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EXPERIMENTAL ARTICLES

Nonlinearity in the Growth of Bacterial Colonies: Conditions and Causes

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Abstract—The universally recognized kinetic model of colony growth, introduced by Pirt, predicts a linear increase of colony size. The linearity follows from the assumption that the colony expands through the growth of only such cells that are located immediately behind the moving colony front, in the so-called peripheral zone of constant width and density. In this work, Pirt's model was tested on two bacteria—*Alcaligenes* sp. and *Pseudomonas fluorescens*—having markedly distinct cultural properties and grown on an agarized medium with pyruvate. The colony size dynamics was followed for different densities of the inoculum, ranging from a single cell to a microdroplet of bacterial suspension (10^5–10^6 cells), and for different depths of the agar layer, determining the amount of available substrate. A linear growth mode was observed only with *P. fluorescens* and only in the case of growth from a microdroplet. When originating from a single cell, colonies of both organisms displayed nonlinear growth with a distinct peak of K_r (the rate of colony radius increase) occurring after 2–3 days of growth. The growth of *P. fluorescens* colonies showed virtually no dependence on the depth of the agarized medium, whereas the rate of colony size increase of *Alcaligenes* sp. turned out to be directly related to the medium layer thickness. The departure from linearity is consistently explained by a new kinetic scheme stipulating a possible contribution to the colony growth not only of peripheral cells but also (much more distinct in *Alcaligenes*) of cells at the colony center. The colony growth dynamics is determined not only by the concentration of the limiting substrate but also by the amount of autoinhibitor, the synthesis of which is governed by the age of cells. The distinctions of growth from a single cell and microdroplet could also originate as a result of dissociation into the R- and S-forms and competition between the corresponding subpopulations for oxygen and the common substrate.

Key words: bacterial colonies, radial growth rate, inoculum density.

Investigating colonial growth of microorganisms is of considerable fundamental and practical importance. Unlike homogeneous growth, which at present can be fairly well predicted by structured kinetic models [1], no satisfactory quantitative theory was so far proposed to describe the development of bacterial colonies. The kinetic model by Pirt [2] found wide recognition because of its straightforward nature and mathematical elegance. Basically, this model states that the colony size (radius R) increases through the growth of only those cells that are located at the colony periphery. Assuming the width of the peripheral zone w to be constant, the rate of colony radius increase K_r is given by the equation

$$\frac{dR}{dt} = K_r = \mu w = \text{const},$$

where t is time and μ is the specific growth rate of cells within the peripheral zone determined by the cultivation conditions and, above all, by the local concentrations of the growth-limiting substrate and toxic metabolic products. The value of K_r remains constant as long as the growth conditions in the peripheral zone do not change, and this gives rise to the so-called *linear phase* of growth. This phase is preceded by a short phase of exponential growth of the microcolony and followed by a phase of decelerated growth, when it is no longer the colony radius but the colony area that increases at a constant rate. Later on, all growth stops entirely [11].

It is worth noting that the mere necessity of introducing several phases with their peculiar growth laws (exponential or linear growth laws or the areas law) is clear enough evidence of the inadequacy of the kinetic model that lacks two basic things: universality and predictive power. Unfortunately, growth phases can be identified only post factum, when the growth of an individual colony has already been followed. In addition, it is impossible to calculate the growth dynamics in advance given the initial conditions and the growth characteristics of an individual organism. In our view, the weak point is not the lack of a special mathematical apparatus but the want of factual experimental findings able to clarify the mechanisms of colony development. Specifically, there is no convincing evidence that the linear growth law indeed holds for a wide range of microorganisms and cultivation conditions such as the inoculum density, the initial distribution of cells across

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the agar surface, the depth of the agar layer, etc. [2–7]. Following the initial introduction of solid media by Koch, a view took root that a colony on agar can grow equally well from a multicellular aggregate and from a single cell. The question of whether the development of the colony, the durations of separate phases, and rates of growth are influenced by the number of cells at the initial point of colony growth was very seldom asked. In one experimental study that we know of [8], it is concluded that the morphogenesis of a colony from a single cell and from a droplet with the volume of 1 μl, containing about 10^5 cells, followed basically the same pattern. It cannot be ruled out, however, that the dependence on the initial conditions could be more pronounced for some species and less marked for other species. For instance, as shown in our paper [9], *Alcaligenes* sp. and *Pseudomonas fluorescens*, two organisms with significantly different colony structures, displayed significantly different kinetics of growth of their bacterial lawn. The growth of colonies of *Alcaligenes* sp., possessing extracellular gas channels [10], was more active, stable, and efficient (with lower maintenance requirements) than that of *Pseudomonas fluorescens.*

