The last generation of bacterial growth in limiting nutrient

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Abstract

Background: Bacterial growth as a function of nutrients has been studied for decades, but is still not fully understood. In particular, the growth laws under dynamically changing environments have been difficult to explore, because of the rapidly changing conditions. Here, we address this challenge by means of a robotic assay and measure bacterial growth rate, promoter activity and substrate level at high temporal resolution across the entire growth curve in batch culture. As a model system, we study E. coli growing under nitrogen or carbon limitation, and explore the dynamics in the last generation of growth where nutrient levels can drop rapidly.

Results: We find that growth stops abruptly under limiting nitrogen or carbon, but slows gradually when nutrients are not limiting. By measuring growth rate at a 3 min time resolution, and inferring the instantaneous substrate level, s, we find that the reduction in growth rate \( \mu \) under nutrient limitation follows Monod’s law,

\[
\mu = \frac{\mu_0 s}{k_s + s}
\]

where \( \mu_0 \) is the growth rate at saturating substrate, and \( k_s \) is the substrate level at which growth rate is half maximal [6]. Many subsequent theoretical and experimental studies with several substrates and different bacteria found similar growth laws, and suggested several modifications to this law [7-13].

Most of these studies on growth laws measured bacteria in a steady-state situation, using a chemostat or balanced growth in a batch culture inoculated at high dilution [6,9,10,14]. Measuring growth laws in dynamical concentration. Therefore, the total generated biomass in a density interval along the growth curve allows one to infer the amount of substrate utilized. Monod found a simple mathematical relation connecting the bacterial growth rate \( \mu \) to the concentration of a growth-limiting substrate \( s \), known as the Monod law:

\[
\mu = \frac{\mu_0 s}{k_s + s}
\]

Conclusions: The observed sharp stop of growth accompanied by a pulsed expression of assimilation genes allows bacteria to compensate for the drop in nutrients, suggesting a strategy used by the cells to prolong exponential growth under limiting substrate.

Keywords: Bacterial growth, Monod law, Gene expression, Nutrient limitation, Nitrogen assimilation

Background

In recent years there has been a resurgence of interest in bacterial growth laws, a field of study initiated over 60 years ago but not yet fully understood [1-4]. Studies on microbial growth kinetics were initiated by the seminal studies of Monod [5,6] who measured the relation between sugar concentration and bacterial growth rate. For high sugar levels, where the substrate concentration was in excess, Monod directly measured the exponential growth rate. At low initial sugar levels, that declined during growth due to bacterial consumption, Monod was unable to measure the substrate levels directly, and relied on the assumption that bacteria grow with a constant yield – that is, a unit increase in biomass corresponds to a constant times a unit decrease in sugar concentration. Therefore, the total generated biomass in a density interval along the growth curve allows one to infer the amount of substrate utilized. Monod found a simple mathematical relation connecting the bacterial growth rate \( \mu \) to the concentration of a growth-limiting substrate \( s \), known as the Monod law:

\[
\mu = \frac{\mu_0 s}{k_s + s}
\]
situations - such as batch cultures which deplete substrate and enter the stationary phase [5] - is much more complicated. A highly dynamical situation occurs in the transition phase between exponential growth and stationary phase where growth stops (also called the deceleration phase). This period, in which μ is strongly influenced by s, is brief, making it difficult to analyze [3,11], both in terms of substrate-growth relations, and gene expression: most studies on gene expression lack the temporal resolution to address such rapidly changing situations [15-18].

Here, we study the last stages of growth in limiting nutrient, in which nutrient levels drop dramatically. To achieve this, we use a robotic assay that allows for the measurement of bacterial growth and gene expression at high temporal resolution (~3 min). We also calculate the measurement of bacterial growth and gene expression at different situations [15-18].

We find similar results for glucose as a limiting substrate. Cells grown on M9 minimal medium with ample nitrogen (18.7 mM NH4Cl) and low levels of glucose (less than ~0.5 mM) stop growth abruptly, going from maximal growth rate to zero growth within 30 ± 3 min (Figure 1d, yellow, pink and purple lines). An abrupt stop of growth in glucose limitation for E. coli was previously observed qualitatively [19,20]. At high glucose levels (more than 11 mM), growth slows gradually over about 4 h (Figure 1d green line). At intermediate glucose levels cells show a transition between gradual slowing and abrupt stop of growth (Figure 1d brown line).

