Applied and Environmental Microbiology

Scanning Electron and Phase-Contrast Microscopy of Bacterial Spores

L. A. Bulla, G. St. Julian, R. A. Rhodes and C. W. Hesseltine
***Appl. Microbiol.* 1969, 18(3):490.**

Updated information and services can be found at:
http://aem.asm.org/content/18/3/490

These include:

CONTENT ALERTS

Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), more»

Information about commercial reprint orders: **http://journals.asm.org/site/misc/reprints.xhtml**
To subscribe to to another ASM Journal go to: **http://journals.asm.org/site/subscriptions/**

Journals.ASM.org

Downloaded from http://aem.asm.org/ on June 4, 2013 by guest

APPLIED MICROBIOLOGY, Sept. 1969, p. 490–495
Copyright © 1969 American Society for Microbiology
Vol. 18, No. 3
Printed in U.S.A.

Scanning Electron and Phase-Contrast Microscopy of Bacterial Spores

L. A. BULLA, G. ST. JULIAN, R. A. RHODES, AND C. W. HESSELTINE
Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Illinois 61604

Received for publication 11 June 1969

The three-dimensional images of free and intrasporangial spores produced by scanning electron microscopy show surface structures not visible by phase-contrast microscopy. Although fine surface detail is not elucidated by scanning electron microscopy, this technique does afford a definitive picture of the general shape of spores. Spores of *Bacillus popilliae*, *B. lentimorbus*, *B. thuringiensis*, *B. alvei*, *B. cereus*, and *Sarcina ureae* have varying patterns of surface ridge formation, whereas spores of *B. larvae*, *B. subtilis*, and *B. licheniformis* have relatively smooth surfaces.

Phase-contrast and transmission electron microscopy have been widely used to investigate the morphology of bacterial spores (14, 17, 19). The technique of carbon replication has shown that the surface of bacterial spores varies from smooth to ridged or dimpled (6). Freeze-etching has revealed that the fine structure of spore surfaces differs among species (11, 15). Other methods of microscopic examination have proven to be inadequate for detailed study of the spore surface.

Wall ornamentation and surface features of fungal spores have been studied with the scanning electron microscope (4, 8, 9, 12). To the best of our knowledge, only one comparable study has been published on bacterial spores (13). In this investigation, we examined spores of certain *Bacillus* species, as well as *Sarcina ureae*, by scanning electron and phase-contrast microscopy. The techniques developed and adapted for the preparation and examination of spores by scanning electron microscopy are also presented.

MATERIALS AND METHODS

Organisms. Nine strains of aerobic sporeforming bacteria from the Agricultural Research Service Culture Collection were selected: *B. popilliae* NRRL B-2309, *B. lentimorbus* NRRL B-2522, *B. thuringiensis* NRRL NRS-996, *B. larvae* NRRL B-3555, *B. alvei* NRRL B-384, *B. cereus* NRRL B-569, *B. subtilis* NRRL B-543, *B. licheniformis* NRRL NRS-1264, and *S. ureae* NRRL B-286. The first four organisms listed are insect pathogens and are discussed by Heimpel and Angus (10), Angus (1), and Rhodes (16). *B. popilliae*, *B. lentimorbus*, *B. alvei*, and *B. larvae* have swollen sporangia; the others do not. *S. ureae* is a sphere containing a centrally located endospore (*Bergey's Manual*, 7th ed.).

Medium and cultural conditions. Actively growing cultures of all organisms were maintained in a liquid medium composed of 1.5% yeast extract, 0.6% K_2HPO_4, and 0.2% glucose. The medium, except for glucose, was dispensed into 300-ml Erlenmeyer flasks in 50-ml amounts and was sterilized for 15 min at 121 C. A 50% (w/v) glucose solution sterilized separately by membrane filtration was added aseptically before inoculation to give a final concentration of 0.2%. The *p*H of the medium was 7.4.

A 1% inoculum was used. Flask cultures were aerated by rotary agitation at 250 rev/min at 28 C in an incubator shaker.

Definition and production of spores. Spores contained within sporangia are referred to as "intact" spores; those released from sporangia are referred to as "free" spores.