The purpose of this study was to test the validity of Pirt's model (the linear growth law) using as an example colonies of *Pseudomonas fluorescens* and *Alcaligenes* sp., which are bacteria with essentially different cultural properties. We shall identify growth conditions under which nonlinearity occurs and give an explanation of this fact.

MATERIALS AND METHODS

A culture of *Pseudomonas fluorescens* 1472, obtained from the All-Russia Collection of Microorganisms (VKM), Institute of Biophysics and Physiology of Microorganisms, Russian Academy of Sciences, and strain *Alcaligenes* sp. d_2, isolated by Lebedinskii and Duda of the Institute of Microbiology, Russian Academy of Sciences, from bottom sediments of a freshwater lake [10], were used.

The bacteria were grown on an agarized medium containing 20 g/l agar (Bacto Agar Typ USA, Ferak, Berlin) and a mineral base described elsewhere [9]. Unless otherwise stated, sodium pyruvate at an initial concentration of 5 g/l was used as a sole source of carbon and energy. The suspension of cells from a homogeneous batch culture grown in flasks on a shaker (180 rpm) at 25°C served as inoculum. In the case of *P. fluorescens*, the compositions of the liquid and agarized media were identical except for the presence of agar. To obtain inoculum of *Alcaligenes* sp., yeast extract (10 mg/l) was added to the liquid medium as a growth factor (yeast extract was not required by bacteria growing on the agarized medium).

Inoculation and subsequent monitoring of colony growth were performed by two methods:

(1) five microdroplets (volume, 10 μl; droplet area, about 2 mm^2) of bacterial suspension were applied with a loop onto the surface of solid medium in standard Petri dishes (diameter, 10 cm), which were then incubated at 25°C. During the next 8–10 days, the size variation of the growing colonies was followed by measuring the radius R of each colony in two directions (along the larger and smaller diameters) with a help of the eyepiece micrometer of a binocular magnifier and by averaging the result over ten colonies (five colonies in each of two dishes);

(2) a droplet of a diluted cell suspension (density, about 10^2 CFU/ml) was spread over the surface of solid medium in a Petri dish. As a result, no more than five colonies emerged in each dish and these were assumed to develop from individual cells. The colony size dynamics was determined as in the previous method.

In order to determine the colony growth rate as a function of the agarized medium thickness, standard Petri dishes containing 15, 20, 30, and 50 ml of the medium were used. After the end of tests, the height of the agarized medium was measured with a ruler. In all other experiments, dishes with 20 ml of the medium were used.

RESULTS AND DISCUSSION

Radial growth of *P. fluorescens* and *Alcaligenes* sp. as a function of inoculum density. In all, three density levels (x_0 mg bacterial mass per liter of liquid culture) were tried for each organism in the case of microdroplet plating (Fig. 1). The reaction of *P. fluorescens* to the change of x_0 was fairly weak. Thus, the linear growth velocity increased by a mere 10–15% with a fourfold increase of x_0 (from 150 to 600 mg/l). For a fixed x_0, the colony radius R appeared to increase linearly with time (Fig. 1, top). At the same time, an analysis of the dynamics of K_r calculated as the derivative of the raw data ($K_r = \Delta R/\Delta t$) revealed some departure from linearity, consisting in decelerated colony size growth after 150–200 h of incubation. The curvature of the radius dynamics could be well described by a fitted parabola of the form

$$R = -at^2 + bt + c,$$

where t is time and a, b, and c are constants. Even so, the statistical merit of using the parabolic regression rather than a linear one was very small, given that the coefficients of determination r^2 were equal to 0.9992 and 0.9984, respectively. Therefore, the growth of colonies of pseudomonads plated on agar in microdroplets can be assumed to follow the linear law at least to a fairly good approximation.

