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Scanning Electron Microscope Study of *Pseudomonas putida* Colonies

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Pseudomonas putida **colonies were examined by scanning electron microscope. A variety of cell morphologies, multicellular arrangements, and extracellular materials were observed in the fixed material. Different regions of a single colony showed characteristic organizations of these architectural elements. In some cases, the detailed microstructure of the fixed colony surfaces observed by scanning electron microscopy could be correlated with macroscopic patterns visualized by histochemical staining and surface relief photography of live colonies. Extracellular materials were seen to extend onto the agar surface beyond the boundaries of the cell mass, and the final structures of these materials, after fixation and desiccation, were colony specific. The significance of these features of colony microstructure for formulating hypotheses about the control of colony morphogenesis is discussed.**

Macroscopic observation by means of histochemical staining and surface relief photography has made it possible to visualize organized phenotypic heterogeneity in living bacterial colonies (15–17). Groups of bacteria expressing different biochemical activities or aggregation patterns could be seen to be organized according to cell lineages in radially oriented sectors and according to cell positions in concentric bands. Many of these sectors and bands further showed visible internal organization, and the shapes of some sectors indicated that clonal expansion was subject to nonclonal positional regulation. Frequently, bacterial cultures were composed of differentiated cells which formed two or more distinct colony types when plated as individual colony-forming units, but these same heterogenous cultures repeatedly produced the same colony patterns when used as sources of multicellular inocula. Observations such as those just outlined have led to the conclusion that the morphogenesis of bacterial colonies is the result of many highly coordinated and regulated multicellular processes and can therefore serve as a valuable subject for developmental analysis.

To find out more about the fine structure of the observed macroscopic patterns, I undertook an analysis of *Pseudomonas putida* colonies with the scanning electron microscope (SEM). Previous SEM examinations of bacterial colonies have indicated regularities in cellular organization and extracellular features of colony architecture (1, 2, 6, 8, 19). However, these earlier studies were limited in two respects relevant to developmental analysis. (i) They had not used colonies produced by genetically well-defined cultures, and (ii) they had not included detailed comparisons of different regions within a colony. To overcome these limitations, I had studied the heredity of pattern formation in the strains chosen for this report for some time before SEM analysis (J. A. Shapiro, *in* J. R. Sokatch, ed., *The Biology of Pseudomonas*, in press).

MATERIALS AND METHODS

Bacteria. Strains PPS2491 and PPS2532 were derivatives of *P. putida* carrying CAM-OCT::Mu dII1681 plasmids, and their origins have been described previously (15; Shapiro, in press). β-Galactosidase synthesis resulted from reading of the *lacZ* sequences in the Mu dII1681 element from outside transcription and translation signals (3).

Bacteriology. Methods of strain cultivation, media, plating, and histochemical staining for β-galactosidase activity have been previously described (16). For plating on polycarbonate filters (Unipore; 0.2 μm [pore size]), the filters were sterilized by immersion in methanol, soaked in a 1% solution of bovine serum albumin, blotted dry, and placed on the surface of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (XGal)-glucose agar. Appropriate dilutions of a PPS2491 culture were then spread over the filter.

Photography. These methods have been previously described (15, 17).

SEM. All samples were prepared for SEM analysis by fixation with OsO_4 vapors (several drops of solution on a piece of filter paper in the lid of a petri dish) for 48 h followed by another fixation with glutaraldehyde (introduced by filling ditches cut in the agar with a 20 or 50% solution to just below the level of the agar surface). After about a week of infiltration of glutaraldehyde (with additions of fresh solution to maintain the level), the fixed colonies were desiccated. The samples illustrated here were all air dried, those shown in Fig. 2 by removing a portion of the filter from the petri dish, gluing it to the SEM stud with silver paint, and leaving the stud at room temperature for several hours before gold plating. The samples shown in Fig. 3–8 were allowed to sit in a closed petri dish at room temperature for about 8 weeks and then in a vacuum jar for several hours; after drying, the colonies and agar were hard and solidly bonded to the plastic dish so that samples had to be cut out with a saw, glued to the SEM stud, and grounded to the stud with foil strips before gold plating. All samples were plated with gold by vacuum evaporation and examined in an ELTEC microscope.

RESULTS

Preparation of colonies grown on filters. A major concern in this work was to prepare the colonies for SEM with as little structural disruption as possible. In the earliest attempts,

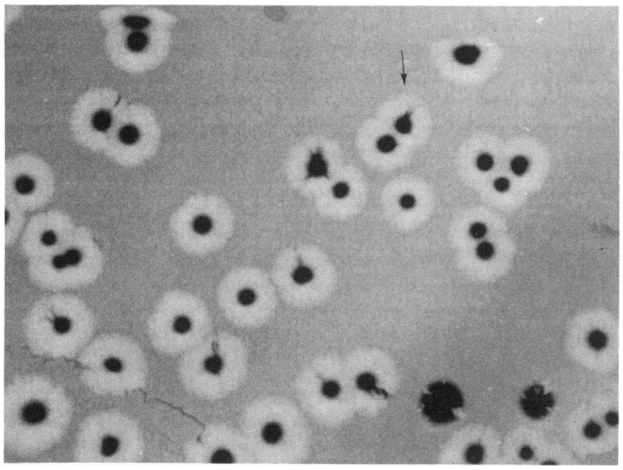


FIG. 1. Colonies of strain PPS2491 grown on a bovine serum albumin-coated polycarbonate filter on XGal-glucose agar. The colonies measured between 1 and 2 mm in diameter. Note the sectors showing radial streaks of β-galactosidase activity. The colony marked with an arrow is the one illustrated in Fig. 3b through e.

