Bacterial growth properties at low optical densities

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Bacterial growth properties at low optical densities

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Abstract A method for accurate quantification of growth rate and yield of bacterial populations at low densities was developed with a modified version of a stepwise linear model for fitting growth curves based on optical density measurements, and adapted to measurements at low optical densities in 96-well microtiter plates. The method can be used for rapid and precise estimates of growth rate and yield, based on optical density measurements of large numbers of cultures of *Escherichia coli*. *E. coli* B lines were serially propagated at low glucose concentration during a long-term evolution experiment. Growth rate and yield of populations sampled from each of 12 lines that evolved for 20,000 generations under these conditions and two ancestral clones was measured. Populations were grown at three different glucose concentrations. Consistent with earlier findings, statistical analysis showed that both exponential growth rate and yield per unit of glucose differed significantly between the three glucose concentrations tested. Significant adaptation of the evolved populations to the nutrient conditions in which they evolved for 20,000 generations was observed.

Keywords Growth curve fitting · Growth rate · Growth yield · Low glucose concentration · Three phase linear model

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Introduction

Measurements of microbial growth properties such as lag phase duration, stationary phase yield and especially growth rate are crucial for many areas of microbiology, including experimental evolution (De Siano et al. 2006; Lenski 2004; Novak et al. 2006; Shi and Xia 2003). Here precise measurements of growth properties are especially important because they are used to quantify the fitness consequences of evolutionary adaptations (Cooper et al. 2001; Dykhuizen and Dean 1990; Elena and Lenski 2003; Vasi et al. 1994).

Experiments that measure the properties of microbial growth are typically conducted in rich nutrient

media at high population densities, owing to the ease of making the necessary measurements. However, the growth conditions for microorganisms in rich broth medium are often very different from those in their natural environment (Egli 1995; Morita 1993; Muenster 1993), which is usually characterised by much lower levels of carbon or other limiting resources (Franchini and Egli 2006). It is therefore important to measure growth characteristics under conditions that are similar to those under which the bacteria evolved. This consideration in turn necessitates the development of accurate methods for quantifying the growth of microbial populations at low nutrient levels. Applications that demand measurements obtained under conditions of low nutrients and low population densities (Augustin et al. 2000; Baranyi and Pin 1999; Feijoo et al. 1997; Llaudes et al. 2002) encounter difficulties.

Previous studies measured bacterial growth rate from low starting concentrations mostly with indicator dyes and plate counts (Augustin et al. 2000; Baranyi and Pin 1999; Feijoo et al. 1997; Llaudes et al. 2002). These methods are either only indirectly measuring population size (in the case of indicator dyes), or they are very laborious (in the case of viable cell counts). Consequently there is a great interest in finding more convenient methods for estimating bacterial growth parameters (Baranyi and Pin 1999).

Models can be fitted to optical density (OD) measurements of microbial populations growing in liquid culture (Dalgaard and Koutsoumanis 2001; De Siano et al. 2006; Lenski 2004; Membre et al. 2005). Here, a reliable method to estimate growth properties from OD measurements for microbial populations at low nutrient levels is presented. Problems in estimating growth properties in such conditions arise from the low optical density of the population, where experimental noise (e.g. air bubbles and scratches at the bottoms of wells) can have large effects on the parameter estimates. To address these issues, a modified version of a simple three-phase linear model and the corresponding fitting software to measure growth rate and yield at glucose concentrations as low as 12.5 μg/ml were developed. The method was applied to bacterial growth in 96-well microtiter plates. This approach enables extensive statistical replication of estimates of growth parameters and high throughput screening of large numbers of samples.