The present assay allows estimation of the substrate level at each time point. Instead of a direct measurement of the substrate level, which is challenging to perform at high temporal resolution and accuracy at low substrate levels, we inferred the substrate level from the bacterial density. To do this, we assume, that the substrate removed from the medium by the cells is incorporated into their biomass with a constant yield [5] ; and that the total biomass (or cell volume) produced is proportional to the OD, as previously demonstrated [10,22]. Indeed, we found that as long as the substrate is limiting, the final OD reached by the culture is proportional to the initial substrate level (Figure 2a). Since under-limiting conditions the final OD is significantly lower than the maximal OD (reached by the non-limited culture) it is unlikely that growth is limited by factors other than the limiting nutrient (that is, effects of the bacteria on the medium other than depletion of the limiting nutrient can be safely neglected due to the low bacterial concentration). The relation between final OD and initial substrate level only begins to saturate when the substrate become non-limiting (initial NH4Cl concentrations higher than about
2 mM (Figure 2a inset)). The slope of the proportionality line, c, allows one to translate OD units into substrate units. In this way we calculate the substrate at each time point s(t), using the OD reached at time t:

\[ s(t) = s(t=0) - c \ OD(t) \]  

Next, we plotted the observed growth rate as a function of the inferred substrate at each time point. We found that for low substrate media – in which growth stops abruptly - the decline in growth rate in the deceleration phase is well described by the Monod law, with \( K_s = 2.6 \pm 0.4 \ \mu M \) for nitrogen (Figure 2b). Similar results are found for glucose, with \( K_s = 5 \pm 1 \ \mu M \) (Additional file 1: Figure S1).

The \( K_s \) values estimated here can be compared to those estimated in steady-state exponential growth. To our knowledge, the value of \( K_s \) for nitrogen has not been previously reported. The value for glucose lies within the large range of previously measured \( K_s \) which spans almost 3 orders of magnitude (from ~0.5 mM to 0.4 \( \mu M \), [11,13,23,24]). These large differences were attributed to strain variations, differences in growth methods, bacterial density, length of exposure to low glucose concentrations, or the history of the inoculi [1,2,11].

Promoter activity of nitrogen and carbon assimilation genes rises sharply in the last generation before growth stops

The present assay allows measuring, along with the growth rate at each moment, the promoter activity of selected genes. For this purpose, we used reporter strains in which the promoter of interest controls the expression of a green fluorescent protein (GFP). Reporter strains were taken from a comprehensive library of \( E. \ coli \) reporters, in which promoters control the expression of the fast folding and highly stable GFPmut2 [25,26]. We studied the
dynamic expression from selected promoters at a time resolution of 8 min. Promoter activity is measured by the rate of accumulation of green fluorescence per OD unit as described [25,27].

We studied the $glnA$ promoter which controls an operon of genes essential for ammonia assimilation (glutamine synthetase $glnA$, the nitrogen regulator $ntrC$ and its regulatory partner kinase $ntrB$ [28-30]). We find that under nitrogen limiting conditions the $glnA$ promoter during exponential phase had moderate activity that is independent of nitrogen levels. Then, about one generation before growth stopped, promoter activity rose sharply by about 4–6 fold (Figure 3). Promoter activity dropped back to low levels when growth stopped. The level of nitrogen at which the rise occurs is about the same, 0.25 ± 0.04 mM, for the three lowest nitrogen levels tested (Figure 3).

In contrast to the pulse of activity at the end of growth on limiting nitrogen, $glnA$ promoter activity remained roughly constant throughout growth on non-limiting nitrogen (M9 standard formula, 18.7 mM NH$_4$Cl), and it gradually declined during entry to stationary phase (green line in Figure 3). We also tested the $glnK$ promoter which controls other genes involved in ammonia assimilation (the nitrogen regulatory protein $glnK$, and

**Figure 2** Under nutrient limitation decline in growth rate is well described by Monod equation. (a) Final OD correlates linearly with initial substrate (NH$_4$Cl) level: $OD_f(s) = a*s + b$, with $a = 0.066 ± 0.003$ and $b = 0.006 ± 0.001$. This relation only saturates at high (more than 2 mM) initial nitrogen level (inset). The intersect of the linear graph is not zero probably due to low levels of nitrogen which were transferred to the media from the over-night culture together with the bacterial inoculum. (b) Growth rate as a function of substrate level, calculated using the linear relation between OD and substrate levels in (a). Line: fit to Monod law, with $K_s = 2.6 ± 0.4 \mu M$. The 0.31 mM curve deviates from the Monod fit more than other curves for unknown reasons.
the ammonia transporter-\textit{amtB} [31-33]). We found very similar results: a sharp rise of promoter activity one generation before growth halts on limiting nitrogen, and a basal level that is nitrogen independent during exponential growth (not shown). This is consistent with previous experiments by Ninfa and colleagues who showed that \textit{glnA} and \textit{glnK} are activated when \textit{E. coli} is starved for ammonia [18]. In contrast, non-related promoters (e.g. \textit{crp} and sigma70 synthetic promoters [34], \textit{clpD, serA, cysD} promoters) showed no increase in promoter activity at the end of growth under nitrogen limiting conditions (not shown). Moreover, the abrupt stop in growth upon nitrogen limitation is not accompanied by increased activity of promoters controlling known stationary phase genes (e.g. \textit{wrbb}, \textit{uspB}, as well as a sigmaS synthetic reporter, not shown)[35].