B. popilliae and *B. lentimorbus* do not sporulate appreciably in liquid medium. Intact spores of these species were obtained from the hemolymph of diseased Japanese beetle larvae, *Popillia japonica* (18). The spore-laden hemolymph, which had been dried in thin films on glass slides, was reconstituted in sterile water. Spore formation was initiated within 48 hr by all other species, and maximal sporulation (90 to 100% spores) was achieved within 3 to 7 days. All spores were harvested by centrifugation, washed five times, and finally resuspended in sterilized distilled water.

Preparation of free spores. Washed intact spores were centrifuged and resuspended in sterilized distilled water at a concentration of 4×10^{10} spores per ml. Of this suspension, 3 ml was placed in thick-walled test tubes and sonically vibrated (Sonifier, model S110, Branson Instruments, Inc.) while the tubes were partially immersed in an ice bath. Spores were sonically treated until more than 95% were freed of sporangia. The percentage of free spores was determined periodically during sonic treatment by direct microscopic counts made with a Petroff-Hausser bacteria counter and a Zeiss phase-contrast microscope. Each spore concentrate required a specific time of sonic treatment to remove the sporangium. *B. thurin-*

Downloaded from http://aem.asm.org/ on June 4, 2013 by guest

giensis, *B. lentimorbus*, and *B. popilliae* were sonically treated for 30 to 60 min; all others were sonically treated for 5 to 15 min.

After sonic treatment, spore suspensions were allowed to stand for 20 min. Most of the sporangial debris settled to the bottom during this time, whereas free spores remained in suspension. Free spores were removed with a Pasteur pipette and were washed with distilled water three times by centrifugation at 1,800 × *g*; the spores then were resuspended in distilled water at a concentration of 5 × 10^9 spores per ml. A 1-ml amount of this suspension was combined with 3 ml of 1% sodium dodecyl sulfate, and the mixture was incubated at 37 C with constant shaking for 10 to 15 min. Spores then were removed by centrifugation and washed in 4 ml of 1 M NaCl by shaking with a Vortex tube mixer for 2 to 3 min. The spores were washed in this way three times with NaCl and then five times with sterile distilled water. Intact spores were washed in the same manner as free spores.

Both intact and free spores were centrifuged from the distilled water suspensions into pellets containing 2 × 10^9 spores. The supernatant fluid was decanted, and the spore pellet was stored at −10 C until needed. Pellets were thawed and diluted in distilled water to 10^6 spores per ml for microscopic examination.

Microscopy. For phase-contrast microscopy, mounting slides were prepared by spreading a thin film of 1% Noble agar evenly over the surface of glass microscope slides. Exactly 0.05 ml of a spore suspension was placed on the solidified agar surface and covered with a cover slip. Spores were photographed on Panatomic-X film through Neofluar phase optics of a Zeiss WL microscope.

Two mounting procedures were used for scanning electron microscopy. In the first, four rectangular strips (3 by 5 mm) of transparent double-coated Scotch tape were placed radially on special aluminum mounting stages (Fig. 1b). In the second procedure, squares (10 by 10 mm) cut from glass microscope slides were placed on mounting stages (Fig. 1c). In both procedures, 0.05 ml of diluted spore suspension was spread over the mounting surface and allowed to dry. Du Pont conductive silver paste was used to anchor specimens to the stage.

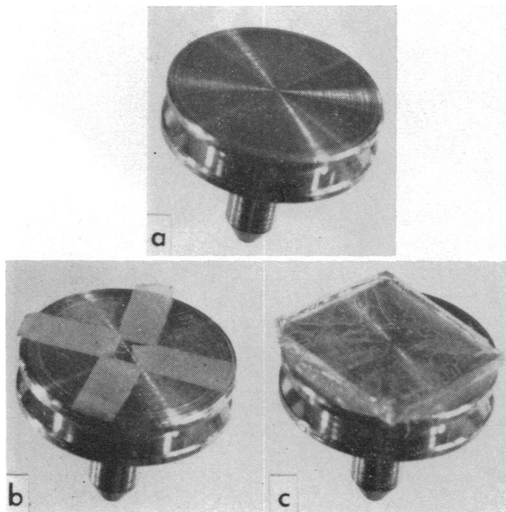

FIG. 1. *Aluminum specimen stages for scanning electron microscopy:* (*a*) *stage alone,* (*b*) *stage mounted with four strips of transparent double-coated tape,* (*c*) *stage mounted with glass square.*

