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EXPERIMENTAL ARTICLES

Cell Division across the Volume of Colonies of *Flavobacterium* sp. 22

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Abstract—*Six*-day-old colonies of *Flavobacterium* sp. 22 were studied by electron microscopy. Direct evidence was obtained of bacterial cell division across the entire colony volume, indicating that the colony growth of *Flavobacterium* sp. 22 is not purely peripheral. It is argued that the colony shape is determined not only by peripheral growth but also by physical forces acting upon a droplet of liquid on the surface. For bacterial colonies developing on solid nutrient media, the intercellular matrix plays the role of such a liquid.

Key words: electron microscopy, division of cells, colony growth, *Flavobacterium*

INTRODUCTION

Cultivation of microorganisms on solid media is a basic method of investigating the behavior of microorganisms in laboratory studies [1]. It is generally accepted that the development of colonies occurs by way of peripheral growth [1, 2]. For example, in a discussion of bacterial growth depending on the plating pattern, it is argued in [3] that "in the case of a bacterial lawn, the notions of the peripheral zone and the center have no straightforward geometrical interpretations. This contrasts with the case of a single colony, where its edge is indisputably the zone of growth." This statement is in agreement with numerous mathematical models [1–4].

According to the definition given by Schlegel, "By growth we mean an irreversible increase in the quantity of live matter, most often accompanied by the enlargement and division of cells. Multicellular organisms grow in size, whereas in unicellular organisms, it is the number of cells that grows" [5]. As stated by Rose [6], "The total number of organisms can be determined either directly, by enumerating the number of cells in an appropriately diluted suspension sample, or indirectly, by measuring some characteristic of the culture or suspension that scales with the number of cells in the culture."

The evidence that the biomass at the center of a bacterial colony does not participate in the radial colony growth was presented by Couper *et al.* and viewed as convincing by Pirt [1]. This evidence, however, is indirect, since it was based on the observation that no change occurred in the relative position of fine and light graphite particles spread over the colony center; according to Rose [6], direct evidence of growth should be based on the determination of a culture characteristic proportional to the number of organisms in the culture. There are no direct demonstrations of peripheral colony growth that we know of.

Microscopic examination of histologic sections of bacterial colonies is a method that preserves the spatial arrangement of cells across the colony volume [7]. By using this method, not only was the ultrastructure of cells described, but important data relating to colony architectonics were also obtained: the presence of morphologically different cells within one colony and a regular arrangement of cells of each morphological type were shown [8–10]; a fairly typical orientation of cells within the biomass was noted [11, 12]; a surface film was shown to form over the colony [13, 14]; and several types of cell contact by means of different membrane formations and slime-like material were described [9, 13–15]. In addition, colonies were shown to have zones of cell lysis [16–18] and, in colonies of *Mycoplasma salivarium* and *Mycoplasma pneumoniae*, zones of cell growth [19].

Given that the division of cells is the only way in which the number of microbial cells can increase, the occurrence of dividing cells is direct evidence of growth in the bacterial culture, and the regions within the colony space where such a division occurs are growth zones. Bearing this in mind, one can hope to obtain direct evidence substantiating or disproving the concept of peripheral growth in bacterial colonies.

The objective of this work was to discover dividing cells in colonies of *Flavobacterium* sp. 22 prepared so as to preserve, as much as possible, the actual placement of cells in live colonies.

MATERIALS AND METHODS

Colonies of bacteria, identified by their physiological and biochemical properties to be *Flavobacterium*

