Critical-Point Drying: Rapid Method for the Determination of Bacterial Extracellular Polymer and Surface Structures

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The relative amount of extracellular polymer which remains about Azotobacter vinelandii, Zoogloea ramigera, Klebsiella pneumoniae, and Diplococcus pneumoniae after critical-point drying was studied by electron microscopy. The results obtained with this technique are compared to those obtained with methods that illustrate extracellular polymer, such as freeze-etching and ruthenium red staining. Comparative results indicate critical-point drying to be a rapid, reliable method for the determination of capsule-like polymer surrounding bacterial cells. In addition, critical-point drying can be used to observe morphogenetic changes, such as vesicle production.

The methods presently employed to examine the ultrastructure of cell surfaces and extracellular polymer surrounding bacteria include negative staining (12), shadow casting (14), freeze-etching (15), and selective staining of polymer for thin sections (3). Although attempts have been made to observe hydrated biological material (9), dehydration is necessitated by the high vacuum required for efficient operation of the electron microscope. The disruptive and distorting effects of air-drying and freeze-drying prior to shadow casting or negative staining have been reviewed by Anderson (2). Surface tension artifacts attributed to drying may be minimized by dehydrating microorganisms by the critical-point method prior to electron microscope observation. Anderson (1) initially used CO₂ to critical-point dry Escherichia coli, whereas Cohen et al. (6) employed fluorocarbons, because of decreased critical pressure, in studies of Pseudomonas fluorescens. Thin-sectional studies of critical-point dried cells by Koller and Bernhard (13) have shown that internal cellular detail is also preserved by this method. Critical-point drying might also be useful in the rapid determination of encapsulation, examination of the arrangement of extracellular polymer, and other structures produced on the surface of bacteria. In this study, critical-point drying was used to examine vesicular appendages and the distribution of extracellular polymer surrounding bacterial cells.

MATERIALS AND METHODS

Vegetative cells of Azotobacter vinelandii (ATCC 12837) were induced to encyst by transferring them to Burk's nitrogen-free medium supplemented with 0.3% n-butanol as previously described (4). Zoogloea ramigera (OSU I-115) was grown in a modified medium of Crabtree et al. (7) as previously described (3). Klebsiella pneumoniae type II (OSU 312) was grown on tryptose blood agar base supplemented with 5% defibrinated sheep blood for 24 to 36 h at 30 C, and then incubated on Worfel-Ferguson agar (8) for 4 to 6 h at 37 C. Diplococcus pneumoniae (OSU 29) was cultured on blood agar in a candle jar at 37 C.

A. vinelandii was prepared for thin sectioning by the modified ruthenium red staining procedure of Cagle et al. (3) and freeze-etched as previously described (5). Cells were dehydrated by the critical-point method by suspending them in distilled water and placing a drop of the suspension on a Formvar-coated grid for 2 to 3 min at 4 C. The cells were then fixed in 3% glutaraldehyde for 5 min, washed with distilled water, and dehydrated by passage through a graded alcoholic series. The bacteria were suspended in an intermediate fluid (amyl acetate) for 5 min and then transferred to the chamber of a Samdri-PVT-3 critical-point drying apparatus (Biodynamics Research Corp., Rockville, Md.) precooled to 10 to 15 C. The chamber was closed and filled with CO₂, and the intermediate fluid was purged by flowing CO₂ through the chamber. After expelling all of the amyl acetate, the cells were dried at critical point by the method of Anderson (1). All cell preparations were examined with a Philips EM300 electron microscope at an 80-kV accelerating voltage.