The growth properties of populations sampled from 12 *Escherichia coli* B lines that independently evolved for 20,000 generations under low glucose conditions (Cooper et al. 2003; Cooper and Lenski 2000; Crozat et al. 2005; Lenski 2004; Rozen et al. 2005), and of the ancestral clones were measured. These lines evolved under a regime of daily 1:100 serial transfers into fresh medium containing 25 μg of glucose per ml. Evolutionary changes in exponential growth rate and yield when populations are grown in the same environment where they evolved were previously quantified (Novak et al. 2006). Here, the growth properties of these bacteria were quantified in that same environment and also with two-fold higher and lower glucose concentrations (50 and 12.5 μg glucose per ml, respectively). Statistically significant differences in growth properties at these different glucose concentrations were found. Of particular interest, the improvement in exponential growth rate relative to the ancestral strain was greatest in the same environment in which the bacteria evolved (i.e. at 25 μg glucose per ml).

Methods

Bacterial strains

Growth properties were measured of two ancestral *E. coli* B clones and 12 evolved lines propagated by a serial transfer regime for 20,000 generations (Lenski and Travisano 1994; Lenski et al. 1991) at 37°C in Davis minimal medium supplemented with glucose at 25 μg per ml. The two ancestral clones differed only by a single marker (arabinose utilization) that is selectively neutral in this environment (Lenski 2004). Every 24 h, the twelve populations were diluted 100-fold into fresh medium, and the resulting re-growth corresponded to ~6.6 generations of binary fission per day. At generation 20,000 aliquots containing population samples were stored in 20% glycerol at −80°C [(Lenski 2004; Lenski et al. 1991), http://myxo.css.msu/edu/ecoli/].

Measurements of growth properties

Growth properties were assayed in 96-well flat bottom microtiter plates (Sarstedt) in order to allow for the screening of large numbers of samples (12 replicate populations from generation 20,000 and 2

ancestral clones at three glucose concentrations = total of 42 samples) with substantial statistical replication: 84 replicates (distributed across seven microtiterplates) for each population and ancestral clone at 12.5 μg/ml glucose concentration, 240 replicates (distributed across 20 microtiter-plates) for the ancestral clones and 40 (distributed across four microtiter-paltes) for the evolved populations at 25 μg/ml glucose and 24 or 36 replicates for the ancestral clone and each population at 50 μg/ml glucose.

Before measuring growth properties, small amounts of ancestral clones and derived populations were taken from the −80°C freezer stocks and acclimated to the experimental environment (see supplementary material). Conditioned cultures were grown in Davis minimal medium at 12.5, 25 and 50 μg/ml glucose at 37°C, while shaking at 400 rpm (shaking radius 3 mm) in the Lab-Term Kühner Shaker, in 96-well flat-bottom microtiter plates. One to 12 wells containing the same bacterial culture were on each plate, and at least four separate plates were used to generate the data for each population or ancestral clone. Several control wells containing uninoculated medium were included on each plate. To prevent evaporation of the medium water holders were placed in the incubator. Optical density (OD) at 600 nm was measured using a SpectraMax 340PC microtiter plate spectrophotometer. Typically, 5–6 measurements were obtained during log phase, 6–8 measurements during stationary phase, and 1 or 2 measurements before the OD_{600nm} increased beyond detection limit.

Data processing

To quantify the OD_{600nm} of the growth media and plastic of the microtiter plate, measurements were taken before the inoculation of the bacterial culture, to serve as "blank" values of each well. To quantify the growth properties, the values of blanks for each well of a plate were subtracted. Measurements that were below the detection limit ($OD_{600nm} < 0.002$ for the SpectraMax 340 PC), or were above a critical OD_{600nm} value that is significantly higher than that reached during stationary phase were discarded. This threshold level was 0.02 for 12.5 and 25 μg glucose per ml and 0.04 for 50 μg glucose per ml. Values higher than this threshold value indicate the presence of air-bubbles or scratches on the bottom of the plastic wells.

Mathematical model

The log-transformed OD measurements of each well were fitted to a three-parameter stepwise linear model for microbial growth (Buchanan et al. 1997). For details on the mathematical model see supplementary material.

