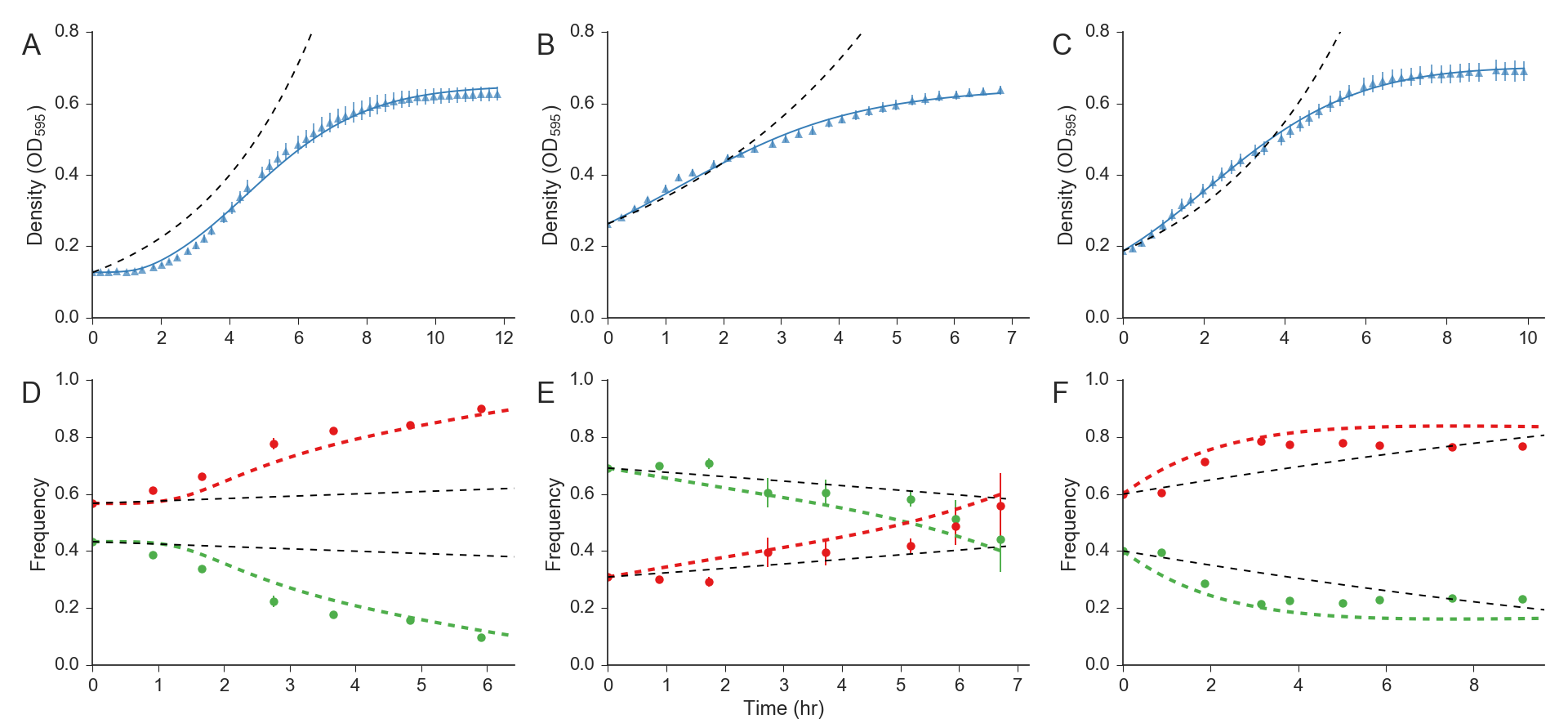


Figure 3. Predicting growth in a mixed culture. Growth of two *E. coli* strains competing for resources in a mixed culture. (A-C) Solid blue lines show the best-fit competition model (eq. 3), markers show growth curve data from mixed cultures. Error bars show standard deviation from 30 replicates (extremely small in B); dashed black lines represent the exponential model prediction (Figure 1). (D-F) Comparison of experimental data (circles) and model prediction (dashed lines; see Figure S3 for confidence intervals) of strain frequencies in a mixed culture. Green and red dashed lines show our model predictions; dashed black lines show exponential model predictions. Error bars show standard deviation (hardly seen in D and F). Root mean squared error of model fit (solid blue line to blue triangles A-C) are 0.011 for A, 0.01 for B, and 0.008 for C. Estimated competition coefficients are a1=10, a2=0.77 for D; a1=3.7, a2=1.9 for E; a1=0.31, a2=0.56 for F. Inferred time-averaged selection coefficients are 0. A, 376 for D, 0.182 for E, and 0.124 for F..

# Discussion

We developed a new theoretical approach to predict relative growth in a mixed culture from growth curves of mono- and mixed cultures, without measuring frequencies of single isolates within the mixed culture. We tested and validated this new approach, which performed far better than the model commonly used in the literature.

Our approach only assumes that the assayed strains grow in accordance with the growth and competition models: namely, that growth depends on resource availability. Therefore, this approach can be applied to data from a variety of organisms, experiments, and conditions.

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 must be determined5,7,8,15. Current approaches to estimating fitness from growth curves mostly use the growth rate or the maximum population density as a proxy for fitness. However, the growth rate and other proxies for fitness based on a single growth parameter cannot capture the full scope of effects that contribute to differences in overall fitness16. In contrast, our approach integrates several growth phases into the pairwise competitions predictions from which fitness can be estimated, allowing a more holistic approach to fitness inference from growth curve data and providing information on the specific growth traits that contribute to differences in fitness.