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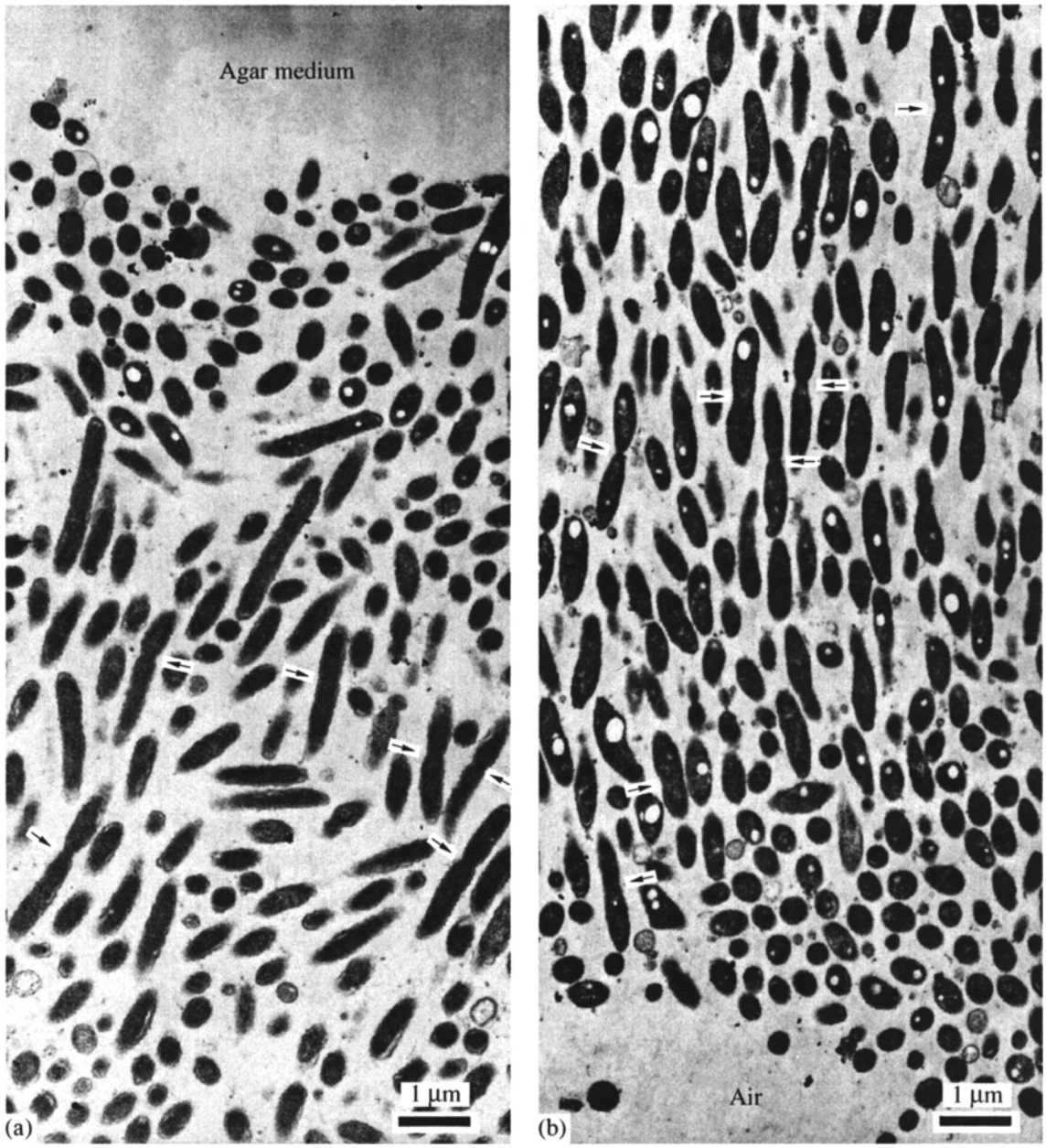

Fig. 1. Fragments of an ultrathin vertical section through the center of a control colony of *Flavobacterium* sp. 22 used to create panoramic images: (a) a part of the colony facing agar medium; and (b) a part of the colony in contact with the air. Dividing cells are indicated with arrows.

sp. 22, were used in this study. The colonies were grown on a solid nutrient medium composed of peptone, 1%; NaCl, 0.5%; and 2% agar in distilled water (pH 7.2–7.4). A replica-plating device [20] was used for inoculation; each test was run in ten repetitions.

Six-days-old test and control colonies were studied. Test colonies were grown in the vapor of *n*-caproic acid, 0.01 ml of which was placed in the lid of a Petri dish, which was covered with the dish itself, containing the nutrient medium and the inoculum. Control colonies were grown under similar conditions except that no caproic acid was added.

Specimens for electron microscopy were prepared as described elsewhere [21]. Ultrathin vertical sections, as big as possible and taken from different regions of three colonies, were obtained using a Reichert UM-03 ultramicrotome (Austria). The sections were examined under a JEM-100C electron microscope (Japan).