The behavior of *Alcaligenes* sp. colonies was essentially different. To begin with, the qualitative nature of the colony size curve was determined by the density x_0 of the inoculum. This curve was concave (exhibiting accelerated growth with time) at low values of x_0, con-

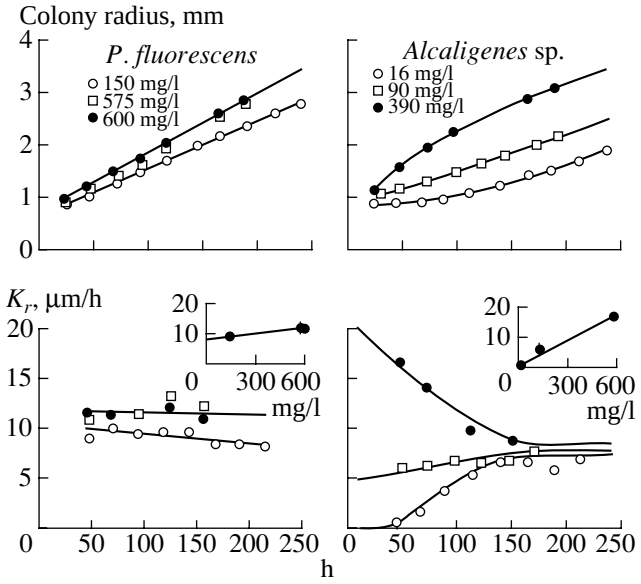

Fig. 1. Effect of inoculum density on the growth of colonies of *P. fluorescens* and *Alcaligenes* sp. Top: mean colony radius as a function of time. Bottom: rate of colony radial growth K_r. Insets: initial values of K_r (calculated from the first three points) plotted against the corresponding density of the bacterial suspension in the inoculum microdroplet.

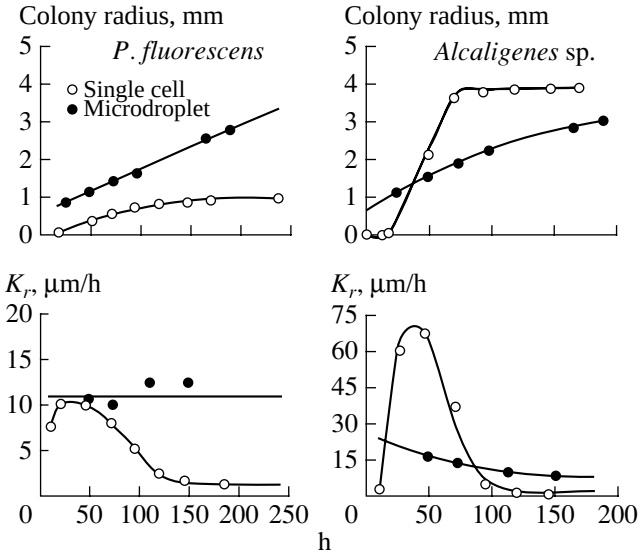

Fig. 2. Growth dynamics of colonies of *P. fluorescens* and *Alcaligenes* sp. as dependent on the inoculation method: growth from a single cell (open circles) and from a microdroplet (filled circles). Top: mean colony radius as a function of time. Bottom: rate of colony radial growth K_r. The cell density in the suspension used for plating microdroplets was 575 and 387 mg/l for of *P. fluorescens* and *Alcaligenes* sp., respectively.

vex (decelerated growth) for the maximal x_0, and showed a close-to-linear radius increase at intermediate values of x_0. The dependence of the initial value of K_r on x_0 was well described by a straight line through the origin (Fig. 1, inset). After the next 100–150 h, however, all the curves gradually converged to the K_r values observed under linear growth (at intermediate x_0), although colonies from denser inoculum always remained larger than their siblings growing from diluted suspensions. We see, therefore, that colonies of *Alcaligenes* sp. lack the linear phase and exhibit a strong positive dependence of the initial growth rate and the final colony dimensions upon the number of cells in the inoculum microdroplet.

