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Surface Features of *Bacillus polymyxa* Spores as Revealed by Scanning Electron Microscopy

JUDITH A. MURPHY AND L. LEON CAMPBELL
Department of Microbiology, University of Illinois, Urbana, Illinois 61801

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The surface features of *Bacillus polymyxa* spores were compared by use of thin sections, carbon replicas, and the scanning electron microscope. Some features of the characteristic ridges, previously reported in ultrathin sections and carbon replicas of spores of this species, were more clearly revealed with the scanning electron microscope. A three-dimensional image is provided because of the greater depth of focus possible with this instrument. End-on views of *B. polymyxa* spores readily illustrate the polygonal porelike structure present.

Bacterial spores have been investigated by many workers with transmission electron microscopes, providing much useful information (17). Details of the internal structures have been obtained from the examination of ultrathin sections (6, 7, 9, 11, 15, 21).

Replica methods which provide a means of studying spore surfaces have been developed (3, 4). Surface sculpturing of spores has been used as an aid in the taxonomy of various species of *Bacillus* and for the study of certain physiological problems such as the effect of heat on spores (8).

To augment information obtained with other methods, in this preliminary study we examine the usefulness of the scanning electron microscope in the study of the bacterial spore surface. The surface features of *B. polymyxa* spores are compared by use of thin sections, carbon replicas, and the scanning electron microscope.

MATERIALS AND METHODS

Preparation of spores. Spores of *B. polymyxa* strain 842 were produced on a potato-agar medium (18) containing, per liter: 200 g of new red potatoes (freshly peeled and chipped), 5 mg of $MnCl_2 \cdot 4H_2O$, and 15 g of agar (Difco). The potatoes were boiled, mashed in a Waring Blendor, and filtered through gauze; the pulp was discarded and the filtrate was diluted. The *p*H was adjusted to 6.8 with 1 N NaOH, Mn^{++} and agar were added, and the final volume was adjusted to 1 liter. The medium was sterilized at 120 C for 15 min and was dispensed into petri plates (25 ml per plate).

The potato agar plates were inoculated with cells grown in Penassay Broth (Difco) for 6 hr at 37 C in a water-bath shaker. The plates were incubated at 37 C, and sporulation was followed by periodic examination of samples with phase-contrast microscopy. After 9 days of incubation, the spores were harvested with cold sterile distilled water and were washed by repeated differential centrifugation followed by a lysozyme treatment (16) to lyse any remaining vegetative cells. The spores were removed by centrifugation and suspended in sterile distilled water. The cleaned spore suspension was stored at 5 C until used.

Preparation of carbon replicas. Two methods of carbon replication were employed, i.e., the replica method described by Bradley and Williams (3) as modified by Bradley and Franklin (2) and the preshadowed carbon replica technique of Dalitz as modified by deBoer and Spit (4).

The preshadowed carbon replica technique was used as follows. A drop of the cleaned spore suspension was pipetted onto a warmed glass slide and a smear was made with a second glass slide. After drying for several hours in a dust-free environment, the slide was shadowed with platinum at an angle of 26° and then carbon-coated at right angles by vacuum evaporation. With a razor blade, the carbon film was scored into 2- to 3-mm squares, and the slide was submerged into 5% hydrofluoric acid for 1 to 2 sec or until the scratches lightened. The squares were floated off onto a distilled water surface and after 1 to 2 min were transferred by means of a wire-mesh screen to the surface of a dichromate-sulfuric acid solution in which oxidation of the spore material occurred (0.9 ml of a saturated solution of sodium dichromate added to 100 ml of concentrated sulfuric acid). After 90 min, the oxidation process was terminated, and the squares were rinsed by transfer through several changes of distilled water. The squares were picked up on bare 200-mesh copper grids and allowed to dry. Preparations were examined in a Jeolco model JEM-T6S electron microscope with an objective aperture of 50 μm and an accelerating voltage of 60 kv.

Preparation of thin sections. For sectioning, a portion of the cleaned spore suspension was immediately fixed with osmium tetroxide and washed with 0.5%

(w/v) uranyl acetate by the method of Kellenberger, Ryter, and Sechaud (12). The fixed material in agar blocks was dehydrated by passage through a graded ethyl alcohol series (to 100%) and then through two changes of propylene oxide and was finally embedded in Epon 812 by the method of Luft (13). Capsules were hardened overnight at room temperature followed by 3 days of incubation at 60 C. Polymerized blocks were sectioned with glass knifes on an LKB 4800A Ultrotome I ultramicrotome. Sections were floated onto either distilled water or 20% acetone in water and were picked up on 200-mesh, carbon-coated, Formvar-filmed grids. Section contrast was increased by staining with 0.25% lead citrate for 1 min (20). Specimens were examined with a Siemens Elmiskop I electron microscope operating at an accelerating voltage of 60 kv and fitted with a 50-μm objective aperture.