*Curveball*, our approach package

## Conclusions

We developed and tested a new approach for analysis of growth curve data and prediction of growth of individual strains within a mixed culture. This approach improves upon the commonly used approach, has a biological interpretation, and can be used to predict and interpret growth in a mixed culture and results of competition experiments.

# Materials and Methods

**Strains and plasmids.** *Escherichia coli* strains used were DH5α (Berman lab, Tel-Aviv University), TG1 (Ron lab, Tel-Aviv University), JM109 (Nir lab, Tel-Aviv University), and K12 MG1655-Δfnr (Ron lab, Tel-Aviv University). Plasmids contain a GFP or RFP gene and genes conferring resistance to kanamycin (KanR) and chloramphenicol (CapR) (Milo lab, Weizmann Institute of Science17).

**Media.** All experiments were performed in LB media (5 g/L Bacto yeast extract (BD, 212750), 10 g/L Bacto Tryptone (BD, 211705), 10 g/L NaCl (Bio-Lab, 190305), DDW 1 L) with 30 μg/mL kanamycin (Caisson Labs, K003) and 34 μg/mL chloramphenicol (Duchefa Biochemie, C0113). Green or red fluorescence of each strain was confirmed by fluorescence microscopy (Nikon Eclipe Ti, Figure S1).

**Growth and competition experiments.** All experiments were performed at30°C. Strains were inoculated into 3 ml LB+Cap+Kan and grown overnight with shaking. Saturated overnight cultures were diluted into fresh media so that the initial OD was detectable above the OD of media alone (1:1-1:20 dilution rate). In experiments that avoided a lag phase, cultures were pre-grown until the exponential growth phase was reached as determined by OD measurements (4-6 h). Cells were then inoculated into 100 μL LB+Cap+Kan in a 96-well flat-bottom microplate (Costar):

* 32 wells contained a monoculture of the GFP-labeled strain
* 30 wells contained a monoculture of the RFP-labeled strain
* 32 wells containing a mixed culture of both GFP- and RFP-labeled strains
* 2 wells contained only growth medium

The cultures were grown in an automatic microplate reader (Tecan infinite F200 Pro), shaking at 886.9 RPM, until they reached stationary phase. OD595 readings were taken every 15 minutes with continuous shaking between readings.