Growth of colonies from single cells. The size dynamics of colonies grown from single cells (see the second plating method in Materials and Methods) is shown in Fig. 2. Strictly speaking, this test variant corresponds to very low densities of bacteria in the inoculum ($x_0 < 10^2$ cells/ml or $x_0 = 10^{-9}$ mg biomass/ml), leading to a high probability of a colony growing from a single cell. At the same time, one cannot totally rule out the possibility of a colony forming from two or more cells stuck together by chance. Microscopic examination of the initial suspension showed that the number of double cells was no more than 2–3 per 100. Therefore, the upper limit of the probability of colony growth from a single cell can be estimated at 0.97–0.98. For the sake of comparison, also plotted in these figures are growth curves for colonies developing from microdroplets of the same inoculum but with the standard densities of 575 and 387 for *P. fluorescens* and *Alcaligenes* sp., respectively.

First of all, it should be noted that the initial growth rates of colonies developing from single cells (Fig. 2) are in very good agreement with the observed relationship between K_r and x_0 (Fig. 1, insets) upon extrapolation to the vanishing density $x_0 = 0$. For *P. fluorescens* such an extrapolation gives 8.25 μm/h, a value close to those yielded by direct diameter measurements at the point of transition from a micro- to a macrocolony (7–10 μm/h). With *Alcaligenes* sp., there was a long lag-phase in the growth of their colonies, which suggests a value of K_r close to zero and is in agreement with a direct relationship between K_r and x_0: $K_r = 0$ at $x_0 = 0$.

The subsequent growth of colonies, however, had several features that were very much different from those observed under microdroplet plating: With pseudomonads, the colony growth started to slow down at a much earlier point, and so no linear phase whatever was observed. By contrast, colonies of *Alcaligenes* sp. exhibited an abnormally strong acceleration of growth, with K_r rising to 60–70 μm/h (compared to the constant rate of roughly 7 μm/h for microdroplet plating), which was followed by fast damping. Therefore, significant deviations from the "linear growth law" predicted by Pirt's model were exhibited by colonies of both organisms developing from single cells.

In the literature available to us, departures from linearity were observed after 30 h of growth of colonies of *Escherichia coli* and *Streptococcus faecalis* developing

from microdroplets. This was explained by the substrate depletion underneath the colony because the duration of the linear phase was observed to increase with the depth of the agar layer [2, 3]. It is for this reason that our test of Pirt's model included measurements of K_r for a wide range of agar layer thickness values.

Effect of agar layer thickness on the rate of radial growth of *P. fluorescens* and *Alcaligenes* sp. colonies. The dynamics of colony growth on media with the depth of the agar layer ranging from 1.5 to 7 mm is shown in Fig. 3. The expansion dynamics of *P. fluorescens* colonies had virtually no connection with the medium depth. A linear growth was observed through the entire duration of the experiment, for about 190 h. With colonies of *Alcaligenes* sp., no linear growth was observed in any test variant. Increasing the depth of the agar layer led to accelerated growth of colonies. At the same time, the shapes of the dynamic curves remained virtually the same for all tried agar layer depths. This means that colonies developing on deep agar displayed no trend to acquire linear growth. In addition, in all test variants, growth started to slow down at about the same time, roughly after 3–5 days of incubation.

Thus, we were able to confirm that colonies of *Alcaligenes* sp. were indeed influenced by the depth of the agarized medium and thus, by the total amount of substrate in the agar layer under the colony, but this influence was far different than that predicted by Pirt's model. There are, apparently, two reasons why pseudomonads are not so much affected by substrate limiting: (i) they have a higher affinity to pyruvate as a sole source of carbon and energy, and (ii) the depletion of substrate under the colony is not so strong because of their lower biomass accumulation.

Dissociation of bacteria and the rate of their radial growth. We now have to describe the last phenomenon needed to put forward an interpretation of the obtained kinetic data, namely the strong influence of the dissociation of bacteria into the S- and R-forms on the colony growth.

In the case of *Alcaligenes* sp., microdroplet inoculation produced 10–30% of colonies with one, or less often, two "blades" at the colony edge. These blades are actually sectors where the cells grow notably faster than the other members of the population. A comparison of the size dynamics of smooth and rough colonies is given in Fig. 4. The R-variants can be seen to grow faster but only during a short period of time, upon which the rates of the radial growth of the S- and R-variants level. This temporary acceleration happens to be more pronounced on a deep agar layer, containing more limiting substrate. In the case of *Pseudomonas*, the dissociation also took place, but the S- and R-variants did not show significant distinctions with regard to the value of K_r (more notable distinctions were observed in the degree of aggregation and adhesion of suspension culture cells to flask's glass walls, but this is a subject of a different communication).

