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Effect of the Volume-to-Surface Ratio of Cultures on *Escherichia coli* Growth: An Experimental and Theoretical Analysis

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Abstract The growth dynamics of bacterial populations are usually represented by the classical S-shaped profiles composed of lag, exponential and stationary growth phases. Although exceptions to this classical behavior occur, they are normally produced under non-standard conditions such as supply of two carbohydrates as sole carbon source. However, we here report variations in the classic S-shaped growth profiles of *Escherichia coli* under standard culturing conditions; explicitly, we found growth during transition to the stationary phase wherein the bacterial growth rate inversely depended on the volume-to-surface ratio of cultures (*V*/*S*); the reasons for this behavior were experimentally explored. To complement our experimental analysis, a theoretical model that rationalizes the bacterial response was developed; simulations based on the developed model essentially reproduced experimental growth curves. We consequently conclude that the effect of *V*/*S* on *E. coli* growth reflects an interplay between auto-catalytic bacterial growth, bacterial growth auto-inhibition, and, the relief of that inhibition.

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Introduction

The growth dynamics of bacterial populations are typically portrayed by the classic logistic pattern of S-shaped growth profiles composed of three phases: the starting phase where growth is delayed, or lag phase; the exponential phase, which is characterized by fast multiplication and lastly the stationary phase where bacterial growth ceases. Nevertheless, a variety of exceptions to this classical growth behavior have been reported. For instance, when *Escherichia coli* is grown in minimal medium with only two sugars as the sole carbon source, so-called diauxic growth curves were observed that are characterized by two exponential growth phases separated by a lag phase [7, 9]. Unusual growth kinetics may also occur under steady-state conditions where the culture is continuously fed with fresh medium under flow conditions [8, 10]. The bacterial growth also responds to surface properties when bacteria are attached to glass beads, sand particles or stones, i.e., subjected to conditions that promote the access of bacteria to nutrients, particularly in diluted culture medium [3]. Likewise, in in vitro cultures of eukaryotic cells, a positive correlation between the culture volume and cell growth has been observed where the inoculum, i.e., the initial number

of cells was kept constant but the volume of the medium was varied [11]. The effect was explained by the higher total amount of some key nutrients in the larger culture volumes.

The above-quoted variations from classical growth behavior refer to specific culturing conditions that are different from typical bacterial studies. In contrast, the experiments performed in our study address standard procedures where bacteria were grown under conventional conditions with fixed initial bacterial concentrations in nutrient-rich liquid medium (batch cultures). Under these conditions, we detected variations in the classic S-shaped growth profiles; specifically, we observed growth after the exponential phase, i.e., growth during transition to the stationary phase; analyses of how culturing conditions may affect such transition phase have not been reported so far.

Materials and Methods

Bacterial Growth Conditions

The EPEC *E. coli* O127:H6 strain [1, 4] was kept frozen prior to use at −70 °C in 15 % glycerol and then plated on Luria–Bertani agar directly from the frozen glycerol stock. After overnight growth at 37 °C under air atmosphere, a bacterial suspension [12] in 0.9 % NaCl adjusted to an optical density of 0.1 at 600 nm was produced and 20 µL of it passed to 10 mL of liquid medium (1.5 % bacto-peptone, 0.4 % yeast extract, 0.5 % NaCl). For growth under variations of the volume-to-surface ratio, different volumes of the inoculated media were transferred into sterilized and thermostated 3.5-mL standard UV quartz cuvettes. The optical density was measured with a HP 8453 UV–Vis spectrophotometer at 37 ± 0.3 °C under gentle magnetic stirring. Before incubation, the UV cells were capped under sterile air atmosphere leaving the screw caps slightly loose to allow for gas exchange. For blockage of the culture medium surface, we used a pre-molded paraffin block that tightly fitted the inside of the cuvette. It may be noticed that in this report growth kinetics are presented in linear–linear axes rather than in the common semi-log plots (logarithmic *y*-axis) because this allowed for better appreciation of post-exponential growth.

Numerical Simulations

Numerical simulations were based on a rate equation approach for bacterial growth dynamics [13], i.e., on the numerical solution of coupled ordinary differential equations, and employed with the non-commercial simulation and adjustment software Simulation and Adjustment 3.3 by Dominique Lavabre (Université Paul Sabatier, Toulouse, France), downloadable at: http://pagesperso-orange.fr/cinet.chim/home_eng.html. The general algorithm for the numerical integration of the differential equations was based on a semi-implicit fourth-order Runge–Kutta method with stepwise control for stiff ordinary differential equations [5].