Scanning electron microscopy. A portion of the cleaned spore suspension was diluted with sterile distilled water until just slightly turbid. The sample was pipetted onto a circular polished-aluminum specimen stub in small droplets and allowed to air dry. After the sample was dried on the stub, it was necessary to evaporate a thin metallic coating onto the stub surface to increase surface conductivity and thus avoid charging of the specimen during observation. This was accomplished by placing the specimen stub in a vacuum evaporator equipped with a rotary table which was situated about 10 cm from the electrode. A thick coating (40 to 50 nm) of gold-palladium (60/40 alloy) was evaporated onto the specimen at angles ranging from 35° to 45°. Specimens were examined in a Cambridge Stereoscan Mark IIa scanning electron microscope operated at 30 kv with a beam specimen angle of 45° and fitted with an 100-μm final aperture.

The use of polished specimen stubs was found necessary in order to prevent the spores from settling in grooves on the stub facing where only partial observation of the spore is possible. The specimen stubs (Engis Equipment Co., Morton Grove, Ill.) were first polished by rough grinding on a fine emery belt to remove the large ridges of the faced stubs. They were then polished on successive metallurgical wheels, 400 grit, 600 grit, and levigated alumina. Finally, they were mounted in a weighted disc in a vibrator polisher with 0.03-μm levigated alumina on a polishing cloth. The polisher was run for 6 to 12 hr depending on the time required to obtain the finish desired. This procedure was used as an alternative to electropolishing, which has a tendency to pit the stub surface because of impurities in the aluminum. For a preferred method, it is suggested that high-purity aluminum be used; the stubs can be machined from this material and then electropolished, resulting in a smoother surface.

RESULTS

Sections. Ultrathin sections of *B. polymyxa* spores have previously been published (5, 11, 19). Figure 1 shows a spore cross section of *B. polymyxa* strain 842. This micrograph is included only to illustrate the characteristic surface structure found in thin sections. The set of ridges, numbering from six to nine, forms an integral part of the spore coat. It is not intended that this micrograph reveal detailed internal structure, although it is apparent that the internal structure of the spore of this particular strain is similar to that of other *B. polymyxa* strains studied. The substructure of the spore was previously revealed by the elegant studies of Holbert (11).

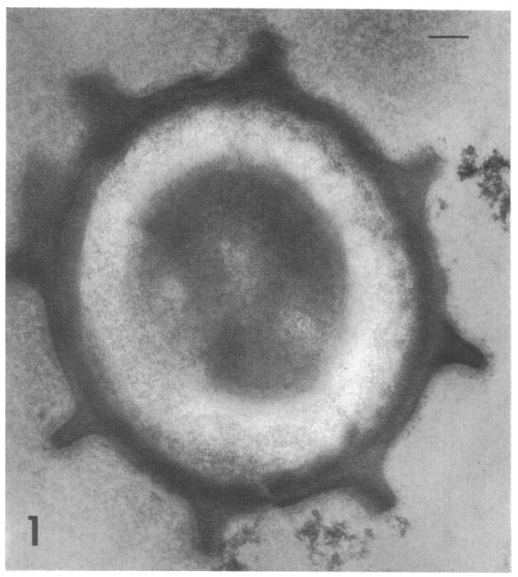


FIG. 1. *Cross section of B. polymyxa spore, illustrating the characteristic ridged surface structure as seen in thin sections examined in a transmission electron microscope. Marker represents 0.1 μm. × 50,000.*

FIG. 2. *Preshadowed carbon replica of B. polymyxa spore, examined in a transmission electron microscope, illustrating parallel surface ribbing coalescing at the end into porelike structures. Marker represents 1.0 μm. × 19,600.*

FIG. 3 and 4. *Preshadowed carbon replicas of B. polymyxa, examined in a transmission electron microscope, illustrating two kinds of random reticulation. Markers represent 1.0 μm. Fig. 3, × 18,000. Fig. 4, × 17,400.*

