­Predicting growth in a mixed culture from growth curves

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# Abstract

Measuring microbial growth in a mixed culture is laborious and expensive, especially in non-model organisms, as it requires unique genotypic or phenotypic markers. We have developed *Curveball* (<http://curveball.yoavram.com>), a new method for predicting growth in a mixed culture from growth curve data using a well-known growth model and a newly derived competition model. We validated *Curveball*'s predictions using growth curve and competition experiments with bacteria. Our method does not only result in a simpler and more cost-effective approach for predicting growth in a mixed culture and inferring relative fitness, but also provide information on the specific growth traits that contribute to differences in fitness. Therefore, our research contributes to the important integration between experimental evolution and population genetics.

Growth curves are commonly used to estimate fitness in microbiology, genetics, and evolutionary biology. Growth curves are usually acquired by measuring the optical density (OD) of one or more cell populations, growing in liquid culture over a period of time. 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 can be 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 lag phase and the stationary phase4 (Figure 1). Thus, it is not surprising that growth rates are often poor estimates of relative fitness5,6.

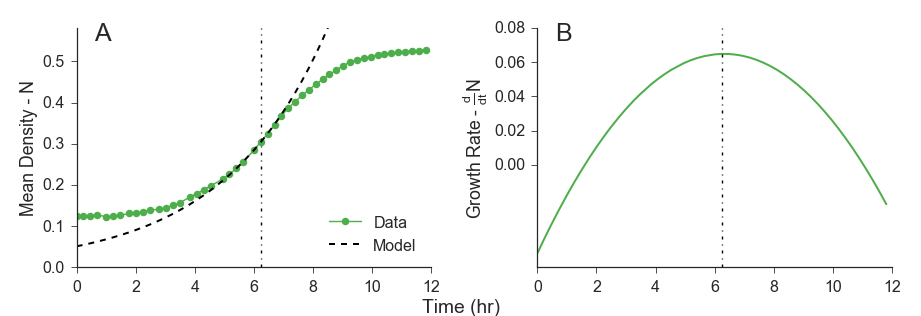


Figure 1. Fitting an exponential model to growth curves data. The growth rate is calculated as the derivative of a polynomial function fitted to the mean of the data *N(t)*: the time point of maximum growth rate *tmax* is found; the 5 points surrounding *tmax* are taken; a line *b+at* is fitted to the log of the mean of the data *log(N(t))* at these time points; the slope and the intercept are interpreted as the initial density *N0=eb* and the growth rate *r=a* in an exponential growth model *N(t)=N0ert*. (A) The green markers represent *N(t)* the mean density in 30+ growth curves. The dashed black line represents the exponential model *N0ert* fitted to the data, with *N0=0.058* and *r=0.27*. The dotted vertical line denotes the *tmax*,thetime of max growth rate. (B) The green solid line shows *dN/dt* the derivative of the mean density. The dotted vertical line denotes the *tmax*,thetime of max growth rate. Data in this figure corresponds to the green growth curves from Figure 2A.

Competition experiments are used by evolutionary biologists to infer relative fitness in a manner that accounts for all growth phases7. In competition experiments, two or more strains are grown together in a mixed culture: a reference strain and one or more strains of interest. The frequency of each strain in the mixed culture is measured during the course of the experiment by assays that distinguish the strains using specific markers7 such as flow cytometry8 and deep sequencing read counts9,10. 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 frequencies over time. However, competition experiments are more laborious than monoculture growth curve experiments and are typically more expensive, requiring the development of genetic or phenotypic assays (see Concepción-Acevedo et al.5 and references therein). Moreover, such assays 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 (for example, in microbial lineages with established markers7), there is a lack of good methods for interpreting and understanding the differences between growth in a monoculture and in a mixed culture. Such differences have a crucial impact on microbial fitness and on the composition of microbial populations and communities.

Here we present a new computational method named *Curveball*, released as an open-source software package (<http://curveball.yoavram.com>). *Curveball* is a predictive and descriptive framework for estimating growth traits from growth dynamics, predicting growth in a mixed culture, and inferring relative fitness.

# 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 growth in a mixed culture, and (iv) inferring relative fitness from the predicted growth.

We demonstrate our method in the following experimental setting.

## Growth curves

In each experiment we grew two *Escherichia coli* strains, each labeled with a different fluorescent marker, green or red, in a monoculture and a mixed culture, and measured their changing density over several hours (Figure 2).