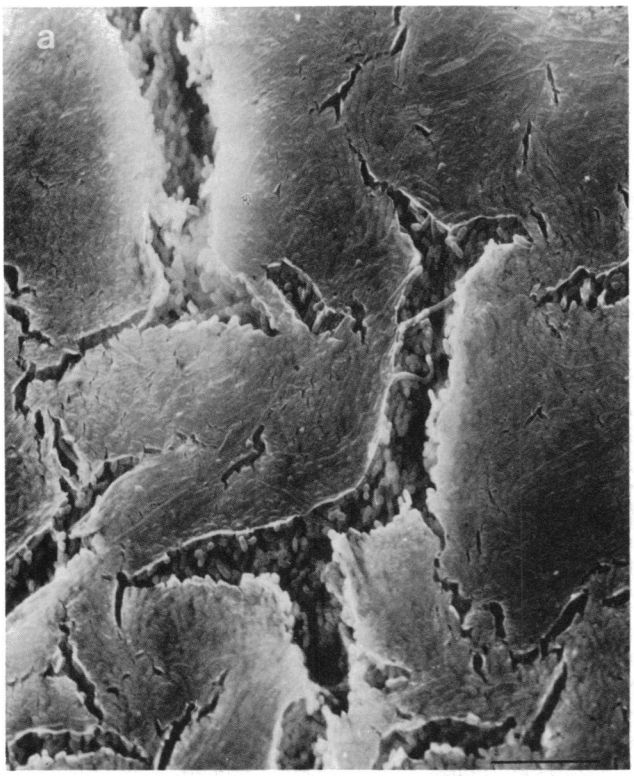
there was signifcant wrinkling of the agar substrate during desiccation. To circumvent this difficulty and have a flat, nondistorting substrate for preparation and SEM, I placed polycarbonate membrane filters coated with bovine serum albumin on agar medium containing the β-galactosidase indicator XGal and inoculated them with dilutions of culture PPS2491. The colonies produced by isolated colony-forming units of PPS2491 showed reduced growth and more regular morphology on the filters compared with those arising after direct inoculation on agar, but there were visible sectors with altered β-galactosidase expression on some of these smaller colonies (Fig. 1). When growth had ceased, the colonies were fixed with OsO_4 and glutaraldehyde with care to avoid any submersion or disturbance of the colony surface. When portions of the filters were removed from the agar after fixation and subjected to rapid air drying at room temperature, the colonies were intact but displayed many cracks and revealed the presence of a thin layer of fixed surface material overlying the cells (Fig. 2). This material was visible over the body of the colony but disappeared near the edges, and there was a striking correlation between the brightness of the colony surface and the presence or absence of the surface covering (Fig. 2c). Colonies prepared in this fashion also showed a variety of cell sizes (Fig. 2b).

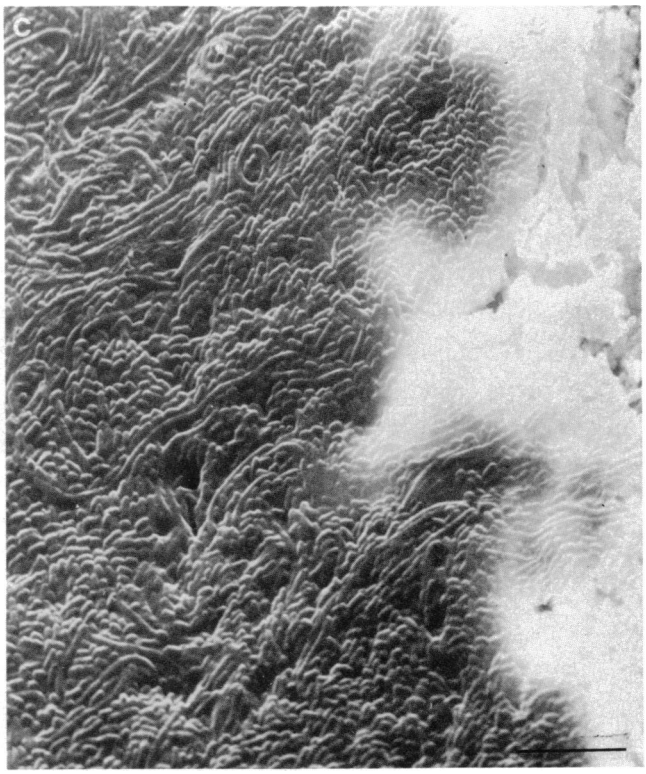
Although badly cracked colonies with a surface coating were not satisfactory for studies of colony microstructure (especially cell arrangements), a chance observation made it possible to develop a reliable method of colony preparation. (Critical-point drying methods used on these colonies had also proved unsatisfactory because, in our hands, they led to explosion of the colony interior and loss of overall colony structure.) A petri dish with fixed colonies on a filter was left closed on the lab bench for over 2 months. At the end of this period, the agar medium was seen to have dried to a flat disk of about 1 mm thickness with a smooth surface and well-preserved colonies still visible. The filter, agar, and plastic dish were bonded solidly together and had to be cut with a saw for mounting and coating. Although the colonies had some cracks, their overall shapes were very well maintained by this method of preparation (Fig. 3a). The particular colony which we examined in detail (Fig. 3b) had a sector in life that was visible as two streaks of β-galactosidase activity (Fig. 1); this sector corresponded to the structurally distinct region observable between 6 and 7 o'clock in the SEM picture. A series of higher-magnification images taken at the crown (Fig. 3c) and at the bottom right edge of the sector (Fig. 3d and e) illustrated several noteworthy features. (i) No covering skin was visible, but there were many small, white balls on the colony surface. Presumably, these balls were composed of the same material as the skin seen on the rapidly dried colonies, and the slower process of desiccation apparently permitted the formation of such spherical structures, leaving the bacterial cells exposed. (ii) The cells in the crown were rather uniform in size and arrangement (Fig. 3c), whereas the cells in the sector were very heterogeneous in size and were often arranged in a complex manner, sometimes displaying visible minisectors (Fig. 3d and e). (iii) As in colonies prepared by critical-point drying (data not shown), many longer cells were visible at the colony edge, often arranged tangentially along the colony perimeter (Fig. 3d). (iv) In the region near the base of the sector, fibrillar extracellular material was seen around the morphologically varied cells (Fig. 3e).

