Scanning Electron Microscopy of Bacterial Colonies

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Received for publication 21 August 1973

A technique is described for observing bacterial colony growth. Bacillus cereus, B. subtilis, and B. cereus var. mycoides were grown on strips of dialysis membrane layered on nutrient agar. Microcolonies of the organisms on strips were fixed in Formalin vapor in situ; the strips then were removed from the agar and secured to scanning microscope specimen stubs without markedly disturbing the cellular arrangement. Scanning electron micrographs clearly depict morphology of individual cells, as well as the spatial orientation of cells within the colony. This technique is reproducible, adaptable, and simple.

MATERIALS AND METHODS

Organisms and cultural conditions. Three strains of aerobic sporeforming bacteria were isolated from soil samples gathered at Peoria, Ill., and characterized as Bacillus cereus, B. subtilis, and B. cereus var. mycoides. The bacilli were routinely grown on 2% nutrient agar (Difco) containing 0.2% glucose; the final pH was 7.4. The glucose was sterilized separately and added aseptically to the medium.

About 40 ml of sterile medium was placed into separate plastic petri dishes (100 by 15 mm). The medium was allowed to solidify before the dishes were placed in an oven at 37 C for about 4 h to dry the agar surface.

Water-soaked strips (13 by 95 mm) cut from cellulose dialysis tubing (size 20, Union Carbide Corp., Chicago, Ill.) were laid on the dry surface and smoothed out with a glass spreading rod. The dialysis strips were cut to these specific dimensions to facilitate removal from the agar plate and mounting on the scanning microscope specimen stub. The width of the strip is identical to the width of the specimen stub; the length allows about 7 mm to overhang the side of the petri dish. This overhanging tab makes it easy to remove strips from the agar (Fig. 1). Before use, dialysis strips were soaked in distilled water overnight, and individual strips were sandwiched between moist filter paper and stacked in a glass petri dish. The petri dish containing the strips was then autoclaved at 121 C for 15 min. The filter paper enclosing the membranes was kept moist to aid handling and storing the sterilized membranes.

Periodic transfer of cells to fresh medium by the method of Collier (4) gave a synchronous population of each organism. Synchronously growing cells were washed three times by centrifugation in the cold and were then resuspended in sterile distilled water. After direct microscope counts were made with a Petroff-Hauser bacteria counter, the cell suspension was diluted serially in water to 10^6 cells/ml. About 0.03 ml of this cell suspension was gently placed onto the dialysis strip arranged as portrayed in Fig. 1. The inoculated strip was incubated at 28 C and examined in situ hourly with a light microscope at 40X. The three cultures studied had grown sufficiently for scanning microscope examination by 4 to 6 h after inoculation.

Scanning electron microscopy. Colonies at the desired growth stage were fixed in Formalin vapor for 24 h. After fixation, the outer edges of the colonies were marked by perforating the strip with a dissecting needle. The strip then was removed from the medium...
by gently lifting the overhanging tab vertically until the entire strip was freed from the agar. An aluminum specimen stub with double-coated adhesive tape on its surface was positioned underneath the marked colony, and excess membrane was cut away. Figure 2 shows (a) an aluminum specimen stub, (b) a stub with double adhesive tape attached, and (c) one with tape and secured dialysis membrane. Mounted colonies were coated with gold-palladium (60:40) to a thickness of about 15 nm while being rotated to 10 rpm in a Denton DV-502 high-vacuum evaporator. After coating, du Pont conductive silver paste was placed on the mounting stub surface adjacent to and touching the dialysis tubing to ensure good electrical contact. The colonies were observed in a Stereoscan scanning electron microscope (mark 2A, Cambridge Scientific Instruments, Co., Ltd., London, England) at a beam specimen angle of 45°. Accelerating voltage was 20 kV; final aperture was 200 μm.

RESULTS

Scanning electron micrographs of bacterial colonies are reproduced in Fig. 3. The outer edge and center of the same colony of \textit{B. cereus} are shown in Fig. 3a and b. At the colony’s outer edge (a), cells one-layer thick are oriented in uniform chains that lie adjacent and concentric to one another. By periodic observation of developing colonies, we noted that the first new cells at the center of the colony divided so that the resultant chains elongated with a counter-clockwise curvature. The curved configuration is seen in Fig. 3a. Continued proliferation of cells at the center masked the initial growth pattern; as seen, the cells are randomly stacked (b). Figures 3c and d present the outer edge and center of a \textit{B. subtilis} colony, respectively. The longitudinal axis of individual cells is perpendicular to the colony’s edge. The center of the colony (d) is characterized by layers of cells bundled together apparently by extracellular material. Figure 3e depicts the edge of a \textit{B. cereus} var. mycoides colony; its cellular arrangement is similar to that of \textit{B. cereus}, with some individual cells of \textit{B. cereus} var. mycoides curved (arrow). The central area of the \textit{B. cereus} var. mycoides colony (Fig. 3f) differs from that of the other two bacteria: it is one cell thick and is composed of uniform, parallel chains of cells.

DISCUSSION

The dialysis membrane technique we developed for observing colonial growth by scanning electron microscopy gives reproducibility and simplicity. Furthermore, the method is adaptable and can be used to examine freeze-dried cells, untreated cells, or cells fixed with other chemicals. Other methods commonly used to examine bacterial colonies involve either excising agar blocks from agar plates containing colonies or growing bacteria on microcover slips

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**Fig. 1. Petri dish set up for growing bacteria on dialysis membrane.** (a) Glass spreading rod; (b) strip of dialysis membrane; (c) nutrient agar.

**Fig. 2. Aluminum specimen stubs for scanning electron microscopy.** (a) Stub alone; (b) stub mounted with transparent double-coated tape; (c) stub mounted with tape plus dialysis membrane strip.
and placing them directly onto specimen stubs (5, 6, 8, 12–14). With these methods, some difficulties arise during drying and chemical fixation. Frequently, cells neither adhere to the agar surface nor to the cover slip. Extended drying, often needed to dehydrate the agar, usually distorts the cells. If the agar is not completely dry, sublimation occurs under high vacuum and the sublimated agar masks the cells.

A variety of membranes can be used for isolation and growth of bacteria. In our work, we selected one that would support bacterial growth; would be readily available, easily handled, and sterilizable; would withstand chemical fixation, drying, vacuum pressure, and metal coating; and would remain undistorted under electron bombardment in the scanning microscope. Of the membranous films and sheets we examined (including various commercially produced cellulose filters of different pore-size and “cellophane papers”) only the dialysis tubing possessed all these characteristics.
ACKNOWLEDGMENTS

We thank G. L. Adams and F. L. Baker for their able technical assistance.


LITERATURE CITED