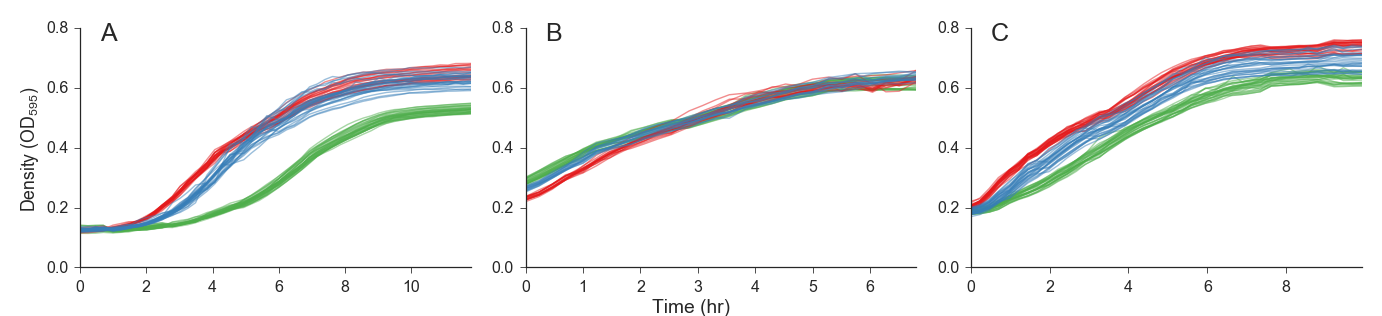


Figure 2. Growth curves data from three experiments with *E. coli*. The optical density (OD) of two strains growing in a monoculture (green lines for green labeled strain; red lines for red labeled strains) and a mixed culture (blue lines). Each experimental replicate is represented by a separate line. (A) Strain DH5α labeled by green, strain TG1 labeled by red. Experiment started by diluting bacteria from overnight stationary phase into fresh media. Therefore, there is a clear lag phase, which is longer for the green strain. (B) Strain DH5α labeled by green, strain TG1 labeled by red. Bacteria were pre-grown in fresh media for 4 hours before the experiment, then diluted into fresh media. Therefore, there is no observable lag phase. (C) Strain JM109 labeled by green, strain K12 MG1655-Δfnr labeled by red. Experimental conditions as in A.

## Growth models

To model growth composed of several stages – the lag phase, the exponential phase, and the stationary phase – we used the Baranyi-Roberts model11. This model follows the growth of a cell population. The population growth rate accelerates as the cells adjust to new growth conditions, then decelerates as resources become scarce, and finally halts when resources are depleted. The model is described by the following ordinary differential equation [see eqs. 1c, 3a, and 5a in11]:

[1]

where is time, is the population density at time , is the specific growth rate in low density, is the maximum density, is a deceleration parameter, and is the adjustment function,. For a derivation of eq. 1 and further details, see Supporting text 1.

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. Following such dilutions, cells must adjust to growth conditions, and this adjustment takes time. The adjustment phase is called the *lag phase*. The specific adjustment function we use was suggested by Baranyi and Roberts11 due to being both computationally convenient and having a biological interpretation: characterizes the physiological state of the initial population, and is the rate at which the physiological state adjusts to growth conditions.

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 a derivation of eq. 2 from eq. 1, see Supporting text 1.

## Model fitting and selection

We estimate the model parameters by fitting the growth model (eq. 2) to the monoculture growth curves data of each strain. The best fit is shown in Figure 3 and the estimated growth parameters are given in Table 1. From this model fit we also estimate the maximum specific growth rate , the minimal specific doubling time, and the lag duration (Table 1).