The strong promoter activity peaks and shut-down follow a hill-like function (Additional file 1: Figure S6)

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{\textit{glnA} promoter is sharply activated in the last generation of growth. (a) Promoter activity of \textit{glnA} as a function of time at several levels of initial nitrogen with a time resolution of 8 min. Promoter activity was calculated by computing the rate of accumulation of GFP per unit time divided by the OD (dGFP/dt/OD). Each point in the graph represents the average promoter activity of 48 experimental replicas with standard error on the order of ~3% at each time-point (b) OD curves for the same experiment, with the position of the peak in promoter activity overlaid on the growth curve (open dots). (c) same as (a) where the x axis is generations instead of time.}
\end{figure}
reminiscent to previous experimental and theoretical studies which found bi-stability and positive feedback loops in the nitrogen assimilation enzymes and transporters under very low nitrogen levels [36,37].

The rise in \textit{glnA} promoter activity in the last generation before growth stops at low nitrogen, is consistent with previous findings that under severely limiting nitrogen (0.19 mM of NH$_4$Cl), \textit{glnA} expressed from a regulated promoter must be over-expressed by 4–5 fold over its wild-type basal levels (at high nitrogen) in order to attain the same growth rate [38]. In that experiment, \textit{glnA} was deleted from the chromosome and placed under control of a \textit{tac} promoter. Thus, the pulsed expression of \textit{glnA} in the last generation of growth is prevented. We tested this mutant strain in the present system, and found that it showed a gradual reduction in growth rate, rather than a sharp stop, at limiting nitrogen levels and low induction levels (Additional file 1: Figure S2). At high induction of \textit{glnA}, the abrupt stop is restored (Additional file 1: Figure S2).

We also tested the effect of removing NtrC- a transcriptional regulator of the \textit{glnA} operon- as well as other operons involved in nitrogen metabolism [28]. An \textit{ntrC} deletion strain which is defective in the regulation of nitrogen metabolic genes showed slower growth rate at low nitrogen levels, and gradual rather than abrupt stop of growth (Figure 4). This finding indicates that transcription regulation by NtrC is essential in order to obtain maximal growth rate and an abrupt stop of growth under nitrogen limiting conditions.

Similar results are also found on limiting glucose (Additional file 1: Figure S3). Here, we studied a reporter for the activity of CRP, a central regulator of sugar metabolism. The reporter plasmid contains a consensus site for CRP controlling GFP expression [34]. We find that CRP activity is moderate and glucose-level-independent throughout exponential growth (4 first hours of growth, Additional file 1: Figure S3). In glucose limitation it shows a rise of about 3-5-fold that lasts about one half of a generation, before growth stops (Additional file 1: Figure S3). In non-limiting glucose (11 mM), CRP activity rises gradually, remaining high in early stationary phase (Additional file 1: Figure S3). Similar results were also found for the \textit{ptsG} promoter, which controls the expression of the PtsG subunit of the PTS glucose permease [39,40] (Additional file 1: Figure S4). It should be noted that in this case the promoter is highly active also in the non-limiting conditions since glucose is the sole carbon source but the increase and decline in promoter activity is moderate compared to the limiting conditions. This observation is in line with recent findings showing a pulse of cAMP level and a sharp increase in the promoter activity of the \textit{acs} gene upon glucose exhaustion [41].

Taken together, these results suggest that up regulation of the relevant metabolic genes in the last generation of growth allows prolonged exponential growth followed by a sharp decline in growth. The pulse of metabolic proteins at the last generation may compensate for the sharp decline in substrate in this phase of growth.

**Discussion**

We used a robotic assay to measure bacterial growth rate, substrate level and promoter activity at high temporal resolution across the growth curve. We find that growth stops abruptly under limiting nitrogen or carbon but slows gradually when these nutrients are not limiting. The abrupt stop is accompanied by a pulse-like up regulation of the expression of genes in the relevant nutrient assimilation pathways. Disrupting the regulation of these genes abolishes the pulse of expression, and turns the sharp stop of growth into a gradual deceleration. Reduction in growth rate under nutrient limitation
follows Monod’s law, evaluated at each moment with the instantaneous level of substrate.

