

Self-Similar Colony Morphogenesis by Gram-Negative Rods as the Experimental Model of Fractal Growth by a Cell Population

TOHEY MATSUYAMA^{1*} AND MITSUGU MATSUSHITA²

Department of Bacteriology, Niigata University School of Medicine, Niigata 951,¹ and
Department of Physics, Chuo University, Tokyo,² Japan

Received 18 September 1991/Accepted 25 January 1992

The ability to form a fractal colony was shown to be common among several species of the family *Enterobacteriaceae*. Bacterial spreading growth in a two-dimensional field of nutrient concentration was indicated to be important for this experimental self-similar morphogenesis. As a basic analogy, the diffusion-limited aggregation model was suggested. Fractal dimensions of colonies were mostly in the range of values from 1.7 to 1.8, similar to those of the two-dimensional diffusion-limited aggregation model. Bacterial characteristics and culture conditions inducing changes in fractal patterns and growth rates were identified. The contribution of the bacterial multicellular nature to fractal morphogenesis is discussed.

Previously, we showed that *Serratia marcescens* exerts fractal morphogenesis in the process of spreading growth on an agar surface (17). Colonies of microorganisms that tend to grow as branching filaments (*Streptomyces griseus* and *Ashbya gossypii*) have also been shown to be fractal (22). Fractals are sets of patterns satisfying the condition that a partial pattern taken out of some pattern is similar (scale invariant) to the original whole pattern. A Koch curve (Fig. 1) is a famous example of such self-similar fractals (12). Mandelbrot reported that such self-similar (in a statistical sense) patterns are abundant in nature (12). Typically observed in the architecture of the lung, fractals are ubiquitous among various biological structures and forms (2, 7, 9, 12, 21, 23).

For the elucidation of the mechanism of fractal morphogenesis by living cells and the biological significance of such structures, bacterial colony fractals seem to be good experimental subjects. Since bacterial colonies can grow in a plastic dish, it is possible to trace the fractal growth under various conditions. Thus, by controlling the nutrient concentration in an agar medium, a *Bacillus subtilis* colony has been shown to have fractal growth through processes which can be simulated by the diffusion-limited aggregation (DLA) model (6, 13, 30), as theoretically suggested by Meakin (19).

As described previously (17), the first fractal colony identified by us was recognized by culturing *S. marcescens*. Surface-active exolipids produced by the bacteria (16) facilitated the development of a giant spreading colony with a fractal morphology. In the investigation described below, the ability to form fractal colonies was shown to be common among gram-negative rods that produce no surface-active exolipids. Some culture conditions influencing fractal colony development were identified and examined for roles in the process of fractal morphogenesis by bacteria.

MATERIALS AND METHODS

Bacterial strains and growth. *Salmonella typhimurium* LT2 and ATCC 14028 and *Escherichia coli* ATCC 25922 were supplied by M. Niwa, Osaka City University Medical School (Osaka, Japan). Strains of *Salmonella anatum*, *S. marcescens*, *Klebsiella pneumoniae*, and *Micrococcus luteus* were

described elsewhere (14, 16, 18, 24). *Citrobacter freundii* NPC 3003, *E. coli* W3110, *Proteus mirabilis* NPC 3007, and *Listeria monocytogenes* EGD are laboratory strains. To get a nonmotile flagellumless mutant (NM-2b) of *S. typhimurium*, nitrosoguanidine mutagenesis was carried out as described previously (18). These strains were point inoculated with a toothpick onto Vogel-Bonner minimum agar (28) containing 1.4% agar (Eiken, Tokyo, Japan) and a specified concentration of D-glucose, L-glucose, or lactose. Ordinary agar media such as BTB (modified Drigalski's medium [Eiken] containing 0.4% meat extract, 1.0% peptone, 1.0% lactose, 0.004% bromthymol blue, and 1.6% agar), nutrient agar (Eiken), and tryptic soy agar (Difco, Detroit, Mich.) were also used. Agar medium (7.0 ml) in a plastic dish (diameter, 9 cm) was air dried at 37°C for 70 min. The inoculated plates were incubated at 37 or 30°C in a wet chamber (relative humidity, 88% ± 2%). Examination for a pH change during cultivation was carried out by using a flat surface electrode of a C1 Cardy pH meter (Horiba Ltd., Tokyo, Japan).