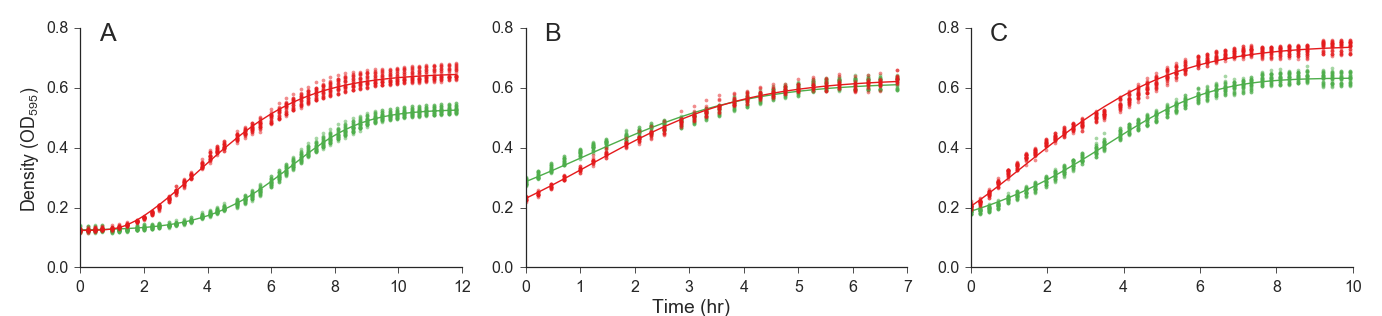


Figure 3. Fitting a growth model to growth curves data. The best model fit (solid lines) for the growth curves data (markers) of two strains (green and red) growing in monoculture. Experiments are the same as in Figure 1.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | Experiment A | | Experiment B | | Experiment C | |
| *Strain*  *Parameter* | **Green** | **Red** | **Green** | **Red** | **Green** | **Red** |
| Initial density | .125 | 0.124 | 0.286 | 0.23 | 0.188 | 0.204 |
| Max density | .531 (.528, 535) | .655 (.649, .661) | .675 (.663, .686) | .634 (.628, .64) | .64 (.635, .645) | .757 (.748, .771) |
| Max specific growth rate | .266 (.261, .271) | .376 (.372, .381) | .258 (.253, .263) | 1.073 (.971, 1.157) | .241 (.238, .244) | .391 (.309, .405) |
| Min doubling time (δ) | 2.739 (2.683, 2.79) | 1.834 (1.809, 1.856) | 6.218 (5.8, 6.632) | .728 (.586, .892) | 2.984 (2.958, 3.008) | 2.218 (2.184, 2.263) |
| Lag duration | 3.871 (3.792, 3.944) | 1.59 (1.546, 1.634) | 0 | 0 | .494 (.462, .524) | .037 (.016, 0.06) |

Table 1. Estimated growth parameters. 95% confidence intervals, calculated using non-parametric bootstrap, are given in parentheses. Densities are in OD595; growth rate in hours-1, doubling time and lag duration in hours. See Table S2 for additional parameter estimates.

## Competition model

For modeling growth in a mixed culture, we assume that interactions between the strains or species are solely due to resource competition. We derived a new 2-strain Lotka-Volterra competition model12 based on resource consumption (see Supporting text 2):

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

is the density of strain and and are the values of the corresponding parameters for strain which we get from fitting the monoculture growth curve data. is a competition coefficient, the ratio between inter- and intra-strain competitive effect.

This 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 the same resource efficiency for both strains, as we use different maximum densities for each strain and competition coefficients .

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 (but not the frequency or density of each individual strain). This fit is performed by minimizing the squared differences between (eq. 3) and the observed OD from the mixed culture; it provides estimates for the competition coefficients (Figure 4A-C).

## Mixed culture prediction

Using the estimated parameters, eq. 3 is solved by numerical integration, providing a joint prediction for the densities and . From the predicted densities, the frequencies of each strain over time can be inferred: .

## Prediction validation

To validate our method, 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 competed DH5α-GFP vs. TG1-RFP; in experiment C we competed JM109-GFP with MG1655-Δfnr-RFP (Figure 2).

In each experiment, we grew in a 96-well plate 32 monocultures of the green strain, 30 monocultures of the red strain alone, and 32 mixed cultures of the green and red strain together, all in the same experimental conditions. The optical density of each culture was measured every 15 minutes using a plate reader. A sample was taken from the mixed cultures every hour for the first 7-8 hours, and the frequencies of the two strains were measured using flow cytometry (see Materials and Methods).

Figure 4 shows empirical results from the competition experiment (colored error bars), our method predictions (in green and red solid lines), and the exponential model prediction (dashed black lines; see Figure 1 for details) for three experiments. Our method performs well and is clearly better than the exponential model for predicting competition in mixed culture.