Results and Discussion

Bacterial Growth Dynamics

We studied the bacterial growth dynamics of enteropathogenic *Escherichia coli* (EPEC *E. coli*), which is a human intestinal pathogen [1, 4]. Our initial kinetic analyses of EPEC *E. coli* growth revealed unusual growth profiles that seemed to depend on the volume-to-surface ratio (*V*/*S*) of the liquid cultures; we hence set out to record growth under different *V*/*S*, i.e., by varying the height of the liquid column of the culture medium at a fixed liquid/gas surface area or under variations of the surface area at a fixed height of the liquid column (Fig. 1).

Standard bacterial growth conditions were employed to obtain experimental data on the growth kinetics of EPEC *E. coli*. Bacterial growth was recorded by continuous spectrophotometrical turbidity measurements under constant and gentle stirring. The experimental approach was oriented to inform the global dynamics of the growth scenarios. For this purpose, only the macroscopic characteristics of the system were evaluated.

In Fig. 2, we show EPEC *E. coli* growth in spectrophotometric cuvettes of the same capacity but containing different culture medium volumes. It may be observed that during the fast bacterial multiplication stage of all experiments, i.e., until about $t = 4.5$ h, the time-evolution of the bacterial growth nearly matched and followed the classical growth behavior. However, after the fast growth stage the growth did not level off completely, as perhaps anticipated

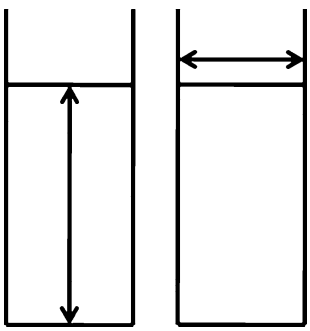


Fig. 1 Change of the scaling properties by variations of the height of the liquid column or by obstructing the liquid/gas surface area. The figure schematically represents experiments performed in 3.5-mL UV cuvettes

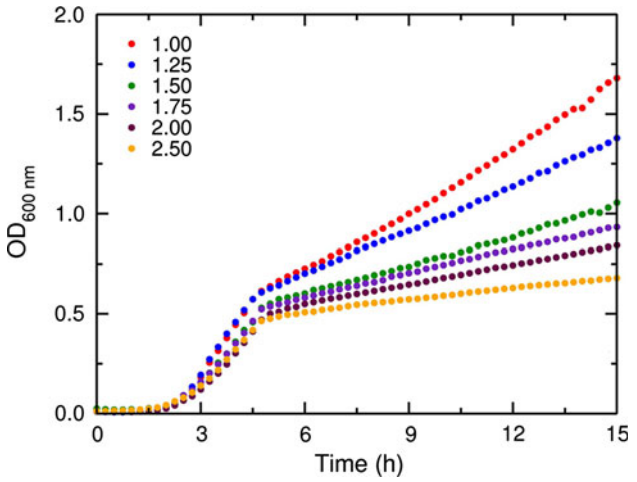


Fig. 2 Time-evolution of the optical densities during the growth of EPEC *E. coli* bacteria. Cultures were carried out in standard UV cuvettes under air atmosphere at 37 ± 0.3 °C and under gentle magnetic stirring. The curves correspond to individual experiments conducted at different volume-to-surface ratios (*V*/*S*) of 1.0, 1.25, 1.5, 1.75, 2.0, and 2.5 cm, respectively (*top* to *bottom*)

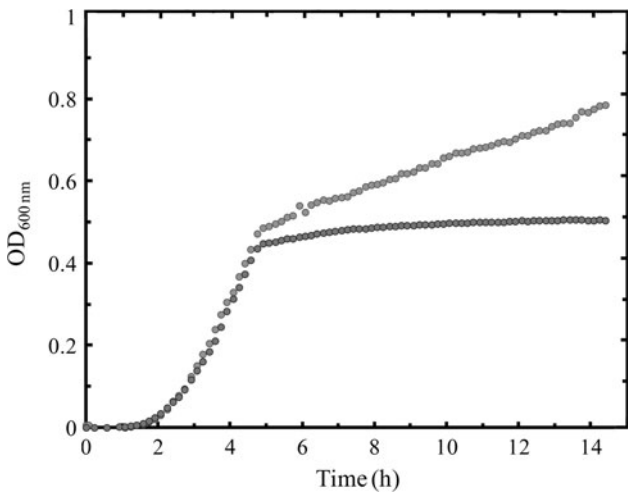


Fig. 3 Kinetics of bacterial growth under obstruction of the liquid surface. Growth of EPEC *E. coli* in a cuvette fitted with a paraffin block to obstruct the culture surface (*lower curve*) in comparison to growth under conditions of a free surface (*upper curve*); same experimental conditions as in Fig. 2

by a simple logistic approach, but instead continued to rise almost linearly over considerable time and with different rates depending on the established *V*/*S*, where the smallest ratios gave rise to the highest growth rates and vice versa.