Preparation of colonies grown on agar. Once the slow drying procedure proved successful with colonies grown on filters, it was applied to colonies grown directly on agar, because such colonies showed greater morphogenetic versatility and because previous work on the control of macroscopic pattern formation had been done with colonies that developed under these conditions. Some very striking colonies on a single petri dish containing XGal-glucose agar (Fig. 4a) were studied in detail because the descendance of each colony had been closely followed (Shapiro, in press). These colonies were each started in the same way. A 1-μl inoculum containing approximately 10^4 CFU of a broth suspension was spotted on the indicator plate. Each suspension had been prepared by suspending a single colony from a TYE agar plate. The plate containing these suspended colonies had itself been inoculated with a dilution of an earlier single-colony suspension labeled PPS2532a11. Thus, the colonies examined here displayed the structural and β-galactosidase patterns produced by nine sibling subclonal cultures (labeled a111 through a119). As reported elsewhere, the pattern produced by each culture was reproducible when it was spotted repeatedly on the same indicator medium (Shapiro, in press). The patterns fell into four classes. Cultures a111 and a114 were similar; a112, a113, a115, a117, and a119 were similar (although a117 and a119 produced distinctive sectors); and a116 and a118 produced unique patterns. These latter two patterns were particularly interesting because they showed converse correlations between β-galactosidase expression and surface texture. In a116 colonies, the sectors of greatest enzyme activity were raised fingers radiating from the colony center, whereas in a118 colonies, the sectors of greatest enzyme activity were radial, petal-shaped depressions. These surface texture differences can be seen in Fig. 4b and c.

It is important to note that the fixation and desiccation of these colonies were entirely performed within the original petri dish, thus minimizing the possibility that structural differences between colonies could be due to variations in their preparation for SEM observation. Of the nine colonies on this plate, a111, a112, a116, and a118 were examined in some detail. The findings on four topics were sufficiently reproducible and specific to deserve particular discussion.

(i) The nature of sectorial differences in a116 and a118.





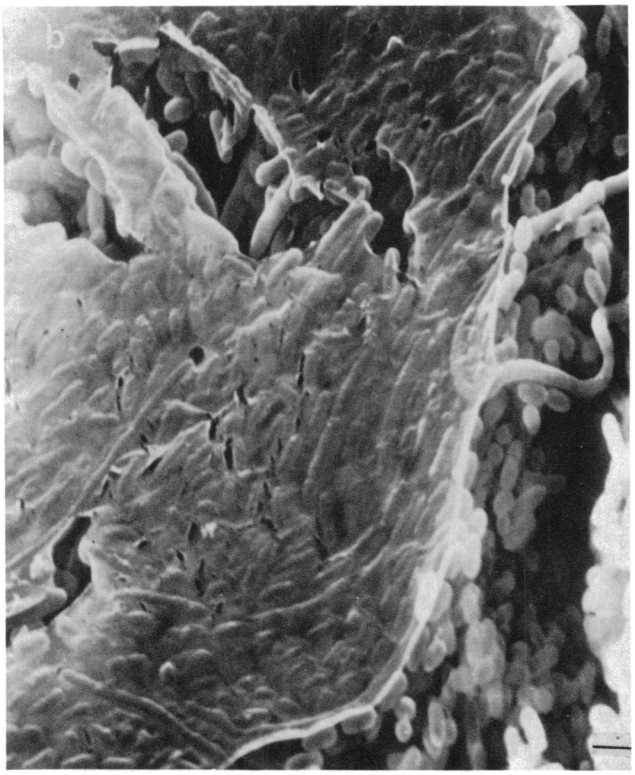


FIG. 2. Filter-grown colonies of PPS2491 prepared for SEM by rapid air drying. (a) A region in the center of a colony (bar, 10 μm). (b) A close-up of the large crack in a, showing the long cell snaking up from and returning to the interior of the colony (bar, 1 μm). (c) A region about 20 μm from the edge of the colony, showing exposed cells at the periphery and the difference in brightness between these uncovered cells and the zone of the surface skin on the right (bar, 10 μm).

Low-magnification views of the a116 colony readily visualized the raised fingers of more extensive β-galactosidase expression (Fig. 5a). Higher magnifications of the sectorial boundaries showed that the cells in the raised areas were exposed and readily visible (Fig. 5b and c), whereas the cells in the depressed zones were largely buried in some kind of extracellular matrix material (Fig. 5d). The a118 colony was more difficult to analyze, because it was fractured into microscopically separated plates by the preparation process. Nonetheless, the petal-shaped sectors could be visualized in some regions of the colony (Fig. 5e), and higher magnifications of the sectorial boundaries showed a difference between raised and depressed zones similar to that observed with a116. Cells were clearly visible in the raised zones, but in the depressed zones of greatest β-galactosidase activity the cells were buried in extracellular matrix material (Fig. 5f). Thus, the distribution of extracellular material correlated with surface structure (absent in raised zones, present in depressed zones) and not with the extent of XGal hydrolysis.

(ii) Different observed cellular arrangements within a single colony. Various regions of the a116 colony were examined in the greatest detail. Figure 6 shows some typical results in two regions near the edge of the colony. It can be seen that a variety of cellular arrangements and morphologies were seen within this colony, often in regions very closely spaced (the two areas shown in panels a and b, on the one hand, and in c and d, on the other, were less than 100 μm apart). Each arrangement had its characteristic features, and sometimes