Nonconductive materials such as bacterial spores must be coated with a uniform layer of metal to prevent buildup of a negative charge on their surfaces by primary electrons during scanning. Mounted spores were coated with aluminum to a thickness of 15 nm in a vacuum evaporator (model CVE-14, Consolidated Vacuum Corp., Rochester, N.Y.) while being rotated at 10 rev/min on a rotary shadower (Ladd Research Industries, Inc., Burlington, Vt.). To ensure uniform coating, metal sources were placed in two platinum wire baskets, one positioned at a 30° angle to the center of the rotary plate and the other positioned directly above the plate. Both sources were located 7.6 cm from mounted specimens.

Mounting stages were then placed in a Stereoscan scanning electron microscope (series I, Cambridge Instruments Co., Ltd., London, England) and were adjusted so that the electron beam would strike the face of the stage at a 45° angle. The spores were examined and photographed at an accelerating voltage of 20 kv.

RESULTS

Scanning electron and phase-contrast microscopy. Figures 2 through 4 are scanning electron micrographs and phase-contrast photomicrographs of each spore examined.

Figure 2 shows intact and free spores of *B. popilliae* and a free spore of *B. lentimorbus*. The sporangium of a sporulated cell of *B. popilliae* contains a parasporal body as well as a spore (Fig. 2a). As can be seen by both electron and phase microscopy, the spore is much larger than the parasporal body. The electron micrograph shows the spore and parasporal body held together snugly in a close-fitting, smoothly textured sporangium. The detailed cytology of *B. popilliae* described by transmission electron microscopy does not conclusively establish whether a common membrane or exosporium ensheaths both (2, 3). The scanning electron micrographs of *B. popilliae* spores released from their sporangium (Fig. 2b, c) show their morphology clearly. Distinct ridges (approximately 170 nm in thickness), which are continuous with each other, extend from one end of the spore to the other (note "tuning fork" pattern in Fig. 2b). The surface of the spore coat appears roughly textured. Black (2) described the sloughing of thin layers from the spore coat of *B. popilliae*. The free spore viewed through phase-contrast optics (Fig. 2c, insert) appears relatively

Downloaded from http://aem.asm.org/ on June 4, 2013 by guest

smooth and ellipsoidal in shape. No surface structures are evident.

The free spores of *B. lentimorbus* (Fig. 2d) possess definite ridges. They are highly pronounced (approximately 170 nm in thickness) as in *B. popilliae* and extend from one end of the spore to the other; the longitudinal ridges are interconnected by short ridges perpendicular to them. The surface of the spore coat appears roughly textured. Surface features of intrasporangial spores of *B. lentimorbus* are largely masked by their sporangia (not shown).