Micrographs were taken in such a way that their series would cover a fairly large part or even the entire section from the colony region of interest. Individual micrographs were input into a computer with a scanner and then edited and glued by appropriate software to obtain panoramic images.

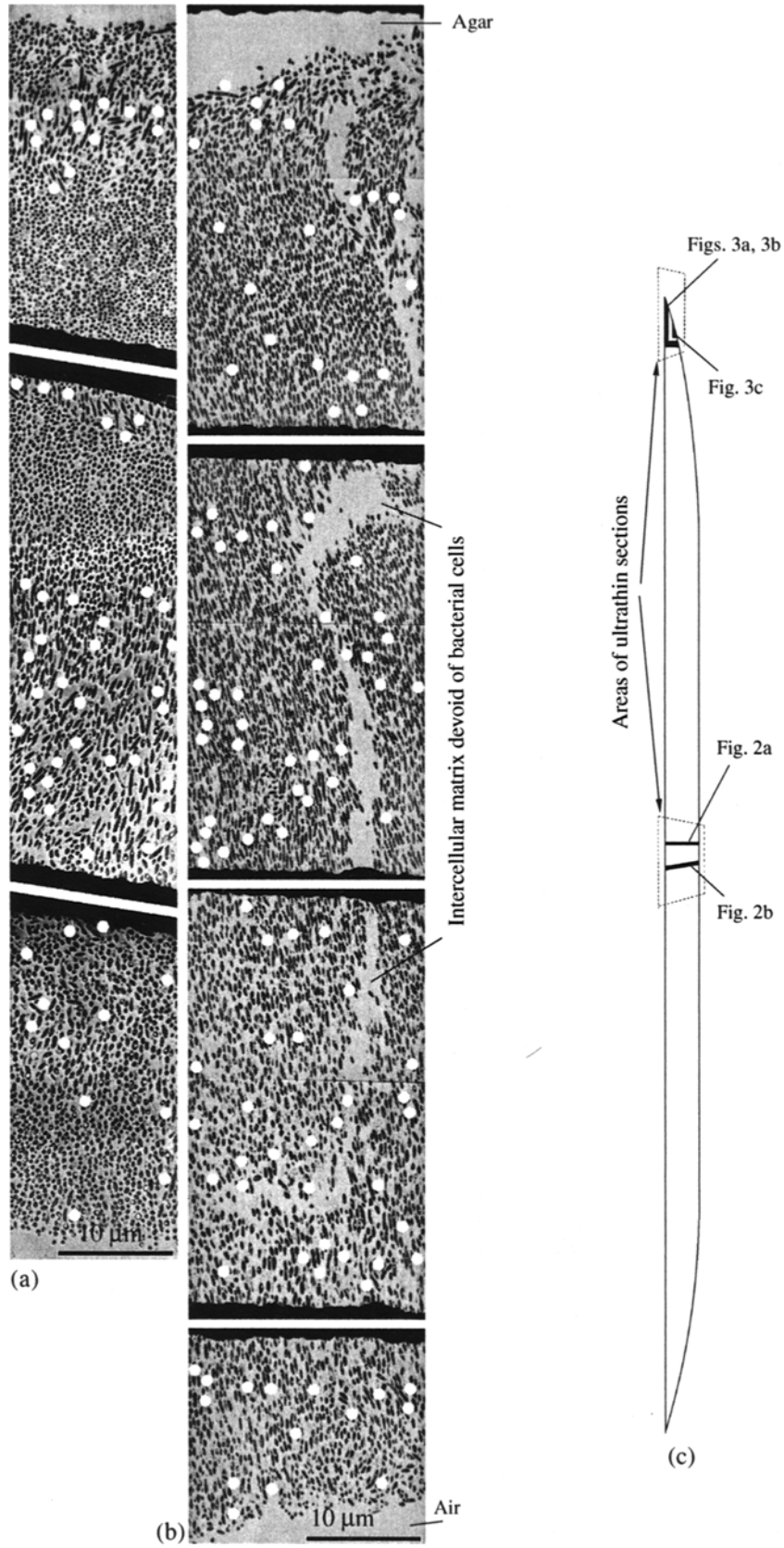

Fig. 2. Panoramic ultrathin vertical sections of control and test colonies of *Flavobacterium* sp. 22 (dividing cells are indicated by labels): (a) the center of the control colony; (b) the center of the test colony; (c) a diagram showing the relation between the areas of colonies, ultrathin sections, and the panoramic images presented in this paper.