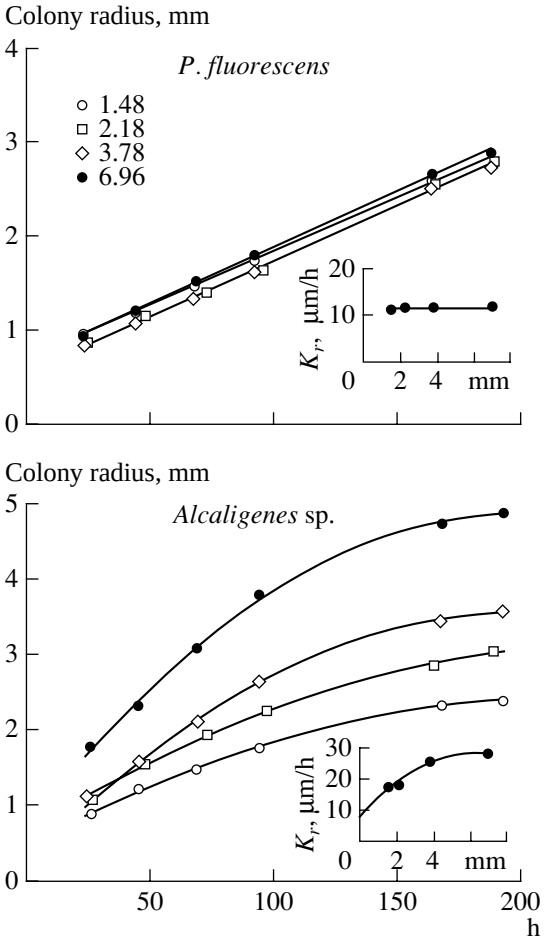

Fig. 3. Influence of the depth of the agar layer on the size dynamics of bacterial colonies. The bacterial suspension density in microdroplets of inoculum of *P. fluorescens* and *Alcaligenes* sp. was 600 and 760 mg/l, respectively. Plotted in the insets are graphs of the initial K_r value versus the agar layer depth.

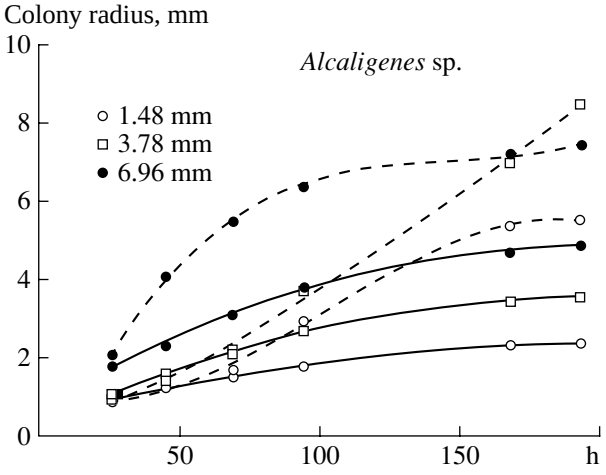

Fig. 4. Growth dynamics of smooth colonies (continuous curves) and colonies with blades (dashed curves) of *Alcaligenes* sp. for different depths of the agar layer. Inoculation conditions as in Fig. 3.