Samples were collected 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 (DPBS with calcium and magnesium; Biological Industries, 02-020-1). These samples were analyzed with a fluorescent cell sorter (Miltenyi Biotec MACSQuant VYB). GFP was detected using a 488nm/520(50)nm FITC laser. RFP was detected with a 561nm/615(20)nm dsRed laser. Samples were diluted further to eliminate "double" event (events detected as both "green" and "red" due to high cell density) and noise in the cell sorter8.

**Data analysis.**  Growth curve data were analyzed using *Curveball*, a new open-source software written in Python14 that implements the approach presented in this manuscript (http://curveball.yoavram.com). The software includes both a programmatic interface (API) and a command line interface (CLI), and therefore does not require programming skills. The source code makes use of several Python packages: NumPy20, SciPy21, Matplotlib22, Pandas23, Seaborn24, LMFIT25, Scikit-learn26, and SymPy27.

**Model fitting.** To fit models to data we used the least-squares non-linear curve fitting procedure21,25. We then calculate the Bayesian Information Criteria (BIC) of several nested models, defined by fixing some of the parameters (see Supporting Text 1, Figure S2, and Table S1). BIC is given by:

,

where is the number of 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 selected the model with the lowest BIC28,29. Other metrics for model selection can be used with our approach, but BIC was chosen for its simplicity and flexibility.

**Selection coefficients estimation.** Selection coefficients were estimated from pairwise competition results using3 where and are the predicted frequencies of the strains and is time. The resulting *st* values were then averaged across time. Note that these estimates can depend on the experimental conditions, such as duration, media, temperature, and strain composition.

**Data availability.** Data deposited on *figshare* (doi:10.6084/m9.figshare.3485984; *for editors and reviewers only:* <https://figshare.com/s/b08c6b975779e03ec48e>).

**Code availability**. Source code will be available upon publication at <https://github.com/yoavram/curveball>; an installation guide, tutorial, and documentation will be available upon publication at <http://curveball.yoavram.com>. *For editors and reviewers only:* installation guide, tutorial, documentation, and source code is available at <https://curveball.netlify.com> (password: *dh5alpha*).

**Figure reproduction**. Data was analyzed and figures were produced using a Jupyter Notebook30 that will beavailable as a supporting file and at <https://github.com/yoavram/curveball_ms>. *For editors and reviewers only:* the notebook is available at <https://dl.dropboxusercontent.com/u/1578682/supp.ipynb>.

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# Author contributions

All authors designed the experiments, analyzed data, discussed the results and edited the manuscript. Y.R. and L.H. developed the model and wrote the manuscript. U.O. advised on statistical analysis. Y.R. wrote the source code. Y.R., E.D.G. and M.B. performed the experiments. M.B. performed fluorescent microscopy. J.B. advised and gave support to all experiments. L.H. supervised all the work.

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# Supporting material

## Supporting Text 1: Monoculture model

We derive our growth models from a resource consumption perspective31,32. We denote by the density of a limiting resource, and by the density of the cell population, both in total mass per unit of volume.

We assume that the culture is well-mixed and homogeneous and that the resource is depleted by the growing cell population without being replenished. Therefore, the intake of resources occurs when cells meet resource via a mass action law with resource intake rate . Once inside the cell, resources are converted to cell mass at a conversion rate of . Cell growth is assumed to be proportional to , whereas resource intake is proportional to a power of cell density, . We denote .

We can describe this process with differential equations for and :

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

These equations can be converted to equations in and :

,

which yields

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

with .

To solve this system, we use a conservation law approach by setting 33. We find that *M* is constant

,

and we can substitute in eq. A2b to get

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

Substituting again , and defining , we get

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

which is the Richards differential equation34, with the maximum population density *K* and the specific growth rate in low density. To the best of our knowledge this the first derivation of the Richards differential equation from a resource consumption perspective.

We solve eq. A4 via eq. A3, which is a logistic equation and therefore has a known solution. Setting the initial cell density we have

.