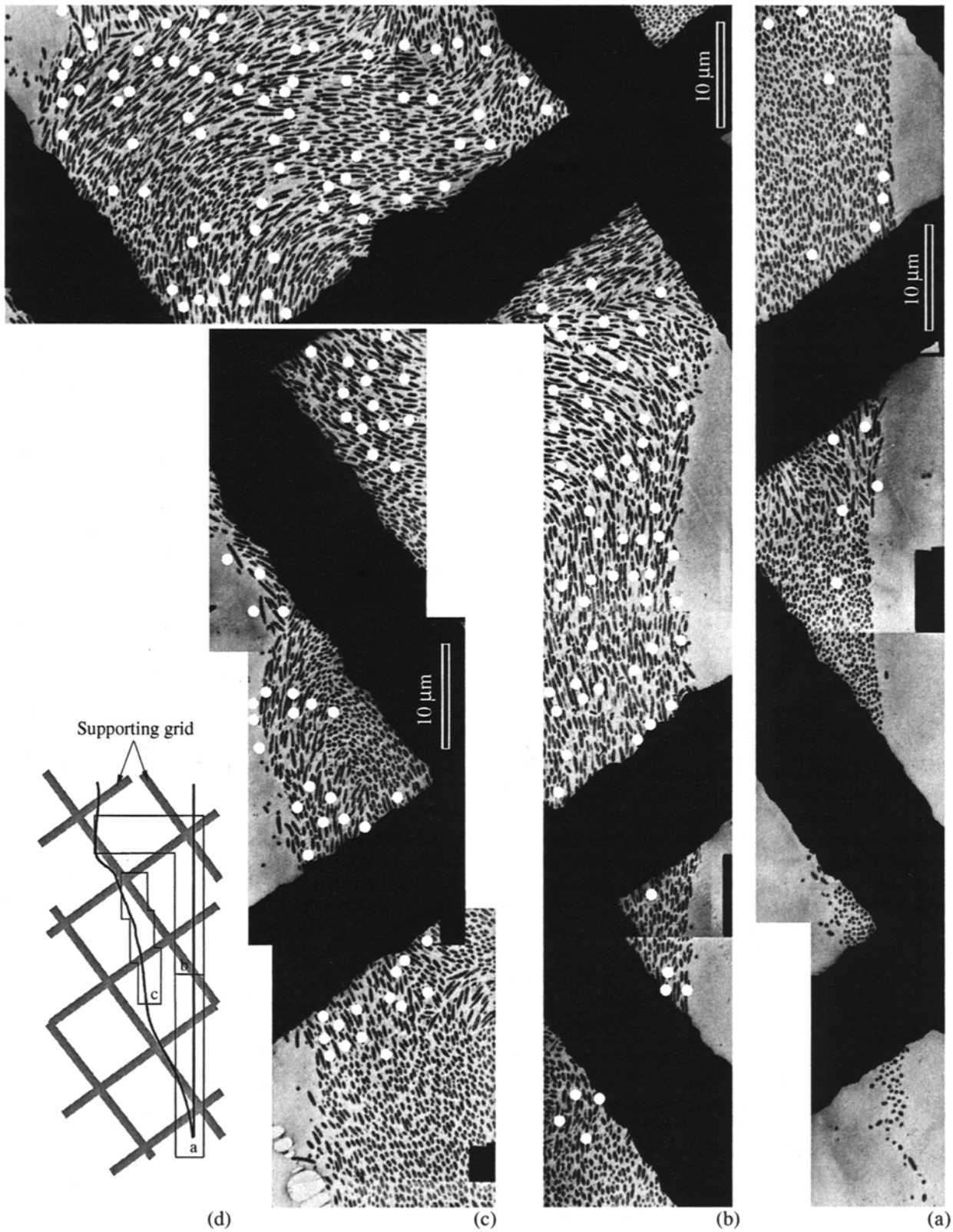

Fig. 3. Fragments of a panoramic ultrathin vertical section in the periphery of a test colony of *Flavobacterium* sp. 22 (dividing cells are indicated by labels): (a) colony edge; (b) colony region adjacent to agar medium; (c) colony region in contact with air; (d) a diagram showing the location of fragments in the ultrathin section.