Fractal analysis. Photographs of colonies were analyzed with a charge coupled device camera (Ikegami, Tokyo, Japan) and a digital image analyzer (EXCEL; Nippon Avionics, Tokyo, Japan) by using a personal computer (PC-9801RX; NEC, Tokyo, Japan). Overall colony size was determined by calculating the radius of gyration (R_G):

$$R_G^2 = \frac{1}{N} \sum_{i=1}^N (\mathbf{r}_i - \mathbf{r}_c)^2 \quad (1)$$

where N is the number of pixels occupied by the pattern, \mathbf{r}_i is a position vector of the i th occupied pixel, and \mathbf{r}_c is a position vector of the center of mass for all occupied pixels:

$$\mathbf{r}_c = \frac{1}{N} \sum_{i=1}^N \mathbf{r}_i \quad (2)$$

If the pattern is self-similar and the values of N and R_G at several stages of the growing pattern are able to be evaluated, we obtain a simple relation:

$$N \sim R_G^{D_f} \quad (3)$$

where the exponent D_f is the fractal dimension of the

* Corresponding author.

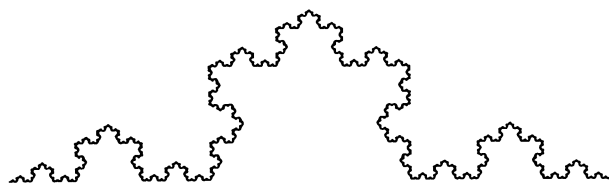


FIG. 1. A Koch curve as a typical self-similar pattern.

pattern. Since we usually had a picture of only one stage of a pattern, we changed the pixel size, p , instead of changing R_G . In this case, R_G is inversely proportional to p ($R_G \sim p^{-1}$), because the length is scaled as p^{-1} . Substituting this relation into equation 3, the number of occupied pixels of size p is scaled as

$$N(p) \sim p^{-D_f} \quad (4)$$

Thus, the self-similarity of the pattern was confirmed by log-log plotting of datum points (N, p). The method of obtaining D_f from this relation is called the box-counting method (4).

RESULTS

Fractal colonies of gram-negative rods. Twelve bacterial strains of gram-negative rods examined showed fractal spreading growth. Fractal dimensions of representative colonies formed by these strains are listed in Table 1. The D_f values varied according to several factors (e.g., a further incubation of the same plate of *S. typhimurium* LT2 for which results are shown in Table 1) but mostly settled in the range of values from 1.7 to 1.8. Characteristic colony patterns of *K. pneumoniae* and *P. mirabilis* are shown in Fig. 2 and 3, with the results of log-log plotting by the box-counting method. It is noteworthy that a nonflagellated bacterium such as *K. pneumoniae* was able to form a spreading colony on the surface of hard agar. The nonflagellated mutant (NM-2b) of *S. typhimurium* ATCC 14028 also showed spreading growth and made a fractal giant colony.

For the development of well-characterized fractal colonies, inoculated plates were incubated for a long time. However, bacterial cells composing the inner parts of a fractal pattern (e.g., presumably aged cells in a spur near an inoculated site) were still viable. In contrast, it was difficult to recover viable cells from the central area of a nonfractal convex colony (diameter, 12 to 16 mm) of *M. luteus* ATCC 9341 which had grown for a long time (4 to 8 weeks).