We confirmed the reciprocal relation between growth rate and *V*/*S* by further experiments in which the liquid surface of the bacterial cultures was physically obstructed with a floating paraffin block. Under those conditions a virtually zero growth rate during the divergence stage was observed (Fig. 3, lower curve), i.e., the kinetic curves were "classically" S-shaped without any post-sigmoidal population increase, i.e., without growth during transition to the stationary phase.

Further experiments under a helium atmosphere (Supplementary Material) demonstrated a similar behavior as the experiments under air shown in Fig. 2. Hence the observed effect is most likely independent of the air present in the headspace. Measurements of dissolved O_2 in the liquid medium during bacterial growth indicated depletion of O_2 close to the time when the divergence in the bacterial growth kinetics began (Supplementary Material), pointing towards post-exponential growth under anaerobic conditions.

Bacterial Growth Model

The experimental data cannot be accounted for straightforward explications. Although complex bacterial growth behavior can be induced, for instance by pre-arranged availability of the carbon source during the bacterial growth evolution [7, 9], the dependence of growth on *V*/*S*, as in the present case, remained a riddle. This is because all experiments shown in Fig. 2 were conducted under the same conditions, i.e., equal initial bacterial concentrations, same temperature and medium composition, same stirring rate and incubation in same-size containers. Hence, one would expect a simple match in the growth curves over the whole time-evolution. For that reason, the observed divergence in the growth rates must be directly related to the scaling properties of the liquid media.

A hint for the possible dynamics leading to the above phenomenon can be extracted from the growth profiles by analyzing the shape of the kinetic curves. These are characterized by a typical auto-acceleration in the first stage that eventually damps off. In the second stage, this typical behavior is followed by a particular and slow growth phase where the time-evolution of each experiment describes a different trajectory depending on the adjusted *V*/*S*.

The inspection of the shape of the growth profiles could guide us first to two opposing mechanistic clues:

(i) The bacterial growth includes a switch to a second and slower growth process that could lead to the flattening of the kinetic curve.
(ii) The fast growth slows down because of the formation of an inhibitor species during the growth.

Since the observed effect varies with *V*/*S*, both of the two mechanistic clues are to be related to the scaling property of the system. The first possibility can be rather ruled out to be associated to effects controlled by the surface–volume properties: for instance, a switch from aerobic to anaerobic growth [2] could possibly cause a transition in the growth curves but would not depend on *V*/*S*. In contrast, the second option, i.e., the formation of an inhibitor

species, could lead to a possible rationalization of the observed phenomenon and at the same time can give an explanation for its dependence on *V*/*S*. In the following, we will try to express this second idea in form of a simplified mechanistic network.

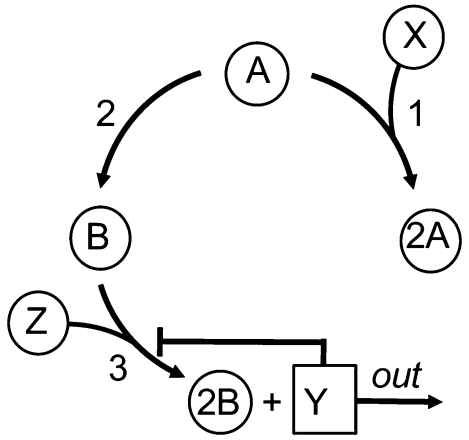
Our general outline, in spirit of Occam's razor and from a nonlinear dynamics perspective, is a schematized bacterial system in which both twofold bacterial auto-replication and certain generalized auto-inhibition occur. Furthermore, to account for the divergence in the growth curves, we suggest the involvement of a relief of auto-inhibition that can be physically related to the variations in the *V*/*S* of the cultures.

The minimal set of species for such a mechanism includes aerobic bacteria (A), O_2 (X), the nutrient (Z), anaerobic bacteria (B) that are formed related to the depletion of O_2 [2], and an inhibitor compound (Y). The species A, X, and Z are implicit to the experiments, species B is likely to be formed, while Y is so far a suggestion that, however, appears essential for any dynamic rationalization of the experimental data.

We propose this outline as a four-step mechanism that can reflect the global dynamics of the experimental observations in terms of an interplay between bacterial growth, growth inhibition, and the relief of inhibition.