Eq. A4 is an autonomous differential equation ( doesn't explicitly depend on ). To include a lag phase, Baranyi and Roberts11 suggested to add an adjustment function , which makes the equation non-autonomous (explicitly dependent on ):

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

Baranyi and Roberts suggested a Michaelis-Menten type of function35

,

which has two parameters: *q0* is the initial physiological state of the population, and *m* is rate at which the physiological state adjusts to growth conditions. Integrating gives

.

Therefore, integrating eq. A5 produces eq. 2.

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 population growth 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 *)*.

We use six forms of the Baranyi-Roberts model (Figure S2, Table S1). The full model is described by eq. 2 and has six parameters. A five-parameter form of the model assumes , as in the standard logistic model, but still incorporates the adjustment function and therefore includes a lag phase. Another five-parameter form has both rate parameters set to the same value (), which was suggested to make the fitting procedure more stable35,36. A four-parameter form has both of the previous constraints, setting and 35. Another four-parameter form of the model has no lag phase, with 1, which yields the Richards model34, also called the -logistic model37, or the generalized logistic model. 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, possibly by pre-growing it in fresh media before the beginning of the experiment. The last form is the standard logistic model, in which and .

## Supporting Text 2: Mixed culture model

We consider the case in which two species or strains grow in the same culture, competing for a single limiting resource, similarly to eq. A1:

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

We define , and (where *j* is 1 when *i* is 2 and vice versa) to find that and therefore so that 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:

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

Here, we notice first that and therefore is a constant. We then substitute in eq. B3 and continue as above. This changes the definition of .

If the intake rates depend only on the resource then

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

Then we define and and again continue as above.

# Supporting figures



Figure S1. Fluorescence microscopy of *E. coli* strains carrying GFP or RFP. Image of a mixture of DH5α-GFP and TG1-RFP cells.



Figure S2. Growth models hierarchy. The Baranyi-Roberts model and five nested models defined by fixing one or two parameters. See Supporting Text 1 and Table S1 for more details.

****

Figure S3. Mixed culture growth predictions with confidence intervals. The green and red lines and markers correspond to the dashed green and red lines and the markers in Figure 3D-F, respectively. The gray area shows the 95% confidence interval, calculated using bootstrap (1000 samples).

# Supporting tables

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Model name | **# Parameters** | **Free Parameters** | **Fixed Parameters** | **References** |
| Baranyi Roberts 1994 | 6 |  | - | 11 |
| Baranyi 1997 | 5 |  |  | - |
| Baranyi Roberts 1994 | 5 |  |  | - |
| Richards 1959 | 4 |  |  | 34 |
| Baranyi 1997 | 4 |  |  | 35 |
| Logistic | 3 |  |  | 38 |

Table S1. Growth models. The table lists the growth models used for fitting growth curve data. All models are defined by eqs. 1 and 2, by fixing specific 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. Note that when , the value of is irrelevant. See also the hierarchy diagram in Figure S2 and a detailed discussion in Supporting text 1.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Experiment A | | | | Experiment B | | Experiment C | |
| *Strain*  *Parameter* | | **GFP** | | **RFP** | **GFP** | **RFP** | **GFP** | **RFP** |
|  | 0.125 | | 0.124 | | 0.286 | 0.23 | 0.188 | 0.204 |
|  | 0.528 | | 0.65 | | 0.619 | 0.628 | 0.633 | 0.741 |
|  | 0.376 | | 0.587 | | 0.304 | 0.484 | 8 | 8 |
|  | 2.636 | | 1\* | | 2.484 | 1.491 | 1\* | 0.164 |
|  | 0.032 | | 0.008 | | **-\*** | **-\*** | 0.039 | 0.393 |
|  | 0.937 | | 3.735 | | **-\*** | **-\*** | 0.188 | 0.104 |

Table S2. Estimated parameters from growth model fitting. \* denotes fixed parameters; - denotes invalid parameter values.