Data fitting

The fitting software consists of two programs. The first program (written in C) essentially reads in the SpectraMax output for the OD measurements and outputs the data in a format that is useful for further analysis. The relevant data are recorded in a number of different text files. The blanks, and each measurement of OD of a given microtiter plate at a given time is stored as one text file. The position of each clone or population on the 96-well microtiter plate is given in a separate "plate design" file. Finally, the time when the OD of a 96-well microtiter plate is measured is recorded in a further file that relates the time of a measurement with the corresponding SpectraMax output file. The C program uses these data to generate a single file containing a line with the identifiers (i.e. the clone or population name), a line with the position on the plate, a line with the blanks for each position, and a line with the point of time and the optical density measurements.

The second program (written in the Open Source statistical software R) reads in the output of the first program for statistical analysis and model fitting. This program subtracts the blanks from the measurements and then removes all OD measurements that are above or below a critical threshold (as described in the section on data processing above). The filtered data are then used to fit the log-transformed OD data for each clone or population to the stepwise linear model in order to estimate the three free parameters of the stepwise linear model (i.e. length of the stationary phase, t_{lag}, the maximum growth rate, v, and the final growth yield in stationary phase, $OD_{stationary}$). The fit of the stepwise linear model is performed by minimizing the residual sum of squares using the general purpose optimizing routine optima in R. In addition to the estimates of these parameters the R program

outputs additional information such as the position of the measured sample on the microtiter plate, the name of the clone or population, the estimated initial population density, the time point of entry into stationary phase, the number of data points used for fitting and the residual sum of squares of the fit. The data transformation program and the fitting program can be obtained from the authors upon request.

Numerical and statistical analysis of fitted growth curves

The quality of the fits were checked and growth curves that were based on too few time points or that had an unacceptably high deviation between measurements and fitted model were removed. Specifically, for the data used here, the exclusion criteria were set to fewer than seven time points and a residual sum of squares larger than 0.05 for the log transformed data. In total, about 5–20% of the fitted growth curves were excluded, depending on the glucose concentration of the medium, with more curves being excluded at low glucose concentration. After applying the quality control 53–72, 27–40 and 16–36 replicates for the evolved populations at 12.5, 25 and 50 μg glucose per ml, respectively, were left for the analysis. For the two ancestral clones, there were 33 and 36, 214 and 224, and 16 and 36 replicates at 12.5, 25 and 50 μg glucose per ml, respectively. To test for changes of growth rate and normalized yield (i.e. yield per unit of glucose, which is here the ancestral medium of 25 μg glucose per ml) across the three glucose concentrations, an analysis of variance (ANOVA) using population and microtiter plate identifier as random effects, and the adaptation status (i.e. ancestral populations versus evolved clones) and the glucose concentration as fixed effects was performed. To reflect the specific experimental design, population was nested in adaptation status, and microtiter plate was nested in glucose concentration. The ANOVA was performed using the statistical software package SPSS.

Results

Growth at different glucose concentrations

To test whether the estimated growth properties are sensitive to small changes in glucose concentration, growth of the evolved lines at the initial glucose concentration (25 μg/ml) in which the lines had evolved for the last 20,000 generations, as well as at a twofold lower (12.5 μg/ml) and a twofold higher (50 μg/ml) concentration was analysed. The mean growth rate of the evolved populations increased from 12.5 to 25 μg/ml glucose but did not increase further at 50 μg/ml (Fig. 1). This pattern was distinct from that of the ancestral clones for which the mean growth rate increased monotonically with increasing glucose concentration. The ANOVA (Table 1) shows that both fixed effects (i.e. adaptation status and glucose concentration) and both random effects (i.e. microtiter plate nested in medium and population nested in adaptation status) were highly significant. Moreover, the interaction between glucose concentration and adaptation status was also highly significant. This significant interaction arises because the evolved populations showed the greatest increase in growth rate relative to the ancestor at 25 μg/ml glucose (Fig. 2a). Hence, the method utilised allowed the detection of a specific adaptation of the evolved bacteria to exactly those nutrient conditions in which the bacteria have evolved during the long-term evolution experiment (Cooper 2002; Elena and Lenski 2003; Vasi et al. 1994; Velicer and Lenski 1999). Of note, if all possible interaction terms are incorporated into the ANOVA, then the interaction between adaptation status and glucose concentration is no longer significant, because of a significant interaction between population and glucose concentration. However, this is due to one outlier population that has the lowest value for growth rate when grown at the concentration at which it had evolved (see Fig. 1a). In a previous study (Novak et al. 2006) this population showed an anomalous decline in growth rate over evolutionary time. It also grows poorly in some conditions where the ancestor and other evolved populations produce normal-sized colonies (R. E. Lenski, personal observation). Some difference between microtiter plates and flasks as growth environments might account for the outlier status of this population (Novak et al. 2006). When this population is eliminated from the analysis, the interaction between adaptation status and glucose concentration becomes significant again. The growth yield (normalized to 25 μg/ml glucose by multiplying yield at glucose concentration of 12.5 and 50 μg/ml by 2 and 1/2, respectively) decreased in some evolved