As illustrated in Scheme 1, step 1 of the mechanism simplifies the typical growth of A in terms of an auto-catalytic process. X is considered as the limiting species for the growth of A. Step 2 stands for the continuous background formation of B from their aerobic precursors A. Steps 1 and 2 are coupled: when during process 1 the concentration of X diminishes, consequently the flux of step 2 increases, i.e., the system switches gradually from a predominantly aerobic to an anaerobic scenario. This is followed by step 3, i.e., the further auto-catalytic generation of anaerobic bacteria B in the presence of the nutrient Z as the limiting agent. Step 3 also denotes the formation of the inhibitor compound Y and expresses the effect of auto-inhibition by this presumed volatile species. The auto-inhibition gains in strength when the concentration of Y increases and therefore slows down the rate of step 3. As depicted by the first-order process of step 4, Y escapes irreversibly from the system that consequently results in a relief of inhibition, i.e., in an increase in the rate of step 3. Hence the loss of the inhibitor cannot be due to a simple decay of the respective component in the bulk system, being invariant to the surface area, but must be associated with a surface controlled process such as evaporation or perhaps a decay exclusively occurring at the gas/liquid interface.

In essence, the formation of the inhibitor compound leads to the flattening of the kinetic curves independent of *V*/*S* while the relief of inhibition accounts for the variations of the kinetic curves depending on *V*/*S*. The latter effect is due



Scheme 1 Minimal growth network composed of auto-catalytic bacterial growth, auto-inhibition (*blocked line*), and relief of inhibition (*out*) related to scaling property

to the physical escape of the inhibitor from the system. In this case, volatile molecules dispersed in the liquid phase can leave the liquid faster if its surface area is increased and/or the volume of the liquid is decreased. This exactly corresponds to our experimental observations: increased culture surface and/or decreased culture volume leads to a faster bacterial growth in the second growth stage due to the faster removal of the volatile inhibitor species. The possible nature of the volatile inhibitor has been investigated and a candidate molecule has been proposed [6].

For the rates of processes (1) → (4), usual kinetic parameters k_a to k_d were established. In the expression of rate (3), apart from the concentration of Y in the denominator, an inhibition factor ϕ was introduced that controls the strength of inhibition. Variations in the first-order rate constant k_d mimic the effect of variations in the volume-to-surface ratio of the experimental system. Thus increasing values of k_d, i.e., faster escape of the inhibitor and resulting increase of rate (3), correspond to decreasing values of the volume-to-surface ratio. The lowercase letters denote the corresponding concentrations of the model species.

$$\text{rate } (1) = k_a a x$$
$$\text{rate } (2) = k_b a$$
$$\text{rate } (3) = \frac{k_c}{(\phi y + 1)} b z$$
$$\text{rate } (4) = k_d y$$

The model follows a chemical kinetics type approach by which the time-evolution of all involved species can be predicted [13]. Changes in the number of bacteria are therefore approximated as changes in species concentrations. Hence the modeling approach cannot be compared to, for instance, the possible linear combination of logistic curves that eventually reproduce the shape of the observed kinetics. Logistic curve

fitting, being a valuable tool for many applications, was not a proper choice for our purposes, namely to reveal the mechanistic origin and the dynamic consequences of our experimental observations. In contrast to a single expression logistic approach, this can be accomplished by taking into account the interconnected kinetics of explicitly defined species—including the inhibitor—that are expressed by the following set of coupled differential equations:

$$\dot{a} = (k_a x - k_b)$$

$$\dot{b} = k_b a + \frac{k_c}{(\varphi y + 1)} bz$$

$$\dot{x} = -k_a a x$$

$$\dot{y} = \frac{k_c}{(\varphi y + 1)} bz - k_d y$$

$$\dot{z} = -\frac{k_c}{(\varphi y + 1)} bz$$

Numerical Simulations

In the numerical simulations the total bacterial concentration $(a + b)$ was assumed to be proportional to the reading of the optical densities. Using arbitrary rate parameter and initial values, the simulations show that experimental data can be qualitatively reproduced with the proposed model.

Results in Fig. 4 demonstrate that the different time-evolutions of $(a + b)$ under variations of k_d match perfectly until the post-sigmoidal diverged growth phase is reached. After this time the differences in k_d become significant and the trajectories of $(a + b)$ start to individualize. Hence the variation in k_d imposes only a difference in the rate on the post-sigmoidal stage. The almost linear shape of those curves originates from the interaction of acceleratory and inhibitory kinetics as expressed by model step 3. After sufficient time and due to the depletion of z $(a + b)$ reaches stationary values where the kinetic curves lose that linearity.