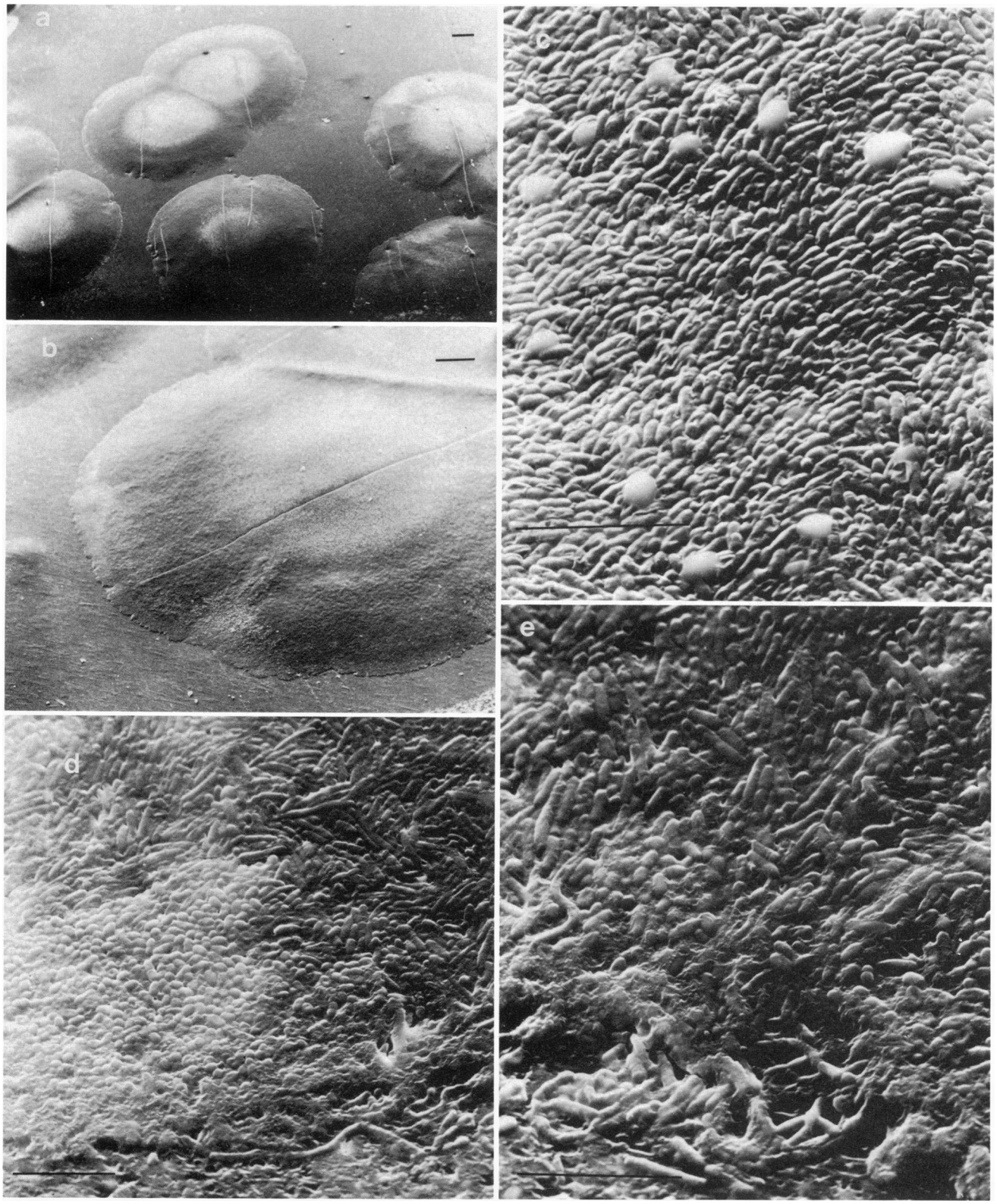
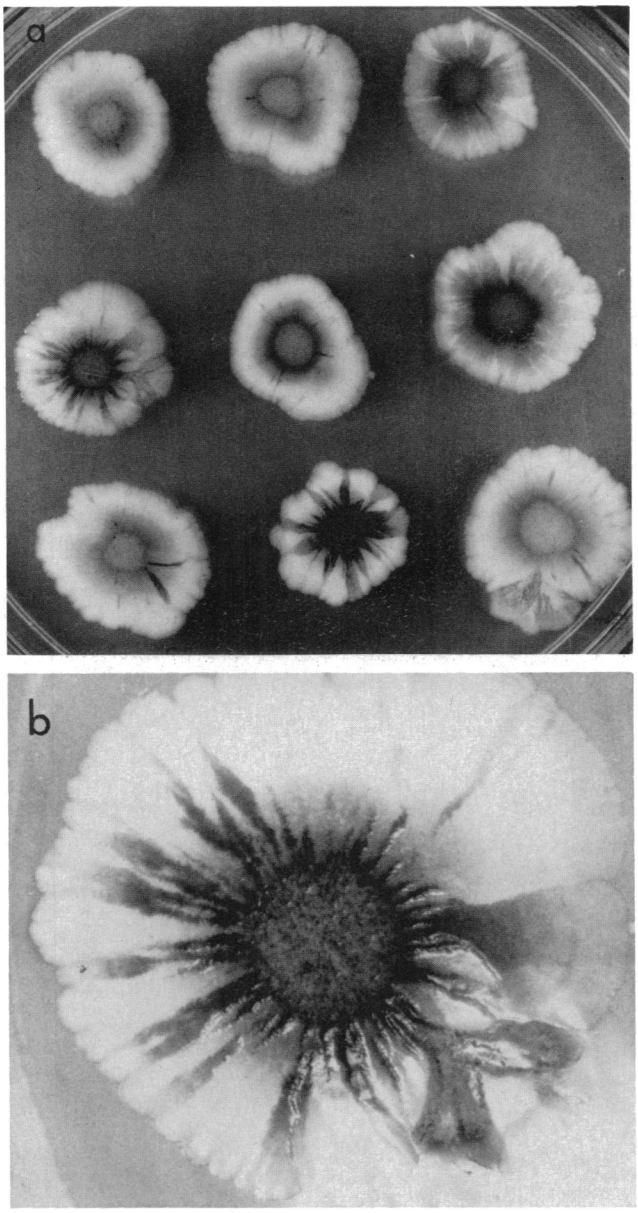


FIG. 3. Filter-grown colonies of PPS2491 after slow air drying. (a) Field of colonies from the area shown in Fig. 1 (bar, 200 μm). (b) Sectored colony examined in detail—the topmost colony of panel a viewed from its unattached side (bar, 100 μm). (c) Region of the sectored colony located just below the crack and above the top of the 7 o'clock sector (bar, 10 μm). (d and e) The edge of the colony at the right-hand extremity of the sector at 7 o'clock; note the filter surface in the foreground of each panel and the puckered bleb at the lower right of d and lower left of e that shows where the two panels overlap (bars, 10 μm).



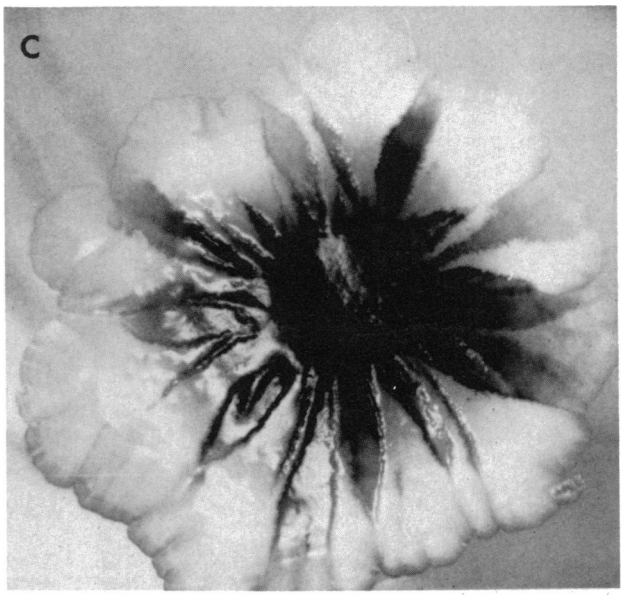


FIG. 4. (a) Colonies produced on XGal-glucose agar by subclonal cultures derived from PPS2532a11. From right to left, the colonies in each row on the dish are a111, a112, a113 (top), a114, a115, a116 (middle), a117, a118, and a119 (bottom). Panels b anc c are close-ups of a116 (b) and a118 (c) with axial plus lateral illumination to highlight surface structure.

microsectors of different patterns could be observed adjacent to one another (Fig. 6d).