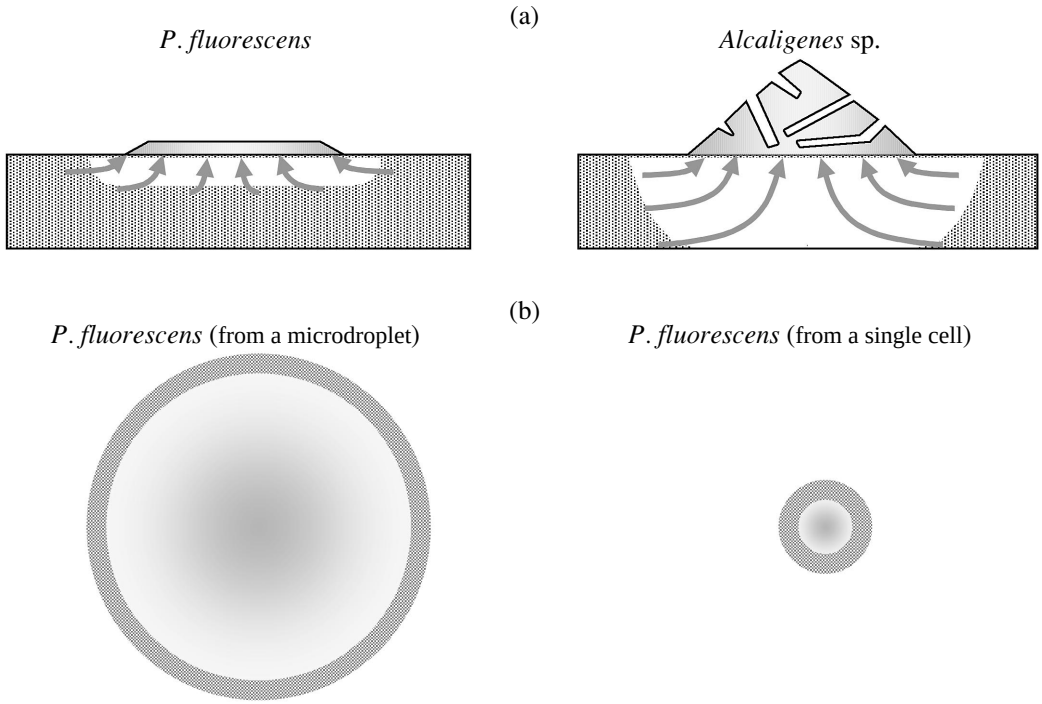

Fig. 5. Schematic representation of the growth of bacterial colonies employed to explain the nonlinearity phenomenon. Top (a): flat colonies of *P. fluorescens* deplete substrate only in a narrow topmost layer because of suppressed growth in the colony center; meanwhile, a more active growth of cells of *Alcaligenes* in the entire colony volume causes substrate to be depleted to the very bottom of the Petri dish, provided the agar layer depth is small enough. Bottom (b): colonies of pseudomonads of the same age growing from (left) a microdroplet and (right) a single cell. The dark ring denotes the zone of peripheral growth, and the internal shading reflects the relative concentration of autoinhibitors. The smaller colony on the right is seen to be more sensitive to the action of inhibitors because of a smaller distance between the peripheral zone and the center of autotoxin production.

A kinetic scheme of growth of bacterial colonies to explain nonlinear growth. Let us summarize our findings.

1. Dynamics of K_r. A linear growth phase occurred only in the case of *P. fluorescens* and only for colonies developing from a microdroplet. When colonies grew from a single cell, the growth dynamics was markedly nonlinear with a salient peak of K_r on the second to third day of growth. Colonies of *Alcaligenes* sp. failed to show linear growth whatever the inoculation method. With microdroplet plating, a gradual slow down of growth of smooth colonies and abrupt changes of K_r for rough colonies were observed. Colonies developing from single cells exhibited an "explosion-like" dynamics of K_r (its values being an order of magnitude greater than in growth from a microdroplet).

2. **Influence of inoculum density** was weakly positive in *P. fluorescens* and strongly positive in *Alcaligenes* sp. Colonies emerging from single cells were always smaller than those developing from microdroplets.

3. **Influence of agar thickness.** Growth of *P. fluorescens* colonies did not depend on the depth of the agar layer, whereas, in the case of *Alcaligenes* sp., it was directly related to the medium layer thickness.