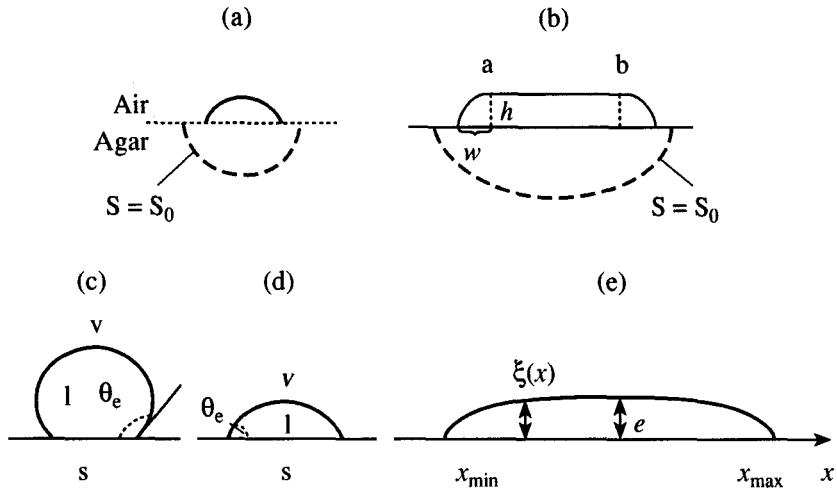

Fig. 4. Two cross sections of a model microbial colony growing on the surface of agar medium: (a) exponential growth; (b) growth limited by substrate diffusion over the length w; S_0 is the initial concentration of the nutrient source (after Pirt [1]); (c) to (e): A small droplet on a horizontal base: (c) and (d) show two variants of incomplete wetting, its degree being lower in (c) than in (d) (v denotes vapor; l is liquid phase; s is solid phase); (e) the final droplet profile resembles a pancake in the case of a complete droplet spread over a dry base (after de Gen [30]).

RESULTS

Cells of *Flavobacterium* sp. 22 are gram-positive rods. In the case of an ultrathin section passing along a bacterial cell, the presence of the septum can tell if this cell was undergoing division at the moment when the sample was fixed. Shown in Fig. 1 are images of two colony parts facing air and nutrient agar; the images were obtained from an ultrathin vertical section passing through the central region of the colony. Dividing bacteria are indicated with arrows.

The panoramic images of colony cross-sections (Figs. 2 and 3) were obtained by computer demagnification of original images similar to those shown in Fig. 1. Because of the low resolution of the panoramic images, it is hard to see the dividing cells. But these can be marked at a higher resolution, as in Fig. 1, and then, in panoramic images, we shall see the distribution of labels assigned to dividing cells. The distributions of labels for sections made in the central region of control and test colonies are shown in Fig. 2; the distribution of labels for the peripheral region of a test colony is shown in Fig. 3.

It was previously shown [21] that colonies of *Flavobacterium* sp. 22 consist of two types of cells of a peculiar ultrastructure. It should be noted that no significant distinctions in the ultrastructure of cells of one type were observed either in different colony parts (center or periphery) or in control and test colonies. Dividing cells were present (in different quantities) in all ultrathin sections obtained from the central and peripheral parts of *Flavobacterium* sp. 22 colonies.

In our pictures of colony sections, it is impossible to see the intercellular matrix enveloping bacterial cells since it is not stained by the electron microscopy dyes employed. Its presence, however, is indicated by a cell-free region extending through virtually the entire depth of the colony (Fig. 2b).

DISCUSSION

Growth of bacterial colonies is, admittedly, a more complex process than that of fungal colonies [1, 2]. Given that it is only the nutrients brought by diffusion that can be utilized, the growth of the colony fairly quickly becomes limited by the nutrient, and this creates heterogeneous conditions preventing growth in the central part of the colony. When a small number of bacterial cells are initially plated on agar medium to form colonies, their multiplication proceeds in such a way that all cells make an equal contribution to the population growth [1]. This process is believed to give rise to the colony's domelike profile (Fig. 4a).