(iii) Colony-to-colony differences in cell arrangements. Although the variety of observed cellular arrangements made comparisons difficult, the patterns seen at the edges were rather characteristic for each colony. For example, in the a116 colony the bacteria were well exposed and arranged in a herringbone pattern, whereas in the a112 colony the bacteria were partially embedded in a matrix material and displayed a more irregular arrangement (data not shown).

(iv) Presence of extracellular material on colony surfaces and on the agar adjacent to colonies. A number of intriguing bulbous structures were visible on top of the colonies, often over cracks or at the sectorial boundaries marking the divisions between scallops of the colony edge, and similar structures could be seen outside the colony perimeter on the agar (Fig. 7). The observed structures were obviously the products of changes that took place in extracellular materials during fixation and desiccation, and their presence at cracks and other discontinuities in the colonies indicated that some of these materials were extruded during preparation. Nonetheless, two important features of these blebs were consistent with the idea that they arose from biological materials produced by the colonies. (i) Their surfaces suggested the presence of extensively polymerized materials, and their connections with the colonies were obvious in many of the photos (e.g., Fig. 7d). (Pictures of colonies produced by related cultures on a medium containing glycerol rather than glucose as the carbon source displayed this connection even more sharply [Fig. 8].) (ii) These extracellular structures appeared to have some kind of biological specificity related to the colony patterns, because their shapes and distributions were relatively uniform on and around each colony but differed from colony to colony. Compare, for example, the dense, irregular, dome-topped structures around a111 (Fig. 7a and b), the well-spaced, ridged disk structures around a112 (Fig. 7c), the puckered, rounded structures around a116 (Fig. 7d), and the densely packed, peaked structures around a118 (Fig. 7e and f).

DISCUSSION

One of the main concerns with morphological studies of fixed materials is to avoid drawing conclusions based on artifacts of preparation rather than on the structures of the living material. This is especially critical with SEM because of the need for desiccation. In the images presented here, three features stood out: extracellular materials, variability in cell morphologies, and variability in cell alignments. Before discussing the significance of these SEM observa-

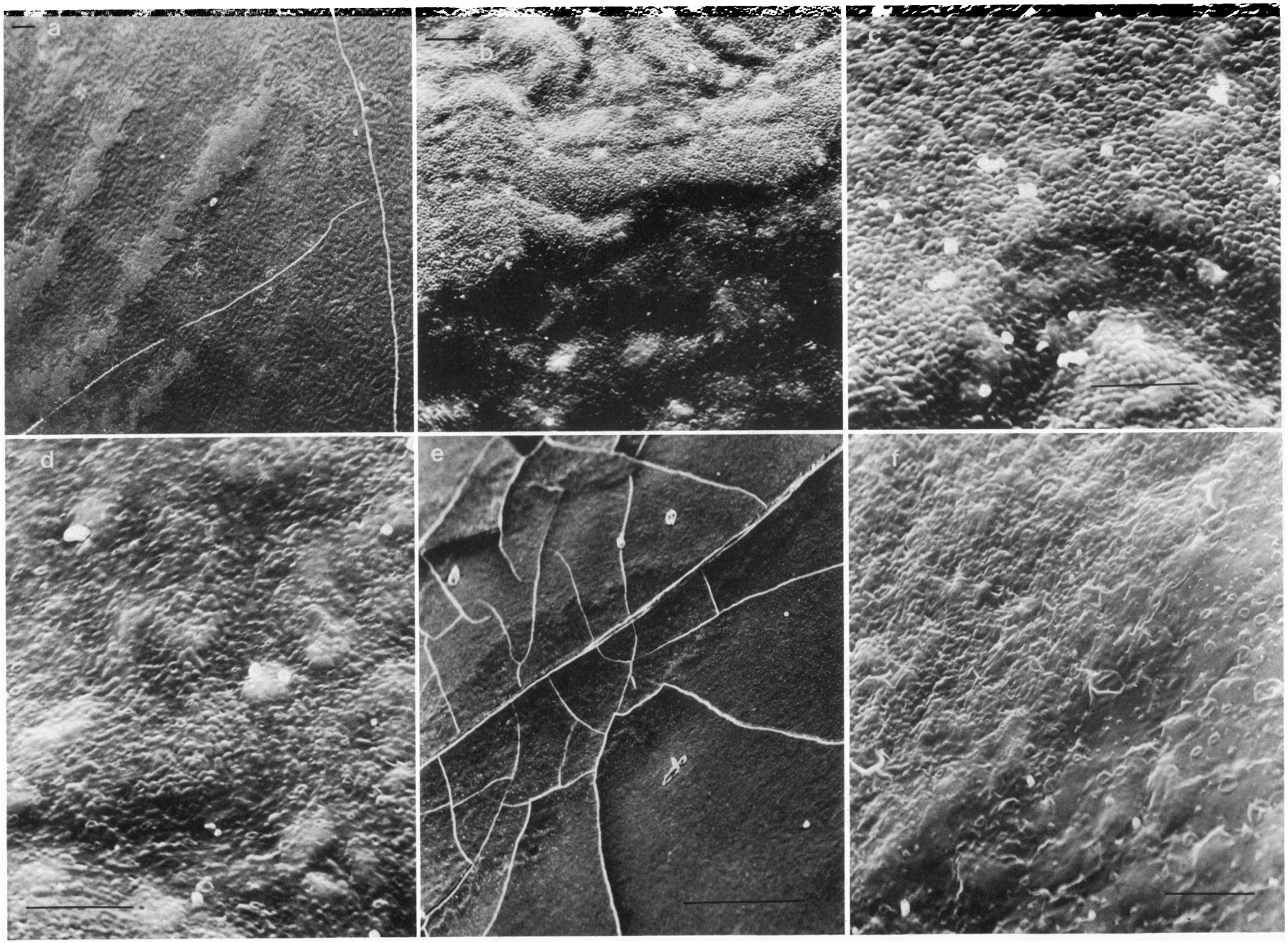


FIG. 5. Sectorial boundaries in PPS2532a116 and PPS2532a118 colonies. (a) Fingerlike sectors in a116 (bar, 100 μm). (b) Edge of a116 sector, the raised sector on top (bar, 10 μm). (c) Detail of raised sector, showing distinct cells (bar, 10 μm). (d) Detail of depressed zone, showing indistinct cells and matrix material (bar, 10 μm). (e) Sector in a118 (bar, 100 μm). (f) Detail of edge of a118 sector, showing raised outside zone above the diagonal and depressed inside zone with extensive matrix material below the diagonal (bar, 10 μm).