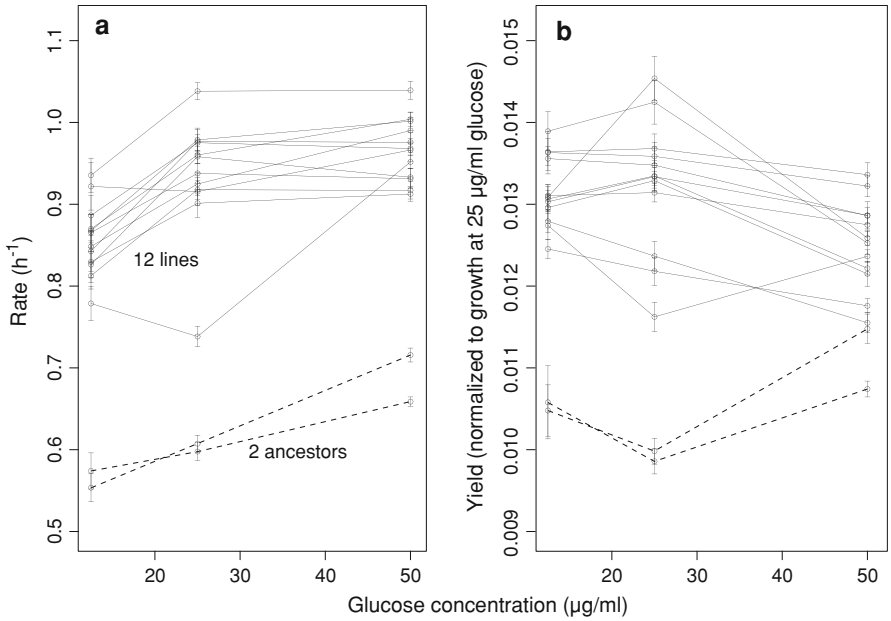

Fig. 1 Growth rate (**a**) and yield (**b**) of 12 evolved populations (*solid lines*) and two ancestral clones (*dashed lines*) for three low glucose concentrations. Growth is normalized to 25 µg glucose per ml by multiplying measurements at 12.5 and 50 µg glucose per ml by 2 and 1/2, respectively. *Error bars* represent the standard error of the mean (based on 53–72, 27–40, and 16–36 replicates for the evolved populations at 12.5, 25, and 50 µg/ml glucose, respectively; 33 and 36, 214 and 224, and 16 and 36 replicates for the two ancestral clones at 12.5, 25, and 50 µg/ml glucose, respectively). The statistical analysis (see main text) shows that rate and yield differ significantly across glucose concentrations in both evolved populations and ancestral clones

Table 1 Nested ANOVA for growth rate obtained for the 12 evolved populations and two ancestral clones in three different glucose concentrations