Effect of glucose on spreading growth. The strains described above (except *S. marcescens*) do not produce surface-active exolipids, which have been reported as promoting agents of spreading growth (17), and the strains make a small inconspicuous colony on minimal hard agar containing 0.1% D-glucose after an ordinary incubation time (a few days). During extended incubation, however, these strains demonstrated remarkable fractal spreading growth on minimal hard agar containing increased concentrations of D-glucose (Fig. 4). L-Glucose failed to show such a promoting effect. Lactose exerted the same effect on *E. coli* but not on *Salmonella* strains that are unable to use lactose.

All the strains in Table 1 except *S. marcescens* showed a similar response to glucose addition. In a Vogel-Bonner liquid culture of *S. typhimurium*, increasing the concentration of glucose from 0.1 to 0.4% did not result in observed growth-promotive effects (data not shown). In the presence

TABLE 1. Fractal dimensions (D_f) of bacterial colonies

Bacterial strain	Medium (% glucose) and incubation time ^a	D_f (box size ^b)	
<i>S. typhimurium</i> LT2	VB (0.4), 2 wk	1.73 (1-100)	
	VB (0.4), 4 wk	1.78 (1-100)	
	BTB, 5 wk	1.76 (1-100)	
	ATCC 14028	VB (0.2), 2 wk	1.80 (1-50)
NM-2b	VB (0.4), 2 wk	1.79 (1-100)	
	VB (0.4), 2 wk	1.78 (1-50)	
<i>S. anatum</i> KS 200	VB (0.4), 4 wk	1.77 (1-100)	
	KS 700	VB (0.4), 8 wk	1.79 (1-50)
<i>E. coli</i> ATCC 25922	W 3110	VB (0.4), 4 wk	1.77 (1-50)
	VB (0.4), 3 wk	1.81 (1-160)	
	TS, 2 wk	1.77 (1-50)	
<i>C. freundii</i> NPC 3003	BTB, 2 wk	1.79 (1-50)	
<i>K. pneumoniae</i> Ful	VB (0.4), 4 wk	1.80 (1-160)	
<i>P. mirabilis</i> NPC 3007	NAS (0.4), 18 h	1.76 (1-100)	
<i>S. marcescens</i> NS 25	NS 38	VB (0.4), 4 wk	1.72 (1-100)
	VB (0.1), 4 wk	1.85 (1-100)	

^a Media: VB, Vogel-Bonner agar; BTB, bromthymol blue agar; TS, tryptic soy agar; NAS, nutrient agar (soft) (0.5% agar). The details are described in Materials and Methods. Times of incubation at 37°C (except for *S. marcescens*, which was incubated at 30°C) are given.

^b Box size ranges are in numbers of pixels.

of 0.8% glucose, the pH around the small colony decreased below 6.4 because of increased acid production by the bacteria. On acidic agar media (pH 6.2), *S. typhimurium* strains failed to grow or formed a tiny colony even in the presence of 0.4% glucose.

Characteristics of fractal colony growth. Small colonies exposed to generous three-dimensional nutrient diffusion did not show fractal morphology at first. Thereafter, branches developed gradually at the colony periphery but were still thick and fusing. When the colony grew further, the branching at the colony periphery became clearer (compare panels a and b of Fig. 5). A growth-inhibitory effect on a small inner spur caused by the surrounding well-growing spurs is noteworthy (screening effect, indicated by an arrow in Fig. 5a and b). Thus, two-dimensional diffusion of nutrients seems to be effective in the fractal growth of a bacterial colony on an agar surface. A growth-inhibitory zone between two neighboring fractal colonies (repulsion effect; Fig. 5c) may also be a reflection of the restricted diffusion of nutrients and toxic metabolites.

The fractal colonies described above were growing two-dimensionally on the agar surface, mostly because of the inability of the bacterial species to grow by submerging into hard agar. When bacteria were able to grow by submerging into hard agar (e.g., *L. monocytogenes*), a three-dimensional self-similar pattern was formed in the agar medium (Fig. 6).