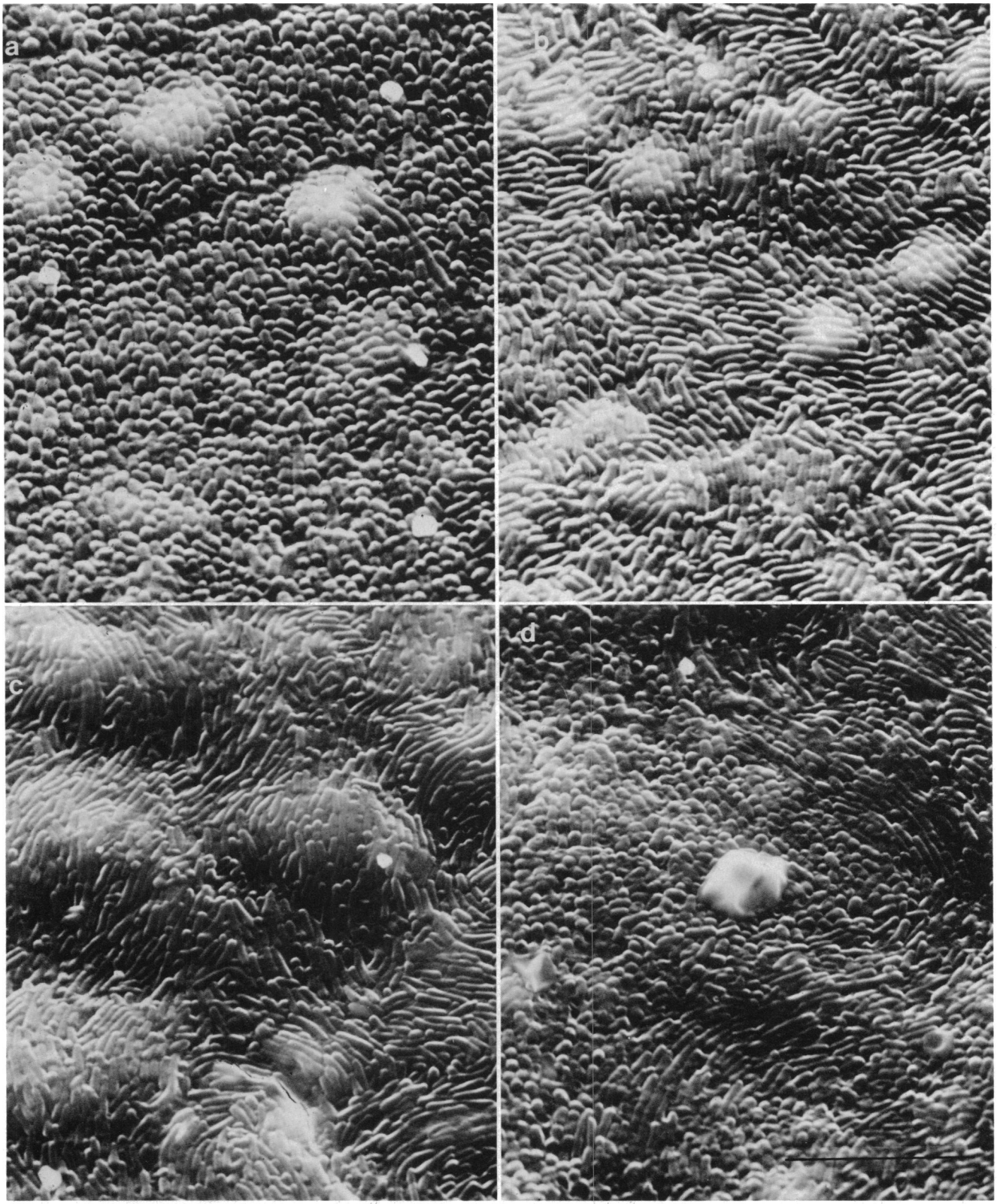


FIG. 6. Different regions of PPS2532a116 colony. All panels are at the same magnification (bar, 10 μm). (a and b) Nearby zones less than 200 μm from the edge of the colony. (c and d) Two nearby zones about 400 to 500 μm from the edge of the colony; note the sectors of different cell sizes and alignments in d.