Factors	*df*	MS	*F*	*P*
Evolved	1	20.058	86.5	<.0001
Medium	2	.770	37.6	<.0001
Population (evolved)	12	.221	39.1	<.0001
Plate (medium)	52	.026	4.9	<.0001
Evolved × medium	2	.114	20.2	<.0001

populations with increasing glucose concentration, with several populations exhibiting a peak at 25 µg/ml glucose (see Fig. 1b). Conversely the ancestral clones had lowest normalized yield at 25 µg/ml glucose. The ANOVA revealed that both fixed effects (i.e. adaptation status and glucose concentration) and both random effects (i.e. microtiter plate nested in medium and population nested in adaptation status) were highly significant also in the case of yield (Table 2). Again the ANOVA revealed highly significant interaction between glucose concentration and adaptation status indicating a specific adaptation of the evolved bacteria to the exact nutrient conditions in which they evolved (Fig. 2b).

Discussion and Conclusions

In this paper, a fitting method for accurate quantification of growth properties of bacterial populations grown at low levels of nutrients (Augustin et al. 2000; Baranyi and Pin 1999; Feijoo et al. 1997; Llaudes et al. 2002) is presented. The method is based on spectrophotometric measurements of optical density (OD_{600nm}) in 96-well microtiter plates and was applied here to data of *E. coli* B populations grown at three different low glucose concentrations (12.5, 25 and 50 µg glucose per ml). At such low glucose concentrations most of the spectrophotometric signal is due to the absorption by the culture medium and the material (i.e. the plastic of the microtiter plate). Moreover, there is considerable variation of the light

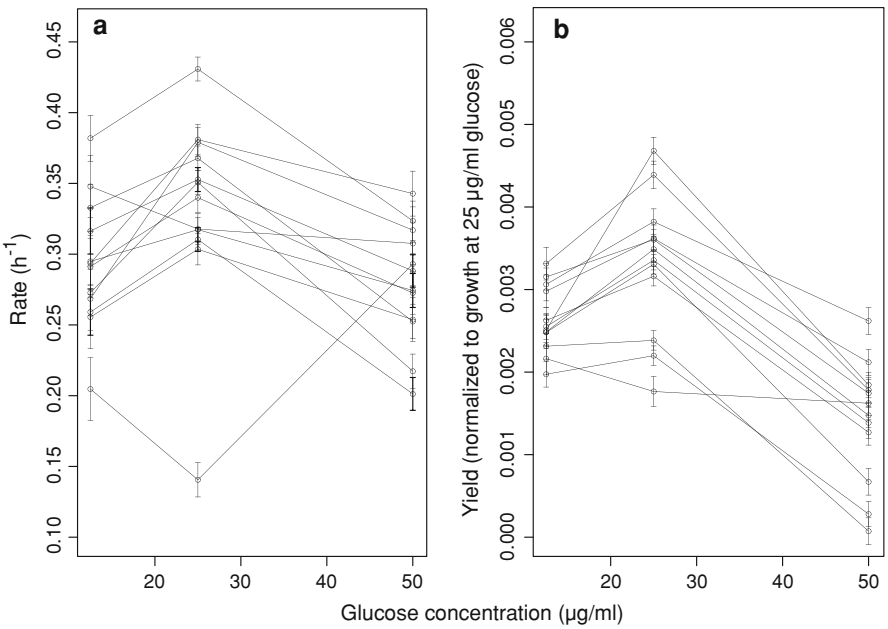

Fig. 2 Increase of growth rate (**a**) and yield (**b**) of 12 evolved populations relative to the ancestral clones for three low glucose concentrations based on the data shown in Fig. 1. The statistical analysis (see main text) reveals a significant specific adaptation of the evolved bacteria to the glucose concentration in which they evolved during the last 20,000 generations

Table 2 Nested ANOVA for growth yield obtained for the 12 evolved populations and two ancestral clones in three different glucose concentrations

Factors	*df*	MS	*F*	*P*
Evolved	1	.001	60.4	<.0001
Medium	2	.0000526	14.4	<.0001
Population (evolved)	12	.0000209	32.6	<.0001
Plate (medium)	52	.00000479	7.4	<.0001
Evolved × medium	2	.0000781	121.8	<.0001

absorption among different wells in a single microtiter plate. This method achieves greatly increased sensitivity by correcting the OD_{600nm} measurements of each well with its absorption in the absence of bacteria.