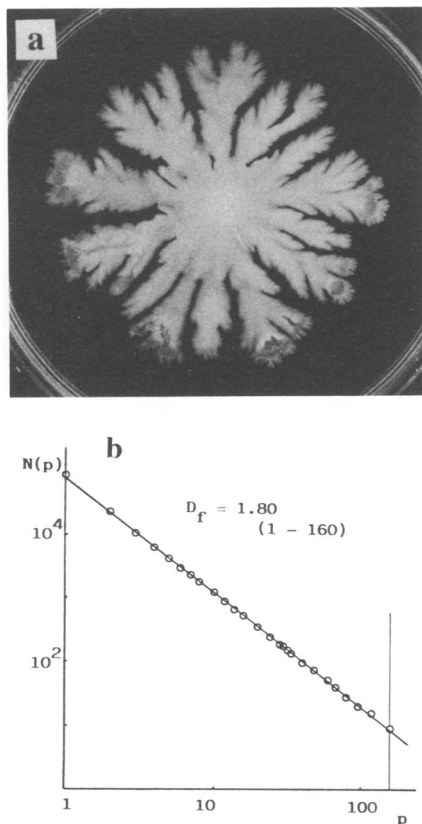


FIG. 2. Fractal nature of a *K. pneumoniae* giant colony. (a) Strain Fu1 was point inoculated onto the center of a Vogel-Bonner agar plate containing 0.4% glucose and incubated at 37°C for 4 weeks. (b) Fractal analysis of the colony by the box-counting method. D_f , fractal dimension in the box size range shown; p , pixel size; $N(p)$, number of boxes.

DISCUSSION

There are a number of species-specific or organ-specific fractal patterns in structures composed of eucaryotic cells. Various self-similar branching patterns of tree shapes are familiar examples. The mechanisms generating such inheritable characteristic structures and the meaning of the random fractality itself have been receiving attention recently in the biological sciences (5, 7, 8). Although bacteria are unicellular organisms, we found that bacteria growing on or in a solid substrate also develop fractal colonies in the process of spreading to their surroundings. Such specific morphogenesis by the bacterial population seems to have some relation to the multicellular behavior of bacteria, which is a topic of current interest (1, 25, 26).

The biological significance of fractality in distributions of living cells (e.g., a bacterial colony) or structures of specific organs (e.g., the bronchial tree) in relation to interactions with the surrounding environments have been discussed previously (7, 12, 17). Since interfaces between fractal structures and their surroundings are geometrically predicted to be extremely broad, the uptake of nutrients and the excretion of toxic metabolites are expected to be efficient, especially under the dynamic conditions of the living body. Although the environment in a solid agar plate is rather static, we could recover viable cells from every part of a thin fractal colony in spite of prolonged incubation. On the other

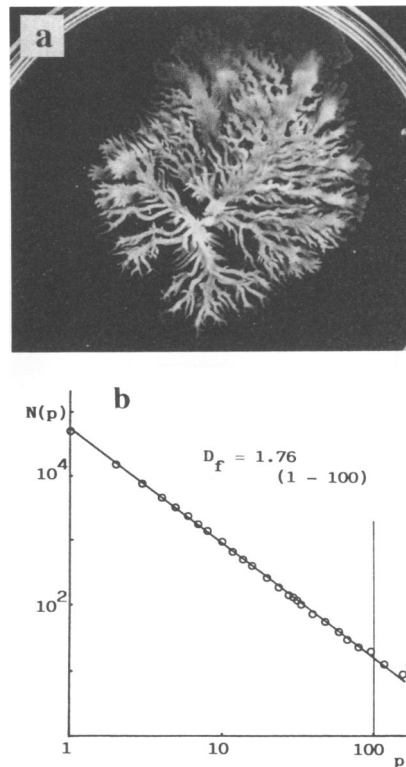


FIG. 3. Fractal nature of a *P. mirabilis* giant colony. (a) Strain NPC 3007 was point inoculated onto the center of a plate of soft nutrient agar containing 0.5% agar and 0.4% glucose and incubated at 37°C for 18 h. (b) Fractal analysis of the colony. Details are described in the legend to Fig. 1.

hand, cells contained in the central area of a convex colony of *M. luteus* seemed to be killed in a crowded bacterial mass.