As shown in Fig. 4, the simulations also agree well with the experimentally observed oxygen depletion at the time at which the time-evolution of $(a + b)$ enters the post-sigmoidal stage. It is also predicted that a, i.e., the concentration of the aerobic bacteria, goes through a maximum and then becomes rapidly small so that the major time of the post-sigmoidal stage is dominated by the growth of the anaerobic bacteria. Simulations further indicate that the quasi-exponential growth of these anaerobic bacteria is damped rather by the effect of auto-inhibition than by the depletion of the nutrient z that is initially present in large excess.

The fitting of experimental data, i.e., a quantitative approach to the observed phenomenon, was not attempted just to avoid the risk of providing parameter values that could be too dependent on the particular model. Certainly, the model will be refined as soon as more detailed experimental data becomes available. Nevertheless, the characteristic shape of the growth curves, considered as a dynamic fingerprint, is firmly believed to originate from the basic elements of the proposed model.

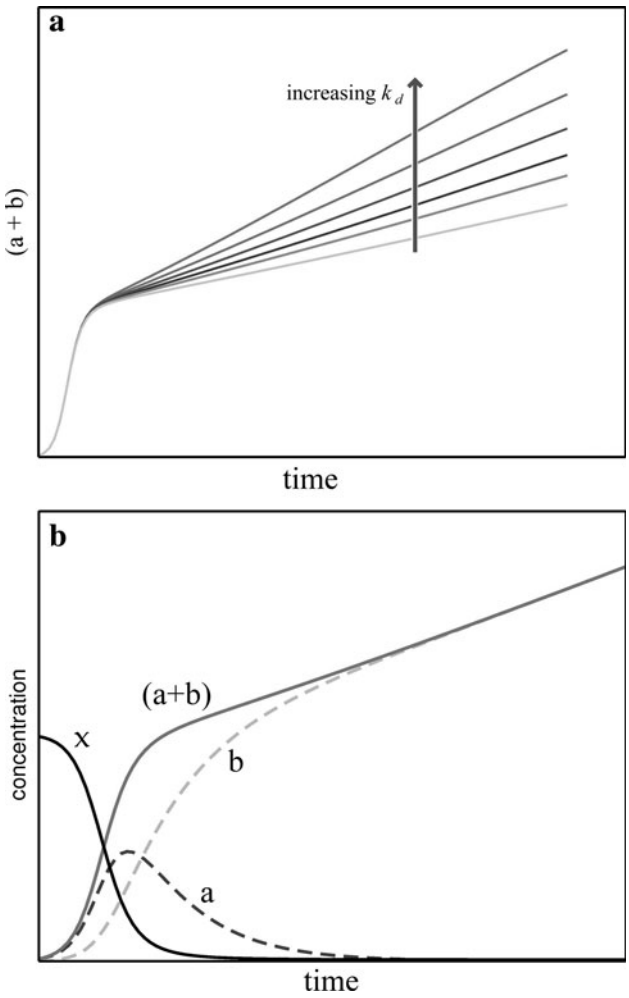


Fig. 4 Simulations of bacterial growth and time-evolution of proposed model species. **a** Series of simulations tracing the total bacteria concentration $(a + b)$ versus time under increases (*upwards arrow*) of the inhibitor escape parameter k_d as given in model step 4. **b** Predicted time-evolution of relevant model species indicating the diverging trajectories for a and b, and the depletion of x occurring at the time at which $(a + b)$ reaches the post-sigmoidal stage. Initial conditions and parameters: $a_0 = 0.01$, $x_0 = 1$, $b_0 = y_0 = 0$, $z_0 = 3$; $k_a = 5$, $k_b = 1$, $k_c = 0.05$, $k_d = 0$–$1{,}000$, $\phi = 1{,}000$; all with arbitrary units

Conclusions

We describe the so far unexplored phenomenon of bacterial growth dependence on a scaling property, i.e., the volume-to-surface ratio of liquid cultures and we also provide the first dynamic analysis of the bacterial growth response. We

hope to stimulate a further experimental examination of this effect in which certainly various ways and different approaches can be envisaged. The suggested mechanism composed of growth auto-inhibition and the relief of that inhibition adds a further facet to the dynamics of bacterial systems and could be the starting point to find additional complex phenomena related to scaling properties and bacterial growth. From a practical viewpoint, our experimental observations that small volume-to-surface ratios improve bacterial growth could be of value in various fields, for instance to increase yields of bacteria-derived man-made products such as recombinant proteins, both in the laboratory and at a larger scale, because we foresee attainment of high cell densities in cultures with small volume-to-surface ratios, even without culture shaking.

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