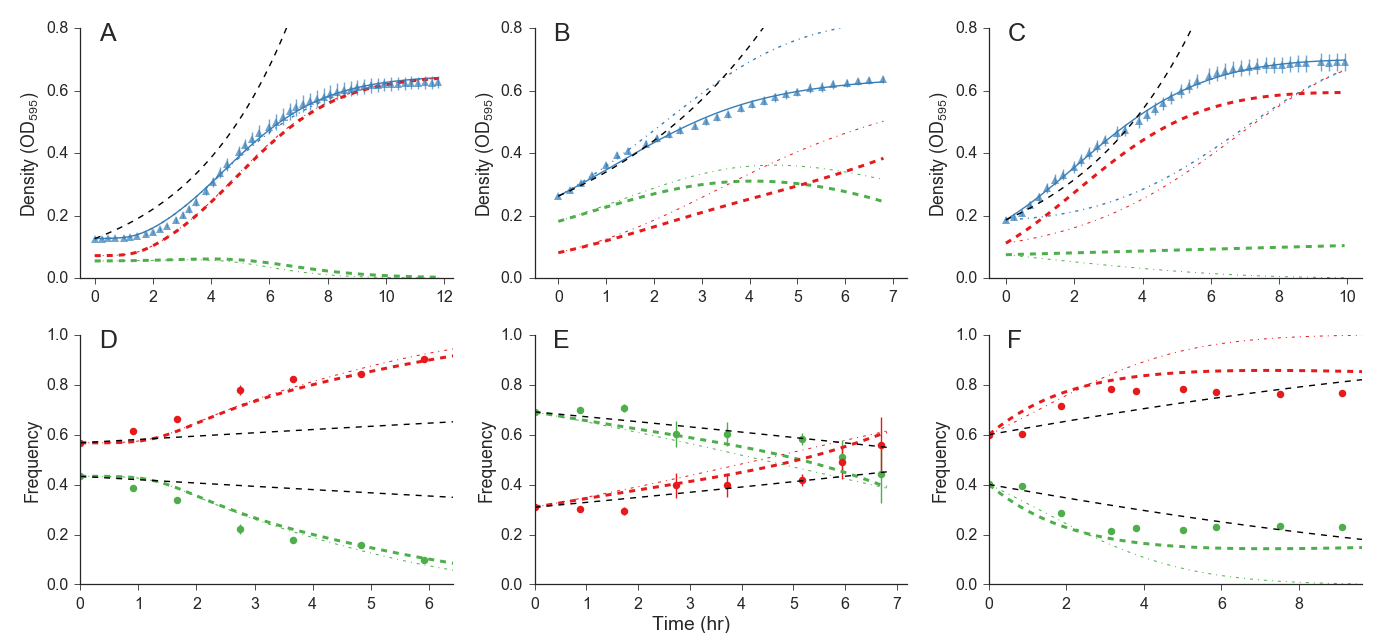


Figure 4. Predicting growth in a mixed culture. Growth of two *E. coli* competing for resources in a mixed culture. (A-C) The blue error bars are the measured total optical density in a mixed culture. The solid blue line is the fitted competition model. The green and red dashed lines represent the competition model prediction for each strain. The black line represents the exponential model prediction (see Figure S2). (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: 1.3·10-4; B: 1·10-4; C: 6.5·10-5. Estimated competition coefficients - D: a1=10, a2=0.77; E: a1=3.7, a2=2; F: a1=0.11, a2=0.53. Inferred mean selection coefficients – D: 0.0107; E: 0.0101; F: 0.0235 (see Figure S3).

## Fitness inference

The best way to infer the relative fitness of two strains is by performing pairwise competition assays7: growing both strains in a mixed culture and measuring the change in their frequencies over time. Using our method, this can be done by simply measuring densities in mono- and mixed cultures, without requiring direct measurement of strain frequencies.

Relative fitness (given by 1+*s*,where *s* is the *selection coefficient* of the strain of interest) can be estimated from pairwise competition results using3:

[4]

where and are the frequencies or densities of the strains and is time. Eq. 4 can be applied to the predicted densities of the strains above to infer the relative fitness of the strain of interest (Figure 3D-F, S3). Thus, our methods allows inferring relative fitness based on densities in mono- and mixed cultures, without requiring actual pairwise competition.

# Discussion

We have developed a new computational method to predict growth in a mixed culture from growth curves of mono- and mixed cultures, without measuring frequencies of single strains in the mixed culture. We tested and validated 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>). *Curveball* is written in Python and includes a user interface so that it can be used by non-programmers. It is free and open, and can be extended by the community: additional data formats, growth and competition models, and other analyses can be added.