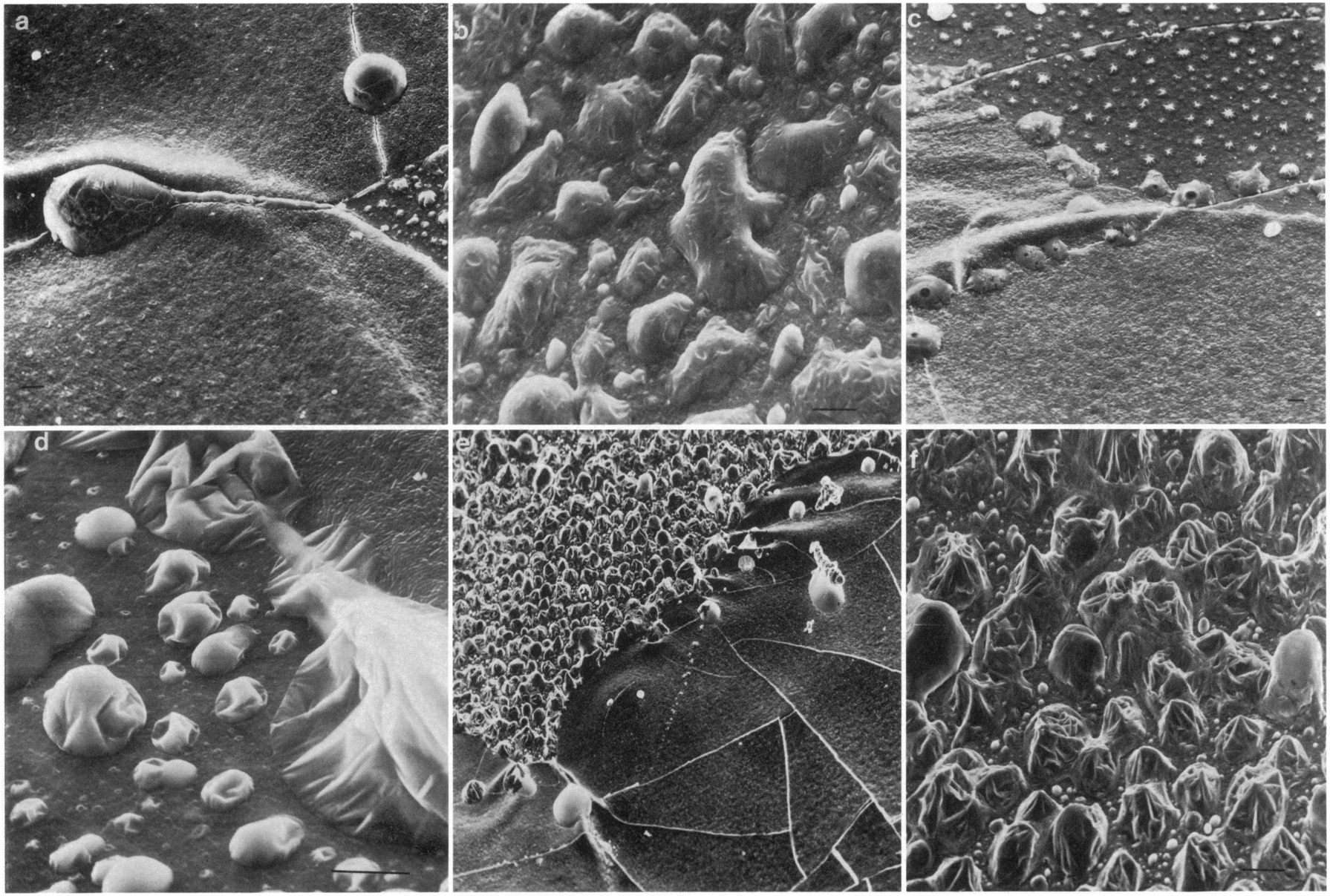


FIG. 7. Extracellular materials near the colony edges (all bars, 10 μm). (a) The boundary between two sectors which marked a scallop in the edge of the a111 colony, showing material over the sectorial boundary and over a crack in the colony surface as well as blebs on the agar next to the colony. (b) Close-up of structures on the agar surface just outside colony a111. (c) The edge of colony a112, showing characteristic blebs on the surface over a crack and at the colony edge as well as the ridged disks on the agar just outside the colony. (d) The edge of colony a116. (e) The edge of colony a118. (f) Close-up of structures on the agar surface just outside colony a118.

tions further, it may be useful to consider the possible role that preparation artifacts played in generating each of these three features.

The visible structures of the fixed extracellular materials clearly depended on the methods of preparation, because changes in one parameter (speed of desiccation) altered what was observed (Fig. 2 and 3). Nonetheless, the observed sheets, balls, and blebs must have arisen from some materials already present, and the observed differences in their shapes and distributions can reasonably be assumed to reflect prior spatial and compositional differences within and around the colonies. As mentioned in the Results section, there was specificity in the shapes and densities of blebs on the agar around each colony. Further, as detailed below, extracellular polymeric materials (generally classed as slimes) have been well documented in living material and shown to have biological function.

The observed variety of cell morphologies (chiefly different cell lengths) was almost certainly very close to what existed in the living material before fixation, because a similar range of cell sizes could be observed by phase-contrast microscopy of bacteria from suspended colonies, and because there was little visible distortion of cell outlines (although some regions showed more puckered cells than others). Moreover, it is difficult to envisage how OsO_4- and glutaraldehyde-fixed bacteria could grow longer or shorter.

The patterns of cell alignment were liable to modification during preparation (especially the shrinkage that accompanied drying), but two factors gave confidence that these patterns were reflective of arrangements that existed prior to fixation: (i) the good preservation of overall colony structure and (ii) the correlation of observed patterns with macroscopic features of colony organization (in particular, sectors and distance from the center or edge of the colony). The patterns were often so well defined and sometimes showed such sharp boundaries (e.g., Fig. 3d and 6d) that it seemed justifiable to conclude that the final observed arrangements, even if not necessarily the same as those in the living state, arose from specific configurations that differed from one region of the colony to another.

The SEM pictures revealed an unexpectedly large repertoire of cellular and extracellular components used in the construction of *P. putida* colonies. These results were consistent with earlier conclusions that bacterial colonies are highly organized, heterogeneous structures. For example, each region of colony a116 (Fig. 5 and 6) was seen to have its own very specific organization of cellular and extracellular components. At least some of the different areas of microscopic organization were, in turn, arranged in ways that corresponded to macroscopically discernible organizational patterns (as in the raised and depressed sectors of PPS2532a116 and PPS2532a118). Other features of microscopic organization occurred at too fine a level to have easily detectable large-scale correlates (e.g., the microsectors illustrated in Fig. 3d and 6d). However, many cases of pattern fine structure could be visualized in XGal-stained colonies (15–17), and it is possible that this fine structure corresponded to localized differences in cellular organization of the kinds documented here. The presence of extracellular material was important, because it showed that colony architecture involved more than just an accumulation of bacterial cells.