For the elucidation of the mechanisms at work in fractal morphogenesis by living cells, the conditions critical for the generation of the fractal colony were examined. We noted first that the size of a colony is important. When the diameter of a colony was smaller than the depth of the agar plate, the colony was round and had a mostly smooth periphery. This small round colony is familiar to most bacteriologists who use agar plates for single-colony isolation. Since we have been interested in bacterial behavior in a surface environment (15–17), we continued to incubate the plate and noted the fractal morphology. Thus, as shown in Table 1, cultivation was carried out for a long time to obtain a large colony. Since bacterial growth is dependent on nutrient supply, the mode of nutrient diffusion influences the bacterial growth. For a large colony on a thin agar plate, a two-dimensional field of nutrient diffusion will be dominant. In such a nutrient concentration field, two-dimensional bacterial growth will progress on the agar surface. The situation is quite similar to that for the generation of a two-dimensional DLA model pattern. The DLA cluster (Fig. 7a) grows through irreversible sticking onto the cluster surface of Brownian particles that are released from random positions far from the cluster one at a time (low concentration limit of Brownian particles) (30). Meakin suggested, through extensive computer simulations, that immotile cells growing two-dimensionally in a field of low nutrient concentration will develop a fractal pattern with the D_f value close to 1.71 and that screening and repulsion effects will be observed among growing branches

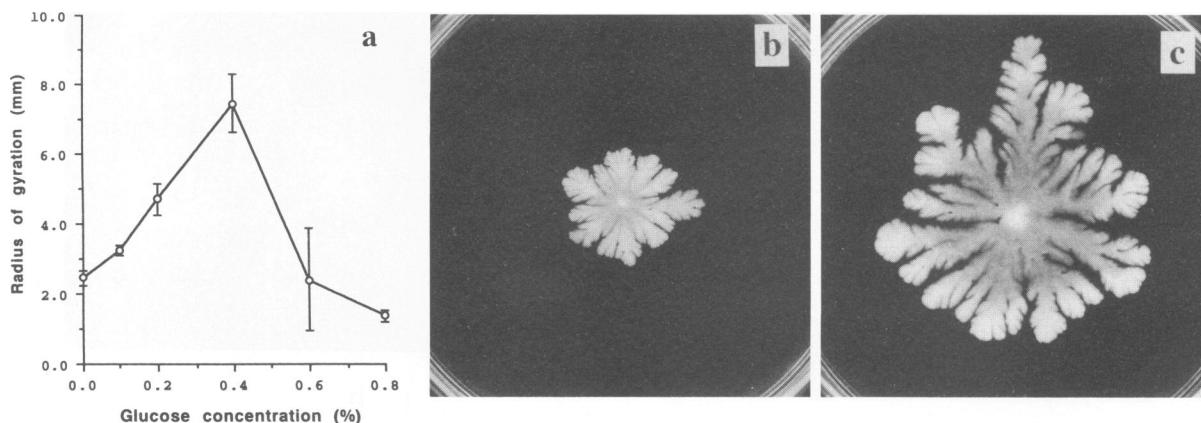


FIG. 4. Effect of glucose on the fractal spreading growth of *S. typhimurium* ATCC 14028. (a) The strain was point inoculated onto the center of a Vogel-Bonner agar plate containing a specified concentration of D-glucose and incubated at 37°C for 1 week. Each point and bar represents the mean \pm the standard deviation for four values. Photographs of colonies were taken after 2 weeks of cultivation in the presence of 0.1% (b) and 0.4% (c) D-glucose.

(19, 20). We think that the D_f values of the bacterial fractal colonies listed in Table 1 are close to the value indicated by Meakin. As shown in Fig. 5, screening and repulsion effects are certainly present in fractal morphogenesis by bacteria.