RESULTS AND DISCUSSION

Methods available for the examination of capsular material in thin sections (Fig. 1 and 2) or freeze-etchings (Fig. 3) can be used to study the structure of extracellular polymer as well as internal cellular detail. The uneven distribution
of polymer (Fig. 1, arrows) exterior to the Azotobacter cyst coat (CC) suggests that a portion of the material was removed during preparation. This problem is routinely encountered in examining thin sections of encapsulated bacteria with the electron microscope. Cyst formation is a morphogenetic process in which bacillary vegetative cells (Fig. 2) incubated on butanol-containing media assume a spherical morphology and produce large quantities of vesicles (arrows) that coalesce to form the exine (Ex). Although ruthenium red-stained thin sections (Fig. 1 and 2) illustrate the amount of polymer surrounding cells more clearly than when other methods of fixation (e.g., glutaraldehyde-OsO₄ or KMnO₄) are used, only sections through cells show the relation of polymer to the bacteria. Frozen-etched cysts of A. vinelandii (Fig. 3) cleaved through the cytoplasm (Cy) are similar to thin sections (Fig. 1). In Fig. 3 several distinct layers of cyst-coat polymer (arrows) indicate that extracellular material is distributed more extensively than observed in thin sections. Critical-point drying can be used to observe extracellular polymer (Fig. 4) that extends from each
cyst (dark arrows), as well as vesicular structures (Fig. 5, arrows) produced by encysting cells which unite to form the cyst coat. On the outer surface of the cyst (Fig. 4), strands of extracellular polymer are involved in the formation of an uneven mesh-like layer (double arrows), previously observed in freeze-etchings (5). Individual strands of polymer approximately 25 to 30 nm thick extend from cysts a distance of 850 nm or more.

Three other bacteria employed in this study possess contrasting types of extracellular polymer. Cultures of *Z. ramigera* produce copious quantities of extracellular polysaccharide. The zoogloeal matrix surrounding *Z. ramigera* has been examined previously to elucidate its structure (11) and functional properties (10). In Fig. 6, the random manner in which the extracellular polymer adheres to the cell indicates that the material is slime (SL) rather than capsule. Critical-point dried *Z. ramigera* also reveals the dimensional structure of entwined polymer

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**Fig. 4.** Polymer extends from the surface of critical-point dried Azotobacter cysts (dark arrows) and is matted on the surface (double arrows). The coat is composed of the intine-exine (Ex-In) which encompasses the electron-dense central body (CB). Marker = 1 μm.

**Fig. 5.** Encysting vegetative cell, similar to Fig. 2, prepared by critical-point drying. Numerous vesicles (arrows) are apparent on the surface of the cell. Marker = 0.5 μm.

**Fig. 6.** Critical-point dried *Z. ramigera* polymer adheres to the cell and forms a large slime layer (SL). The dimensional structure of the polymeric floc appears almost spherical. Marker = 1 μm.
which is responsible for the floc formation of this organism. Ruthenium red-stained thin sections indicate that the capsule of \textit{K. pneumoniae} is composed of fibrils approximately 300 nm in length (16). Cells prepared by the critical-point method reveal that \textit{K. pneumoniae} is heavily encapsulated, as evidenced by the strands of polymer (Fig. 7, dark arrows) extend-

\textbf{Fig. 7.} \textit{K. pneumoniae} dehydrated by the critical-point method. Fibrils of capsular polymer extend from the cell (dark arrows) and are entwined in two electron-opaque structures (double arrows). Marker = 0.5 \, \mu m.

\textbf{Fig. 8.} Critical-point dried \textit{D. pneumoniae}. The characteristic diplococcus is surrounded by thin strands of polymer (arrows) that comprise the capsule. Marker = 0.5 \, \mu m.
ing from the cell and entwined on the surface of the organism. At either end of the cell, tangled fibrillar polymer is observed in approximately spherical structures (double arrows). Strands of polymer surrounding K. pneumoniae are approximately the same thickness (20 to 30 nm) as those observed around A. vinelandii cysts (Fig. 4) and extend from the cell a distance of 500 nm (Fig. 7). The ultrastructure of the capsule surrounding D. pneumoniae has been characterized as an irregular, mat-like polymer (16). However, D. pneumoniae dehydrated by the critical-point method (Fig. 8) possesses polymer that is distinctly fibrillar (arrows). A constant number of thin strands (40 to 55 per cell) extend from each cell more than 725 nm.

Although thin sections (Fig. 1 and 2) and frozen-etched preparations (Fig. 3) possess specific advantages, the critical-point method of dehydrating bacteria maintains dimensional cell structure (1, 6) including extended vesicles (Fig. 5), preserves extensive extracellular polymer (Fig. 4 and 6 to 8), and provides a method for the examination of extracellular polymer following short periods of preparation (2 h or less).

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