Estimates of the growth rate and yield are obtained by fitting a stepwise linear model to the corrected spectophotometric measurements. The accuracy of the estimates of growth rate and yield depends on the number of measurements taken during log phase and stationary phase, respectively. In the data used here, around 12–15 OD_{600nm} measurements, of which around 5–6 measurements were made during log phase and 6–8 measurements during stationary phase (and typically 1 or 2 measurements below detection limit) were typically obtained. To increase the accuracy of the estimates of growth rate, measurements with unusually high OD_{600nm}, which typically are due to air-bubbles or scratches on the plastic of the microtiter plate were eliminated. Moreover, all estimates of rate and yield that were based on less than 7 OD_{600nm} measurements were eliminated as well. This allowed the estimation of growth rate with a coefficient of variation (i.e. standard deviation divided by mean) of 10.6, 5.3 and 4.8 for 12.5, 25 and 50 µg glucose per ml, respectively. The coefficient of variation for growth yield was 6.7, 4.8 and 4.4% for 12.5, 25 and 50 µg glucose per ml, respectively. For example, a coefficient of variation of 5% in growth rate and yield at 25 µg/ml glucose implies that a sample size of 5 would be sufficient to detect a difference of 10% in growth rate and yield as statistically significant (at $P = 0.05$ with a probability of 80%; Van Belle and Martin 1993). As this method is based on measuring growth in microtiter plates, large numbers of replicates can be used to obtain highly accurate measurements of growth rate and yield (Novak et al. 2006).

To test the described method, growth properties of *E. coli* B populations that have evolved for 20,000 generations in a constant regime of daily batch culture at low glucose concentration (25 µg glucose per ml) (Lenski 2004; Lenski et al. 1991) were analysed. Using this method significant differences between growth properties of bacterial populations in three

different low levels of glucose were detected. Specifically, it was revealed that the exponential growth rate and the yield per unit of glucose differed significantly between 12.5, 25 and 50 μg glucose per ml. Note that, the dependence of the exponential growth rate on glucose concentration is in conflict with the Monod model, provided the substrate affinity constant Ks is indeed as low as <0.1 (Vasi et al. 1994). Here, one should also have in mind that estimates of Ks for *E. coli* across literature vary by three orders of magnitude. Also, it has become clear that the Ks constant used in the Monod model is not a constant, but can vary (Ferenci 1999; Kovarova-Kovar and Egli 1998). Moreover, it was shown that the increase in growth rate and normalized yield of the evolved relative to the ancestral populations were greatest at 25 μg/ml glucose. This implies, that the evolved populations have adapted specifically to the glucose concentration used in the batch cultures in which the bacteria have been propagated. The specific adaptation to glucose concentration turned out to be significant despite the relatively small difference between the initial glucose concentrations tested (i.e. a twofold higher and lower initial glucose concentration in batch culture). Thus, this data support earlier findings that document specific adaptation of the bacteria to 25 μg/ml glucose based on fitness measurements derived from competitive growth of evolved versus ancestral populations at different glucose concentrations (Cooper 2002). In contrast to the previous study by Cooper 2002, fitness, which is a relative measure, is split into it's rate and yield components, which are absolute values. This gives a more detailed picture of the adaptation to limited glucose.

The demonstration of significant differences between growth properties at different levels of glucose concentration not only documents the sensitivity of the presented method, but also emphasises the need for accurate measurement of growth properties of bacteria under conditions that are maximally similar to those in their evolutionary relevant environments. This is particularly important for experiments that require accurate estimates of growth properties of bacteria under natural conditions, which are frequently characterised by low concentration of nutrients (Franchini and Egli 2006).

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