In trying to understand the regulation of pattern formation in the morphogenesis of bacterial colonies, two of the principal questions have concerned (i) the mechanisms for coordinating the activities of unrelated cells at similar positions in the colony to generate concentric zones of phenotypic variation and (ii) the mechanisms for maintaining the identity and integrity of distinct clonal populations (16). Although diffusible chemical signals will probably turn out to be very important information carriers in bacterial populations, the present data indicate that there are at least two other candidates as possible physical bases for communicating over large distances (i.e., many cell diameters) within colonies: (i) various polymeric extracellular matrix materials and (ii) long cells. One reason that these possibilities are attractive to consider is that they provide plausible mechanisms for communicating within and between differentiated populations over large distances through dense masses of cells, something which cannot be accomplished readily by diffusion gradients. Nerve cells and vascular tissues provide analogies for long-distance communication systems in the development of multicellular eucaryotes. The extracellular materials also provide a possible physical mechanism for maintaining the integrity of sectorial populations and the boundaries between adjacent sectors (16). Careful picking from colonies under a dissecting microscope showed that a sector often has internal cohesion and can separate cleanly as a single viscous mass from the rest of the colony; such separations may result from a discontinuous, sector-specific distribution of polymeric materials. It will be interesting to test this notion by studying the distribution of different polysaccharides in colonies.

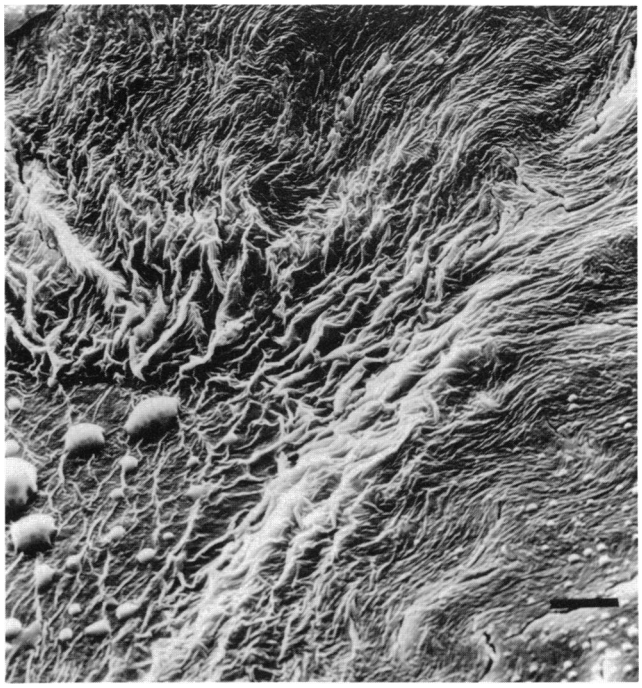


FIG. 8. The edge of a PPS2532 colony grown on XGal-glycerol agar (bar, 10 μm).

The observation that each colony appeared to extrude material onto the agar substrate beyond the limits of cellular proliferation was not without precedent. In natural environments, many organized bacterial populations have been found to be encapsulated, such as *Neisseria* infections (9, 11, 12), antibiotic-resistant reservoirs of recurrent septicemias (10), and biofilms on solid structures and intestinal walls (4). In the laboratory, slimes have been observed to play an

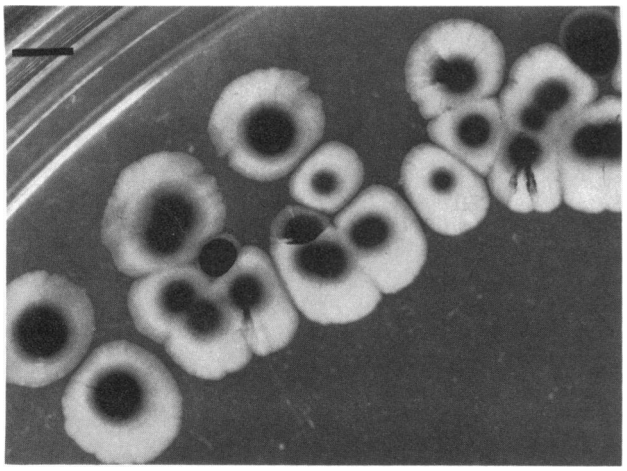


FIG. 9. PPS2491 colonies on XGal-glucose agar (bar, 2 mm). Note fused colony edges when centers were closer than 2 mm but distinct intercolony boundaries when centers were farther than 2.1 mm apart.

important role in the development of both *Proteus* and myxobacteria swarms (14, 18), and, in the case of myxobacteria, slime trails can be seen in time-lapse films to coordinate the movements of large numbers of bacteria (13).

Since no extracolony materials were observed around restricted colonies grown on polycarbonate filters, there may be a correlation between the ability of *P. putida* to spread on the substrate and the ability to extrude polymeric materials. In this connection, it is interesting to note that there are a variety of territorial interactions between nearby colonies formed by nonswarming bacteria which can be observed on crowded agar plates. The most common of these is the formation of intercolony boundary zones where two growing colonies have encountered each other after a day or more of initial growth (Fig. 9). These boundary zones appear to be devoid of bacteria and can be several millimeters in length, showing that the colonies continued proliferation after their initial encounter. Analogous boundaries have been observed to form in many bacterial growths on agar. A particularly illustrative case is that of *Proteus mirabilis*, in which swarms of different strains establish sharp, well-defined boundaries but swarms of the same strain fuse (5). The strain specificity of this phenomenon, the isolation of *Proteus* variants that have lost the ability to establish boundaries (unpublished observations), and time-lapse recording all indicate that boundary formation by this species is a biological process rather than the nonspecific consequence of nutrient depletion. Recent observations on *Cytophaga johnsonae* colonies lend plausibility to the idea that extracolony polymers participate in similar interactions, for there are mutant *Cytophaga* colonies which surround themselves with extensive cell-free sheets of polymeric material, and other colonies are unable to spread into the areas occupied by these sheets (J. L. Pate, personal communication). It may be useful to speculate on the possibility that extracolony polymers play a dual role in these interactions between distinct bacterial populations on agar (i) as carriers of colonial identification and (ii) as part of the sensory apparatus involved in redirecting colonial development when other colonies are nearby.

The preceeding notion that bacterial masses on agar are sentient may seem inconsistent with our standard views of procaryotic behavior. Nonetheless, recent observations on the directed movement of *Myxococcus xanthus* flares toward bacterial clumps and glass or polystyrene latex beads have made it clear that some bacterial populations can perceive objects at a distance and adjust their group behavior accordingly (7). Whereas the social behavior of *Myxococcus* spp. certainly has some very special features, these observations are relevant to the questions raised by colony morphogenesis involving nonswarming organisms, for the regular formation of highly structured colonies indicates that many other groups of bacteria also have the capacity for coordinating the activities of large numbers of cells.

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