Bacterial growth laws have mostly been measured in exponential phase in batch culture or in chemostats (see [3,11] for reviews). Studies of growth dependence on substrate in dynamical situations are scarce due to lack of experimental methods that can accurately probe such situations [5]. The present assay enables measurement of growth laws in a batch culture, including the stages where substrate is rapidly depleted by cells nearing stationary phase. The measurements were enabled by the high temporal resolution of the robotic assay, and the large number of repeats which allowed growth to be measured with a standard error of about 2%. Difficulties in measuring very low substrate concentrations are bypassed by using accurate measurements of OD and a calibration curve relating OD (biomass) to substrate [13] - a method that can in principle be generalized to other substrates that are incorporated into biomass. Using this approach we could add many experimental points to Monod’s original data on glucose limitation and extend it to nitrogen limitation. In both cases we found that Monod equation fits the data well.

The results suggest a mechanism used by the cells to prolong exponential growth under limiting substrate. The cells express a low basal level of assimilation proteins throughout exponential growth (this level is independent of substrate levels). Then, when substrate drops below a critical level (about 0.25 mM in the case of nitrogen in the form of NH4Cl), the cells up regulate the enzymes, regulators and transporters in the assimilation pathway. Such maximal regulator activity only at extreme signal is consistent with the finding that positive feedback regulation in the Pho system is active only at very low signal levels [42]. In our system the pulse of expression allows cells to maintain their rapid exponential growth rate for about one more generation (Figure 5). In this generation, they are able to utilize the remaining substrate. In other words, instead of growth rate declining at 0.25 mM nitrogen, the enzymes allow rapid growth until about 100-fold lower nitrogen levels, on the order of $K_s = 2.6 \pm 0.4 \mu M$. Growth stops when substrate drops below $K_s$.

Conclusions
The present study extends our understanding of the last generation of bacterial growth in batch culture prior to entry into stationary phase. We find that growth stops abruptly under limiting nitrogen or carbon and that reduction in growth rate follows Monod’s law. By following promoter activity of different genes we found that the abrupt stop of growth is accompanied by a pulse-like up-regulation of the expression of genes in the relevant nutrient assimilation pathways. This mechanism allows the cells to maintain their growth rate for about one more generation in which they are able to utilize low levels of substrate. The results presented in this study suggest a strategy used by the cells to prolong exponential growth under limiting substrate.

Methods
Strains and plasmids
All strains in this study were derivatives of NCM3722 strain, a standard wild-type $E. coli$ strain used in studies of the nitrogen system [43]. For growth rate measurements we used the NCM3722 parental strain. For measuring

![Figure 5](http://www.biomedcentral.com/1752-0509/7/27) By increasing the levels of assimilating proteins bacteria reach their final OD faster. A schematic representation for a mechanism for rapid growth in the last generation. A pulse of metabolic proteins allows the cell to compensate for a sharp decline in substrate. This allows continued exponential growth at about the same speed as maximal exponential growth rate. Growth ends abruptly when substrate goes below the Monod constant. If the pulse of metabolic enzymes is prevented (eg. by mutating the regulators), a gradual stop of growth is obtained. In a hypothetical competition between the wild-type and mutant strain, the wild-type would deplete resources faster and outgrow the mutant in the last generation. Over repeats of this competition, the wild-type strain would be selected. This is a basis for the potential selective advantage of the regulatory strategy found in the present study.
resolution of measurements was ~8 min. Promoter activity was calculated by computing the rate of accumulation of GFP fluorescence per unit time divided by the OD (dGFP/dt/OD) as described [27].

Calculation of substrate levels
A conversion ratio between OD and substrate levels was computed using linear regression at different limiting substrate conditions (0.94 mM, 0.47 mM, 0.31 mM, 0.24 mM of NH₄Cl (see Figure 2)). Substrate curves were then calculated by: s(t) = s(0) – c OD(t), with c = 15.1 ± 0.7 for nitrogen and c = 21.3 ± 6.3 for glucose. This method relies on the assumption that conversion rate of substrate to biomass (the yield factor) is constant over the timeframe investigated, and that OD is linear in biomass.

Additional file

Additional file 1: The last generation of bacterial growth in limiting nutrient.

Abbreviations
CRP: cAMP receptor protein; GFP: Green fluorescent protein; OD: Optical density; PA: Promoter activity.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
AB conceived and designed the research, performed the molecular genetics manipulations and the experiments, analyzed data and wrote the paper. YH designed the research, analyzed the data and wrote the paper. ED participated in the design of the study and contributed to the mathematical analysis of the data. DK contributed to the design of the study and helped to write the paper. UA designed the research, analyzed data and wrote the paper. All authors read and approved the final manuscript.

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