FIG. 5. *Preshadowed carbon replica of B. polymyxa spores, examined in a transmission electron microscope, illustrating parallel surface ribbing coalescing at the ends into porelike structures. Marker represents 1.0 μm. × 15,200.*

FIG. 6 and 7. *Carbon replicas of B. polymyxa spores prepared according to Bradley and Franklin (2). Both parallel ribbing and random reticulation are illustrated. Note that spores prepared by this method are not as clean as those prepared by the preshadowed technique illustrated in Fig. 2 to 5. Markers represent 1.0 μm. Fig. 6, × 3700. Fig. 7, × 10,200.*

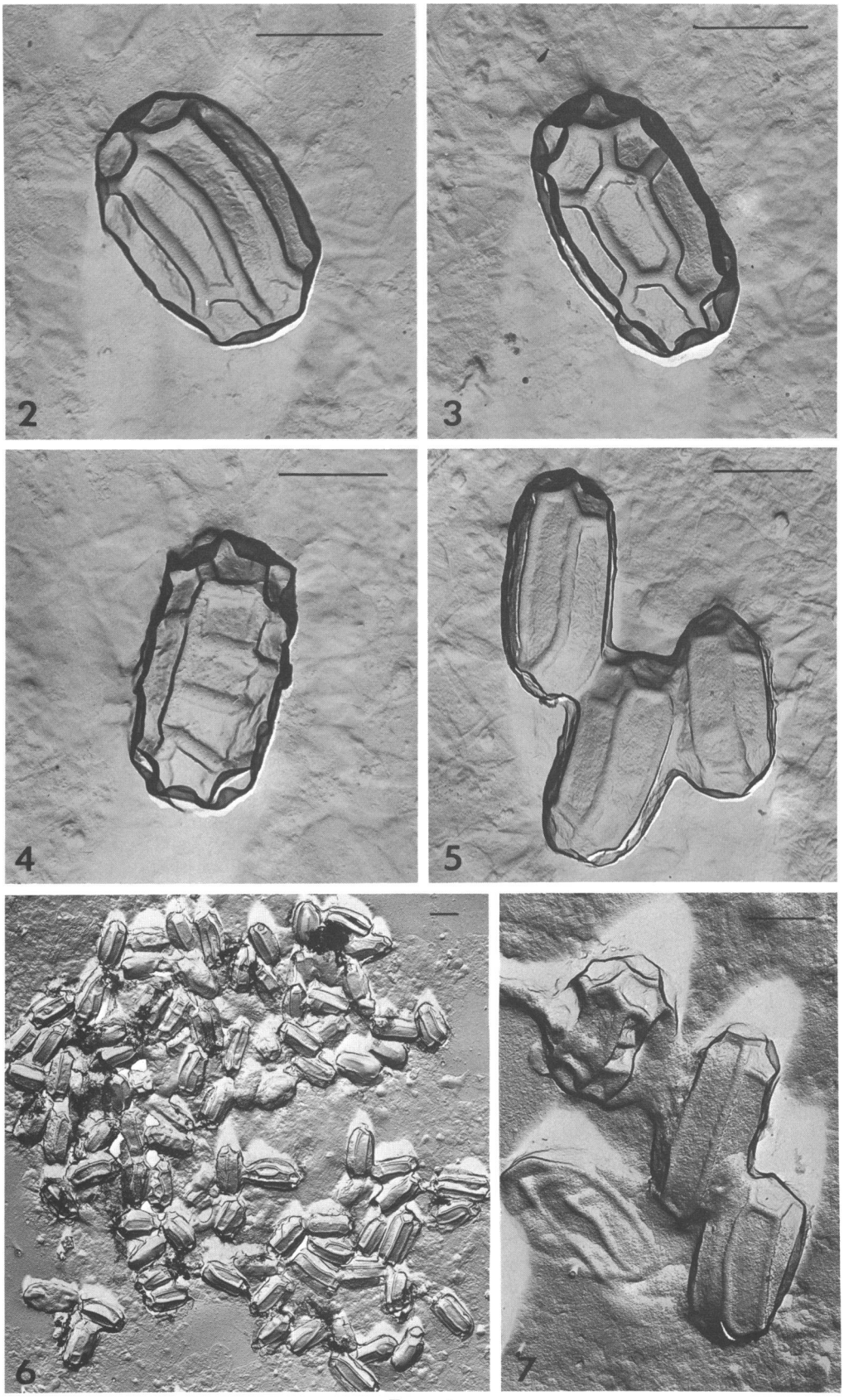


FIG. 2–7

Size measurements made from cross sections and longitudinal sections resulted in an average size of 2.0 by 1.1 μm, with a length to width ratio of 1.8:1. These values are only approximate since slightly oblique sections or cross sections taken from the extreme end of the spore would give distorted results. Measurements were taken only to give a rough comparison of spore sizes obtained from the three methods employed and to calculate a general average.