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 5,7,8,13. Current methods for estimation of fitness from growth curves mostly use the growth rate or the maximum population density as a proxy of fitness. However, the growth rate and other proxies of fitness based on a single growth parameter cannot capture the full scope of effects contributing to differences in fitness14.

In contrast, our method integrates several growth phases into the fitness estimation, allowing a more holistic approach to fitness inference from growth curves data. Our method also provides information on the specific growth traits that contribute to differences in fitness. We hope our research will help to standardize the way fitness is measured, thereby improving communication between empirical and theoretical evolutionary biologists and ecologists.

## Conclusions

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

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# 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 were previously shown to have roughly the same effect on growth15. 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, Figure S1).

**Growth and competition experiment.** Strains were inoculated into 3 ml LB+Cap+Kan and grown overnight at 30°C with shaking. Saturated overnight cultures were diluted into fresh media so that the starting OD was above the OD of blank media (1:1-1:20 dilution rate). In experiments without lag phase, cultures were pre-grown for >4 hours after dilution, in 30°C, their OD monitored until the exponential growth phase was reached. Cells were then inoculated into 100 μL LB+Cap+Kan in a 96-wells flat-bottom microplate (Costar) as following:

* ~30 wells containing a monoculture of a GFP-labeled strain
* ~30 wells containing a monoculture of a RFP-labeled strain
* ~30 wells containing a mixed culture of both GFP- and RFP-labeled strains
* 2 wells with blank media

The cultures were grown at 30°C until reaching stationary phase in an automatic microplate reader (Tecan infinite F200 Pro). OD595 readings were taken every 15 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 (DPBS with calcium and magnesium, BI #02-020-1). 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 eliminate "double" event (events detected as both "green" and "red" due to high cell density) and noise in the cell sorter8.

Fluorescent cell sorter output data was analyzed using R16 with the *flowPeaks* package that implements an unsupervised flow cytometry clustering algorithm17.

**Data analysis.** Growth curve data were analyzed using *Curveball*, an new open-source software written in Python18. *Curveball* implements the method presented in this manuscript. *Curveball* includes both programmatic interface (API) and a command line interface (CLI), and therefore doesn't require programming skills. *Curveball* makes use of several python packages: NumPy19, SciPy20, Matplotlib21, Pandas22, Seaborn23, LMFIT24, Scikit-learn25, and SymPy26.

**Model fitting.** To fit the growth and competition models to the growth curve data we use the *leastsq* non-linear curve fitting procedure20,24. 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 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 with the lowest BIC27,28.

**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 analysed and figures were produced using a Jupyter notebook29 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, analysed data, discussed the results and wrote the manuscript. Y.R. and L.H. developed the model. 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 perspective 30,31. Denote by the density of a limiting resource and by the density of the population cells, 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 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 mass at a rate . Cell growth is assumed to be proportional to , whereas resource intake is proportional to , where is a power of cell density.

We can describe this process with differential equations for and :

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

These equations can be converted to equations in and :

,

which gives us the equivalent differential equations:

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

with . To solve this system, we use a conservation law approach by setting 32. 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 equation33, 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 :

.

Eq. A4 is an autonomous differential equation ( doesn't depend on ). To include a lag phase, Baranyi and Roberts 11 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 34, 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 eq. A5 produces eq. 2 in the main text.

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 *)*.

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 susggested to make the fitting procedure more stable34,35. A four parameter form has both of the previous reductions, setting and 34. Another four parameter form of the model has no lag phase, with 1, which yields the Richards model33, also called the -logistic model36, 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 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.

# Supporting figures



Figure S1. Fluorescent microscopy of *E. coli* strains. Image of DH5α-GFP and TG1-RFP cells taken using a Nikon Eclipe Ti microscope.



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.

# 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 |  |  | 33 |
| Baranyi 1997 | 4 |  |  | 34 |
| Logistic | 3 |  |  | 37 |

Table S1. Growth models. The table lists the growth models used for fitting growth curves 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* | | Green | | Red | Green | Red | Green | Red |
|  | .125 | | .124 | | .286 | .23 | .188 | .204 |
|  | .528 | | .65 | | .619 | .631 | .633 | .74 |
|  | .376 | | .587 | | .304 | .483 | 8 | 8 |
|  | 2.636 | | 1\* | | 2.484 | 1.512 | 1\* | .164 |
|  | .032 | | .008 | | **-\*** | **-\*** | .039 | .393 |
|  | .937 | | 3.735 | | **-\*** | **-\*** | .188 | .104 |

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