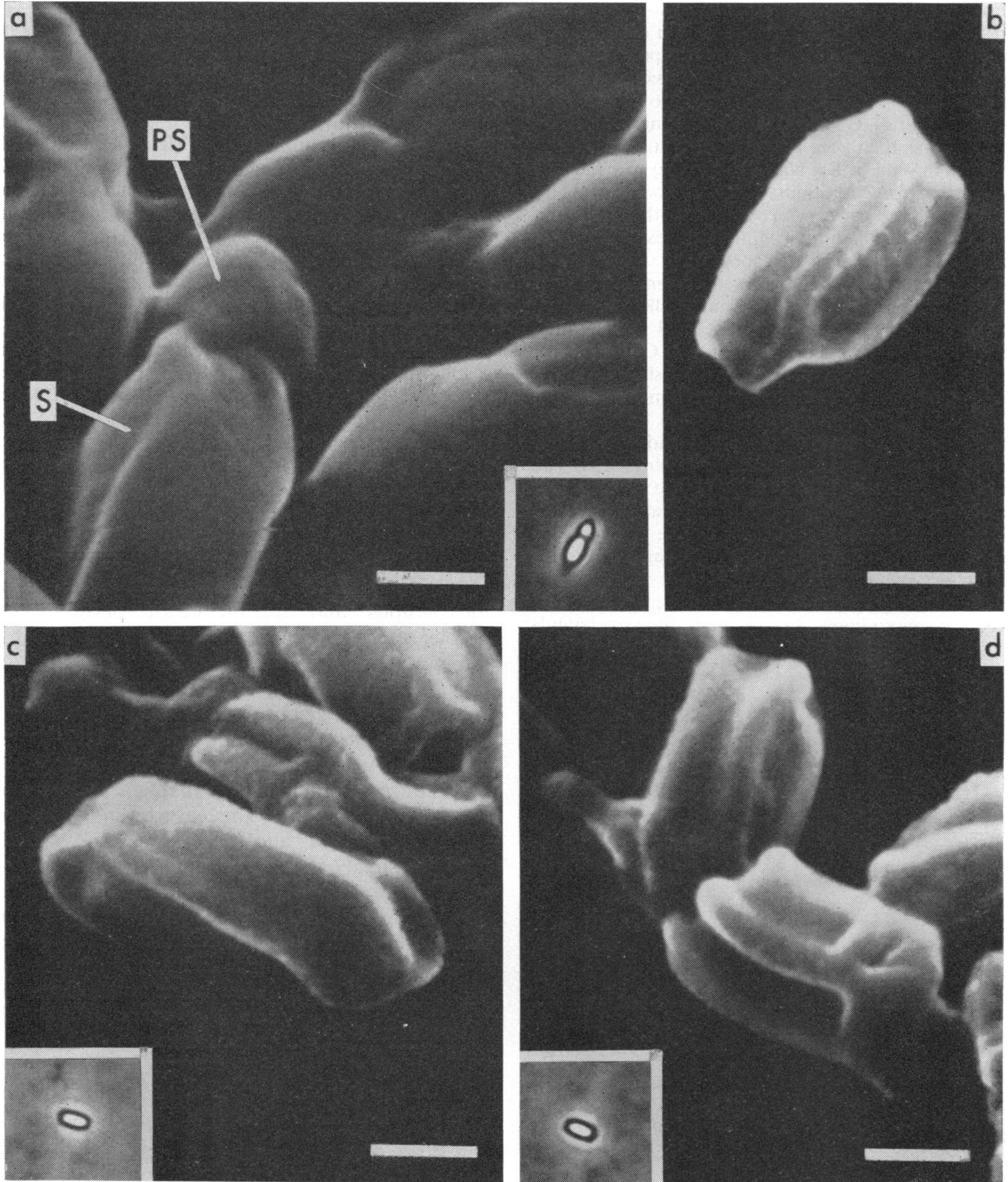

FIG. 2. *Spores of B. popilliae (a–c) and B. lentimorbus (d). Scanning electron micrographs: (a) spore (S) and parasporal body (PS) contained in sporangium; (b–d) free spores. ×30,000. Corresponding phase micrographs inserted. ×1,250. Markers represent 0.5 μm.*

Downloaded from http://aem.asm.org/ on June 4, 2013 by guest

Figure 3 includes scanning electron and phase-contrast micrographs of intact and free spores of *B. thuringiensis* and *B. alvei*. The spore and parasporal body of *B. thuringiensis* are nearly equal in size and lie loosely within the sporangium (Fig. 3a). The sporangium appears as a transparent sheath draped and folded over the spore and parasporal body to form a loose envelope. Hannay (7) reported that the parasporal body is not surrounded by a membrane. The free spore of *B. thuringiensis* (Fig. 3b) has an irregular pattern of surface ridges. These ridges are also