Although details of the internal structure of the spore can be obtained from thin sections, other techniques must be used to obtain a three-dimensional view of the spore and its surface features.

Replicas. The carbon replicas in Fig. 2 to 7 are similar to those obtained by van den Hooff and Aninga (19) and Bradley and Franklin (2). Bradley and Franklin (2) found two strain differences in the surface structure among the *B. polymyxa* strains they studied, one in which the ribs formed a reticulate structure at the ends of the spores and the other in which the ribs appeared in the form of loops. The strain used in this study is structurally similar to the reticulate type. In our study, the reticulation was found randomly over the surface of the spores (Fig. 3, 4, 6, 7) as well as at the ends of the spores (Fig. 2, 5, 6, 7).

Size measurements made from photographic negatives of the replicas resulted in an average size of 2.4 by 1.3 μm, giving a length to width ratio of 1.8:1.

Similar results were obtained with both replication methods employed (Fig. 2 to 5 and 6, 7), although greater difficulty was encountered in obtaining clean preparations (Fig. 6, 7) when using the method of Bradley and Franklin (2). This is thought to be the result of incomplete removal of the manganese dioxide formed by the decomposition of the chromic and permanganic acid mixture used during the replication process (3). Acid decomposition occurred despite the fact that precautions were taken to prevent it, i.e., the acid mix was prepared just before use and kept well covered when not in use. An attempt was made to remove the manganese dioxide by dipping the grid into concentrated hydrochloric acid after the final wash, before shadowing with palladium-gold (3). This procedure, however, was not entirely effective.

Although sample preparation is cumbersome, an overall view of the spore at high magnification and resolution of surface detail can be obtained by the replica technique. Interpretation of a three-dimensional model is still somewhat uncertain, especially with respect to end-on views of the spore, as this orientation is seldom found in replicas.

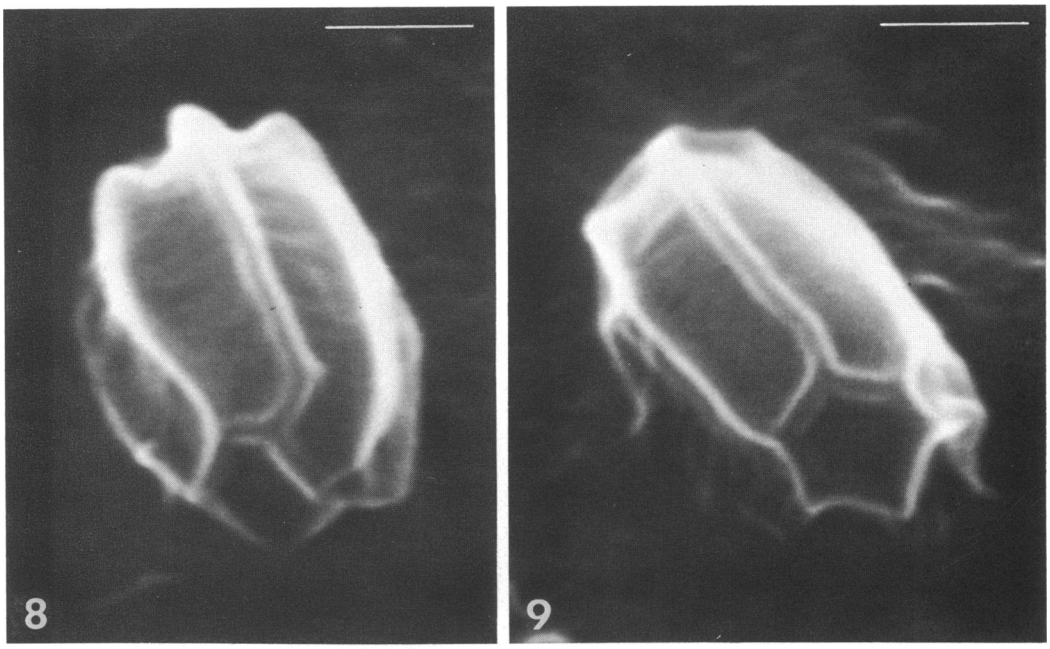
Scanning microscopy. The stellate profile was first reported by A. Meyer in 1897 (14) in his remarkable drawing of a spore cross section. Later the ridged surface was confirmed by van den Hooff and Aninga (19) in sections and by Bradley and Williams (3) in carbon replicas. The ribbed surface structure can now be directly examined by use of the scanning electron microscope. This microscope has a greater depth of focus than the transmission type electron microscope while using a much smaller mean electron-beam current density. The lower specimen current (10^{-12} amp as compared to 10^{-7} amp) used decreases the chance of damage to biological material which can be caused by excessive beam current.