In the case of bacteria that are unable to penetrate agar, nutrient compounds are supplied and metabolites are removed by means of diffusion. The cells located at the colony center are, therefore, under less favorable conditions than those near the colony edge, and this fact results in the cessation of their division. As a consequence, the colony expansion over a solid substrate, e.g., an agar layer, is sustained by growth limited to the peripheral zone of the colony. As the colony becomes bigger, the diffusion flux decreases, the radial velocity of colony propagation drops down to zero, and growth in the colony finally comes to an end [2]. It follows that colony expansion over agar medium is mostly due to cell division occurring near the colony edge, and this must be responsible for the flattened shape of the colony (Fig. 4b). According to Panikov [2], the peripheral area of the colony is analogous to a growing cell suspension in a fermentation vessel, whereas its central

part is similar to a waste cell suspension drained to the receiver.

As previously reported [21], the bacteria making up colonies of *Flavobacterium* sp. 22 are represented by a population of two types of rod-shaped cells differing in their ultrastructure and spatial orientation. The bacteria of these two types do not mix within the colony volume, and, as revealed by ultrathin sections, occupy quite definite colony regions (type 1 faces air and type 2 faces agar). Because the ratio between the length and the diameter of the bacteria studied is as high as 6 : 1, one can readily see the orientation of cells in the section plane. In control colonies, type 2 cells are more stringently aligned than type 1 cells. This explains why almost no labels can be seen in control colonies (Fig. 2a) in the regions where the orientation of type 2 cells is perpendicular to the vertical section plane (and parallel to the plane of the agar medium). They occur only in two small regions where the orientation of cells is for some reason changed.

It is readily seen by comparing panoramic images (Figs. 2a and 2b) that the orientation of type 2 cells in colonies of *Flavobacterium* sp. 22 is changed by the growth in the vapor of *n*-caproic acid, and the fraction of cells lying parallel to the section plane increases. This explains a more uniform distribution of labels assigned to dividing cells in the central part of a test colony (Fig. 2b), where a realignment of type 2 cells is caused by the action of the *n*-caproic acid vapor.

By using the *n*-caproic acid vapor in this study, we saved ourselves a lot of electron microscopy work associated with the need to examine ultrathin sections at different angles to the plane of the agar medium. The label distributions were analyzed only for vertical colony sections.

It should be noted that, on sections passing through the peripheral zone of the test colonies (Fig. 3), where the division of cells must be more active, there are regions containing no labels, and this fact can be explained by the orientation of cells being perpendicular to the section plane. The distribution of labels assigned to the dividing cell in the two important colony parts—the central region (Fig. 2) and the periphery (Fig. 3)—shows that the division of bacterial cells proceeds in the entire volume of both control and test 6-day-old colonies of *Flavobacterium* sp. 22. The observed nonuniform distribution of labels is most likely determined by the orientation of cells in the section plane and not by the presence of specific division zones within the 6-day-old colonies of *Flavobacterium* sp. 22.

The lack of significant distinctions in the ultrastructure of cells of the two types making up a colony suggests that the heterogeneous conditions with regard to nutrients, accumulated metabolites, and *n*-caproic acid vapor were insufficient to cause, after 6 days of growth, a change in the ultrastructure of cells that could be revealed by electron microscopy.

Although, at the moment when a colony was fixed, cells were dividing in the entire volume of the colony, its vertical profile was flattened rather than dome-shaped. It follows that the domelike shape of mature colonies can be determined by factors other than the absence of cell division at the colony center and the occurrence of such a division near the edges of the colony. This conclusion is supported by the comparison of data obtained in experiments with colonies of *Escherichia coli* M-17 growing on agar-containing media [22] and in a special apparatus [23] enabling gas phase control, as well as the continuous supply of fresh nutrient medium and the removal of metabolic products through a semipermeable membrane. Plated sparsely in Petri dishes, the colonies, after 16 days of growth, were 18–20 mm in diameter, while, after 12 to 15 days of growth in the apparatus, the colonies were as big as 70–80 mm and virtually filled all the space intended for their growth. Unfortunately, Pshenichnikov *et al.* [23] failed to present any observations regarding the increased height of the colony. Apparently, even if such a change did occur, it was too insignificant to be reported.