It is worth noting that the obtained empirical evidence, with due caution, can be extrapolated to a wider spectrum of organisms than the two bacterial species studied. It was shown in a number of previous papers [2, 13, 14] that the behavior of colonies of most "cultivated" microbes, such as enterobacteria, bacilli, baker's yeasts, etc., was the same as that of pseudomonads. In an earlier work [1], such organisms were collectively referred to as *r*-selected (i.e., having an advantage under the conditions of an *r*-selection) or opportunistic species, which display a fast reaction and high rate of nonlimited growth. *Alcaligenes* sp. is a *K*-strategist and, therefore, a stronger competitor, inhabiting the chemocline zone. It has developed the capacity for stable and effective colonial growth through a more complex spatial arrangement—the formation of gas balloons—and by synthesizing flavohemoprotein, which is an analog of hemoglobin [12]. It also, apparently, possesses a more efficient system of O_2 transportation to preclude oxygen starvation in the central part of the colony, which, for this reason, is three to seven times higher than the colonies of *P. fluorescens*. In terms of a kinetic model, this feature of the organization of *Alcaligenes* sp. can be formulated as follows: (i) these bacteria maintain their activity not only in the peripheral zone but also in the central part of the colony; (ii) the expansion of a colony is supported by a larger fraction

of cells than in the case of a more common "balloon-free" colony structure.

All the three experimentally observed and outlined departures from linearity can be consistently explained within the framework of a new kinetic scheme, partly illustrated in Fig. 5. To begin with, we assume that cells both on the periphery and in the center of the colony can contribute to its growth. The latter contribution is more prominent in *Alcaligenes* and leads to substrate consumption from greater depths of the agar layer beneath the colony. The depletion zone, in the case of *Alcaligenes*, (but not *Pseudomonas*) extends to the bottom of the Petri dish, and for this reason the agar layer depth has a strong influence on the value of K_r (Fig. 5). The proportionality between K_r and x_0 (the initial colony growth rate and the inoculum density in a microdroplet) in *Alcaligenes* arises from the fact that, with a high rate of mass exchange within the colony, the total diffusion flux of the substrate into the colony, which maintains the growth rate, turns out to be proportional to the total cell mass. In pseudomonads, the inflow of the substrate is limited to the contact area between the colony biofilm and agar, and so the connection between the number of cells in the inoculum and the growth rate virtually vanishes as soon as the (micro)colony becomes multilayered.

Why is a colony from a single cell smaller than the one developing from a microdroplet? An answer to this question can be found in Fig. 5b. The development of a colony on the agar surface underneath a microdroplet proceeds in the form of a microlawn: first exponentially over the entire biovolume and then only in the peripheral zone. Cells in the colony center switch from a trophophase (growth and multiplication) to the idiophase and start to synthesize products of secondary metabolism including autoinhibitors. It is evident that autotoxins are more hazardous to small colonies growing from single cells because, in this case, the peripheral zone is much closer to the colony center. In microdroplet plating, autotoxins are distributed within a larger volume of a colony center, and this increases the chances for their scattering by diffusion and inactivation before reaching the peripheral zone.

Additional kinetic effects (including a peak on the K_r curve) are caused by the dissociation of bacteria into the R- and S-forms and the competition between the corresponding subpopulations for oxygen and the common substrate. Suppose that R-variants grow faster under the abundance of substrate in the medium but cannot cope as well with utilizing diluted substrate (because of their lower affinity to the limiting substrate) and are more liable to dying under starvation. Then, it is evident that different rates of growth from a microdroplet and a single cell can be caused by the following mechanism. Under microdroplet plating, the chances for an R-clone blade to appear are slim, given that as many as 10^5–10^6 cells start to grow simultaneously and contribute to colony propagation over the agar surface. This causes a shortage of pyruvate under which S-variants have a growth advantage. Occasionally, however, due to a play of chance, an R-variant occurs at the edge of a microcolony and serves as a starting point for a sector of accelerated growth, which gives rise to a colony with a blade. A linear growth in the direction of the blade ends when the substrate in the agar layer under the blade becomes noticeably depleted, and, at this point, the R-strategy of growth once again loses its advantage.

When a microcolony grows from a single cell, the substrate remains in surplus for a long period, causing the predominant development of R-variants, which under such conditions produce a rough colony with irregular edges rather than just a few blades. The point when the R-variants start to prevail can be identified by an abrupt increase of K_r (Fig. 2). The subsequent depletion of the substrate puts an end to the domination of the R-forms, and K_r rapidly drops down.

The ultimate test of the consistency of the outlined mechanisms should obviously be based on a quantitative prediction of the available experimental data using the so-called distributed colony model. Such a model should be formulated as a set of equations in partial derivatives (with respect to time and space) that would unambiguously and explicitly represent the postulated mechanisms in the mathematical form.

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