The scanning electron microscope used in this study has a magnification range from 20 to 100,000, but for bacterial spores the most useful range is from 1,000 to 50,000. At the lower magnifications, quantitative estimates of spore populations can be made, whereas detailed surface structure can be seen at higher magnifications ($\geq$10,000).

Typical scanning micrographs of *B. polymyxa* spores are shown in Fig. 8 to 12. By comparing these micrographs with those of spores prepared by the replica methods (Fig. 2 to 7), it can be seen that a more readily interpreted three-dimensional image is obtained by scanning microscopy.

The characteristic ridges are prominently illustrated in Fig. 8 and 9, in which the ribs are seen to run parallel to the long axis of the spore, coalescing at its ends into a polygonal porelike structure. Some random ribbing, such as that found in replicas (Fig. 3 and 4), is also apparent in scanning preparations (Fig. 10 to 12). It would be of interest to study a germinating culture of *B. polymyxa* spores to determine whether the porelike structure present in Fig. 8 and 9 is involved in the germination process. This study would be facilitated by scanning microscopy as end-on views of spores are readily obtained (Fig. 8, 9, and 11). The possibility of such a germination pore is currently being investigated in this laboratory.

The general average size calculated from all measurements made from the various methods employed in this study results in a value of 2.2 by 1.2 μm and gives some idea of actual spore size. The size measurements differ because of the inherent inaccuracies present in the individual methods (1, 10). The length to width ratio is the same with all three methods (1.8:1) and probably reflects the approximate dimension ratio of the actual spore, as method errors would tend to cancel out assuming measurement error to be consistent in both directions.