Although the DLA process seems to be a basic mechanism evoking fractal morphogenesis in bacteria, some environmental factors may modify the growth rate or the pattern itself. The effect of glucose on the spreading growth (Fig. 4) was a solid-medium-specific phenomenon. In a Vogel-Bonner liquid culture of *S. typhimurium*, increased concentrations of glucose did not result in growth-promotive effects. At glucose concentrations higher than 0.004%, *E. coli* is known to grow at a constant logarithmic growth rate in a liquid culture (29). Since L-glucose was ineffective and lactose was effective, medium osmolality and the cyclic AMP-catabolite gene activator protein global regulon of *E. coli* (11) do not seem to participate in the control of the spreading growth. Surface tension working at the growing periphery of the bacterial colony also seems to exert a profound effect on the fractal morphogenesis. Vicsek (27) proposed a modified DLA model by introducing a surface tension parameter as a curvature-dependent sticking proba-

bility, $p_s(\kappa)$ [$p_s(\kappa) = A\kappa + B$, where κ is the local surface curvature and A and B are constants]. Computer simulations with different values of A and B gave patterns with thick branches, the skeletons of which are still fractals, as shown in Fig. 7. We have reported the role of the surface-active exolipid produced by *S. marcescens* (17) or *Serratia rubidaea* (15) in fractal colony formation by the bacteria. The computer simulation figures coincide well with our previous observations, indicating the promotive function of bacterial surfactant in fractal growth.

Biological factors other than biosurfactants are also important in fractal morphogenesis by bacteria. Surface translocation of flagellated *P. mirabilis* organisms seemed to contribute to a characteristic dendritic pattern (Fig. 3) which is quite distinct from that of other fractal colonies. *P. mirabilis* formed such a self-similar pattern after an 18-h incubation. It was possible to examine the spreading colony branch with a microscope. Although a great deal of rapid cell translocation (mostly two directional, back and forth to the extending end) in the axis of each branch was observed, cells located at the end and in the sheath of each branch seemed reluctant to translocate to the outside of the population.

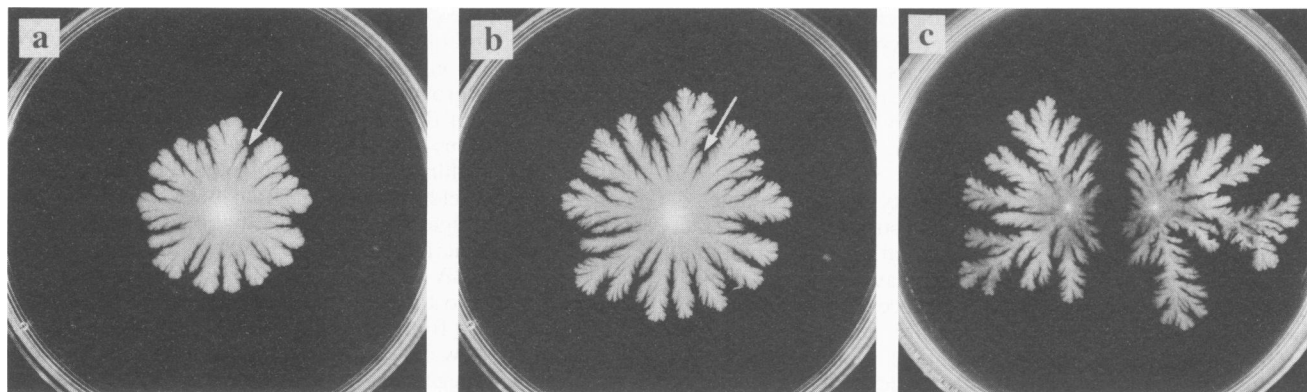


FIG. 5. (a and b) Fractal spreading colonies of *Salmonella* spp. *S. anatum* KS 200 was point inoculated onto the center of a tryptic soy agar plate and incubated at 37°C for 2 (a) and 4 (b) weeks. Each arrow indicates a representative spur under the influence of a screening effect by surrounding spurs. (c) Two neighboring giant colonies of *S. typhimurium* ATCC 14028 point inoculated onto a Vogel-Bonner agar plate containing 0.2% glucose. The plate was incubated at 37°C for 3 weeks.