A bacterial colony is composed of at least two components: the bacterial cells themselves and the intercellular matrix, or bacterial glycocalyx, synthesized by the bacteria of the given community and serving as their environment. Glycocalyx is a polysaccharide-containing component located on the external side of the cell wall [24, 25] and plays a broad spectrum of roles in the colony. Among other things, it may be responsible for the virulent properties of a culture [26]; by having extracellular hydrolytic enzymes, it contributes to the degradation of large macromolecules into smaller ones [27]; and it serves as a barrier for heavy metal ions. After attaching to a surface, bacteria envelop themselves with additional glycocalyx material and multiply within this matrix. Having originated inside the hydrated exopolysaccharide matrix, the daughter cells remain in its internal part, and this leads to the formation of a microcolony [24, 28].

The intercellular matrix is a highly hydrated polymeric material with a 99% content of water [24], a kind of "liquid," and, as such, must play an important role in giving the colony its form. On the one hand, it is spread on the surface of a denser medium and, like any liquid, must wet it. On the other hand, it is surrounded by air, and, therefore, surface tension forces must be at work. Thus, the form of a colony in its growth is also determined by the intercellular matrix, which must obey the laws of physics for a droplet of liquid on a surface.

The amount of the intercellular matrix produced by bacteria should be minimally sufficient to maintain their activity, given that its synthesis draws on the resources of bacterial cells. Therefore, especially at the early stage of colony development, when bacteria are covered with a thin layer of glycocalyx, the surface tension forces tend to draw the cells together and press

them to the agar surface. This contention is in agreement with the colony growth model developed by Rodin and Panikov [29], who claimed that "Microcolonies develop in such a way as if the microorganisms followed two rules of microcolony construction: (a) out of all possible mutual arrangements of cells, they select that which provides their closest possible contact with each other; (b) they try, at the same time, to keep as close as possible to the nutrient medium surface."

Figure 4 is an illustration of two entirely different processes: a biological one—the formation of a colony—[1] and a physical one—the behavior of a droplet of liquid on a base [30]. According to [30], for a small droplet of liquid on a planar surface of a solid body (suspension, porous medium, or cloth), there can be a case of incomplete wetting, characterized by a steady-state (Figs. 4c and 4d) with a nonvanishing edge angle. In the case of incomplete wetting, the base region covered by liquid is bounded by a contact line, which, in our case, is a circumference. This is indeed observed at the initial stage of colony growth on agar (Fig. 4a). In the case of spreading, the droplet takes on the form shown in Fig. 4e. This form is typical of later stages of bacterial colony growth (Fig. 4b).

In this paper, our analysis was limited to just two profiles commonly used to explain peripheral growth [1], although it is clear that the variety of forms of bacterial colonies is much more diverse. For example, a student's guide to practical work in microbiology [31] describes 12 forms and 8 profiles of bacterial colonies. There may, of course, be some discordance between the outlined considerations and the real processes effective during colony growth, due to the mere fact that, even with extensive electron microscopy work, only a small number of cells making up a population are examined, and the results obtained for relatively small regions are extrapolated to the whole colony (Fig. 2c).

It is encouraging that the architectonics of bacterial colonies grown under certain conditions is reproducible and specific [19, 21]. This holds the promise that additional data will be later obtained and that their accumulation will lead to a better and fuller understanding of the processes occurring in bacterial colonies. Evidence obtained for other types of colonies will show whether the division of cells over the entire colony volume is characteristic of all bacteria or if the colonies of *Flavobacterium* sp. 22 are in this respect an exception. Such evidence will probably also support the hypothesis that colonies with true peripheral growth (when cells in the central colony region either do not divide or the number of dividing cells there is negligible) are colonies with lysis zones [16–18].

On the assumption that the division of bacterial cells is a criterion for growth of a culture of microorganisms, the fact that dividing cells were observed in the central colony part of the culture under investigation is direct evidence of growth in the entire volume of *Flavobacterium* sp. 22 colonies. The colony profile observed with the most mature colonies of this species warrants the conclusion that the intercellular matrix, along with other functions [24–28], plays an important part in shaping bacterial colonies.

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