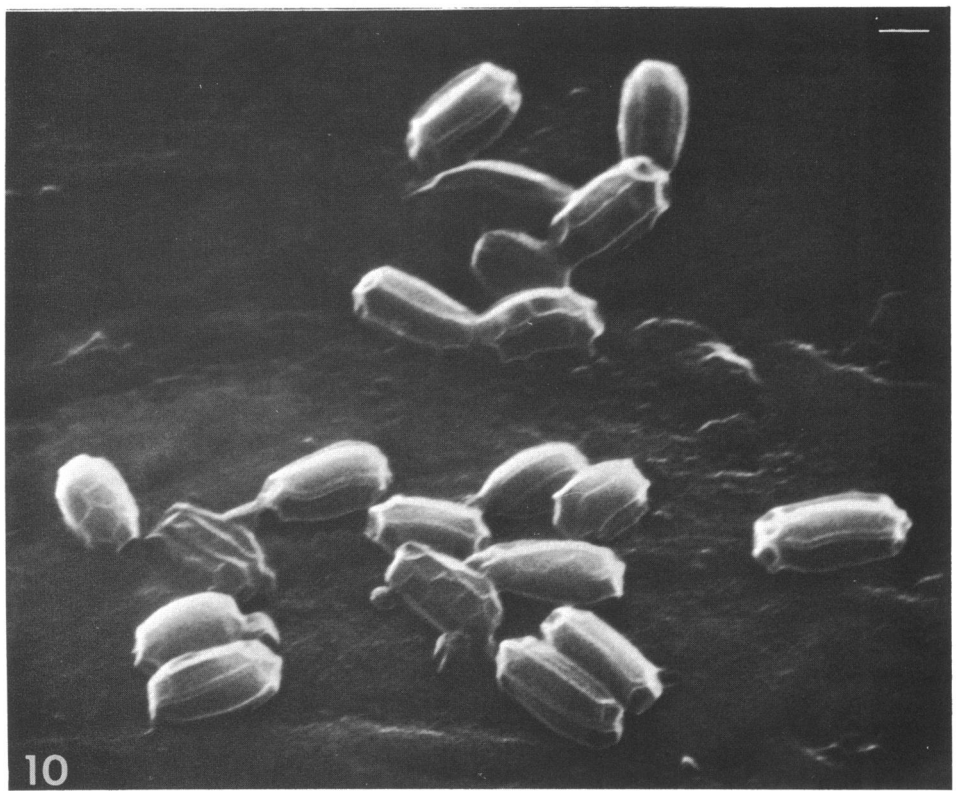


FIG. 8 and 9. *B. polymyxa spores examined in a scanning electron microscope. Parallel ribbing of the spore surface coalescing into a polygonal porelike structure is well defined in these two figures. Compare these end-on views with replica views in Fig. 2 to 4. Note the depth of focus made possible with the scanning microscope. Markers represent 0.5 μm. Fig. 8, × 38,800. Fig. 9, × 38,800.*

FIG. 10. *B. polymyxa spores examined in a scanning electron microscope. Both parallel ribbing and random reticulation of the surface structure are illustrated in scanning preparations, as was also found in replicas. Marker represents 0.5 μm. × 9,600.*

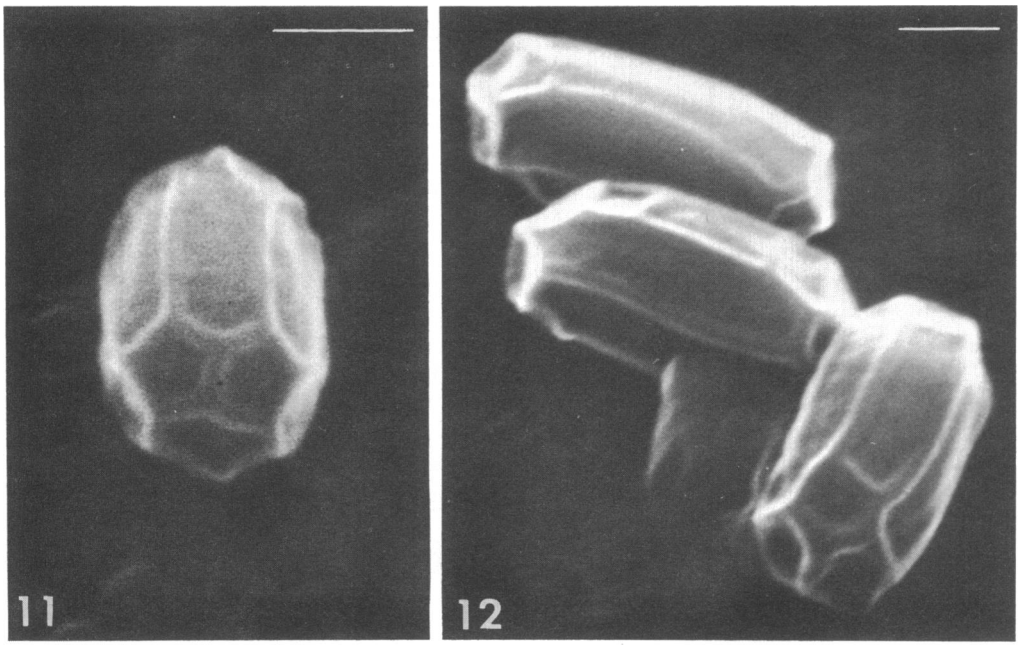


FIG. 11 and 12. *B. polymyxa spores examined in a scanning electron microscope. End-on views showing reticulation at the end of the spore. Markers represent 0.5 μm. Fig. 11, × 35,500. Fig. 12, × 25,600.*

DISCUSSION

With the scanning electron microscope, surface views of spores at high magnifications can be obtained. The alternative method for obtaining surface views of these electron-dense structures at high magnification is by examination of carbon replicas with the transmission electron microscope. Although the resolving power of such microscopes is greater than that of the scanning microscope (less than 1 nm as opposed to 15 nm), the preparation of carbon replicas requires much time and effort and does not provide the depth of focus seen in scanning micrographs.

Several applications of the scanning electron microscope to the study of spores can be suggested from this preliminary work. Because sample preparation is so simple and rapid, routine examination of spore surface structure for taxonomic purposes could be easily carried out. The scanning microscope also would be a useful tool for studying the germination process, especially when used to augment information obtained from observation of thin sections in the transmission electron microscope. Other possibilities include quantitative estimates of spore populations, the study of the effects of various physical and chemical agents on gross spore morphology, and the study of crystals associated with spore formation in certain bacterial species.

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