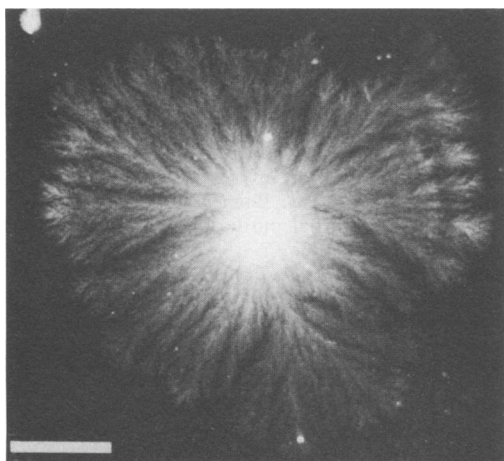


FIG. 6. Self-similar pattern made by *L. monocytogenes* growing in a three-dimensional agar space. Strain EGD was point inoculated onto the surface of a nutrient agar (1.5% agar) and incubated at 30°C for 7 weeks. Colony growth progressed initially on the surface. After 3 weeks, however, growth in the agar became dominant. The diameter of the surface colony is 5 mm. Bar, 5 mm.

Consequently, extension of the branch was significantly slower than the movement of the cells in the axial area (data not shown). Thus, instead of independent behaviors of each bacterium, multicellular behaviors were observed in the fractal growth of the *P. mirabilis* population.

The bacterial behaviors on agar plates observed in the

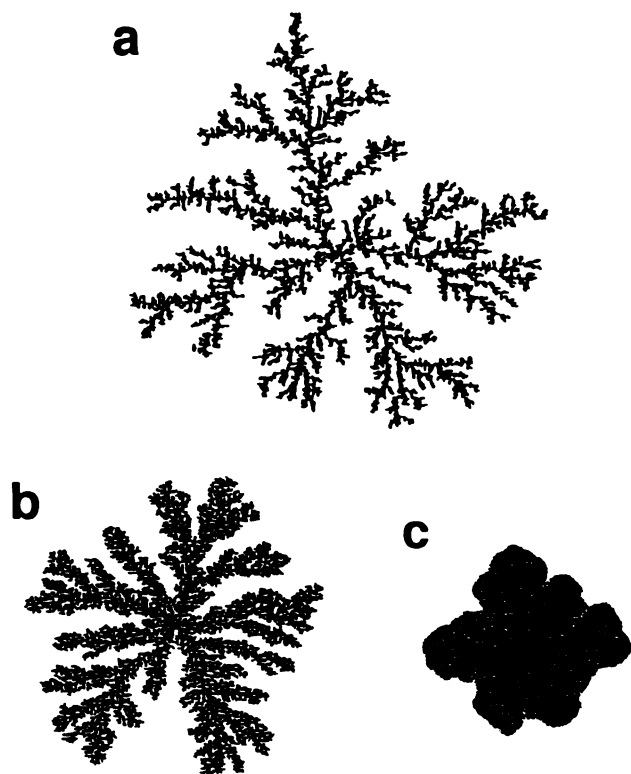


FIG. 7. Computer simulation of a DLA model with curvature (κ)-dependent sticking probability [$p_s(\kappa) = A\kappa + B$]. (a) $A = 0$; $B = 0.5$. (b) $A = 1.5$; $B = 0.5$. (c) $A = 6.0$; $B = 0.5$.

present study are cultural phenomena. However, as described by many bacteriologists (3, 10, 25), bacterial life on surfaces is common in nature. In such environments, factors limiting the distribution and diffusion of nutrients and toxic metabolites are commonly present. Thus, situations inducing DLA-type fractal distribution of bacteria seem to be ubiquitous in nature.

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