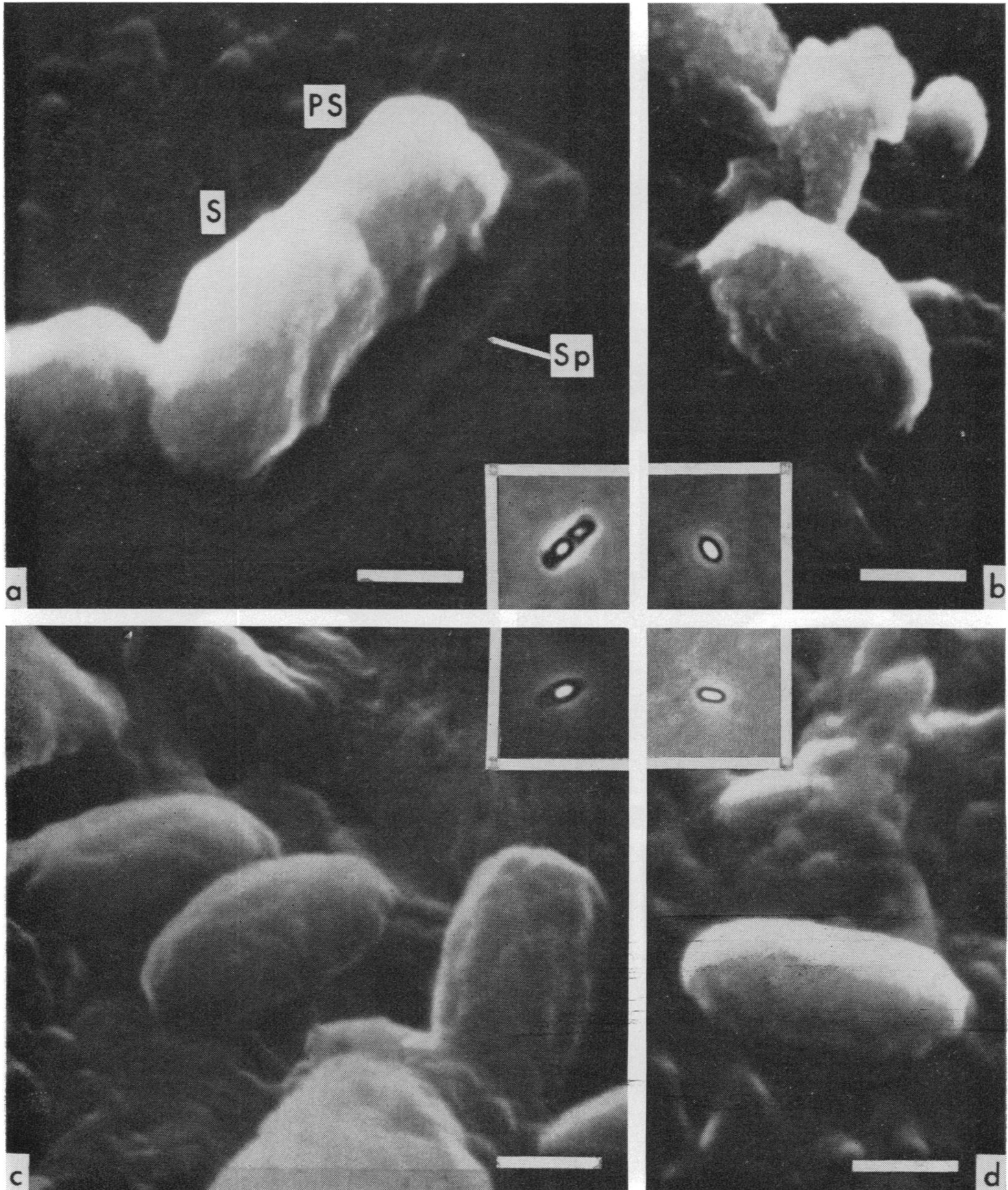

FIG. 3. *Spores of B. thuringiensis (a, b) and B. alvei (c, d). Scanning electron micrographs: (a) spore (S) and parasporal body (PS) contained in sporangium (Sp); (c) intact spore; (b and d) free spores. ×30,000. Corresponding phase micrographs inserted. ×1,250. Markers represent 0.5 μm.*

Downloaded from http://aem.asm.org/ on June 4, 2013 by guest

visible through the sporangium of the intact spore (Fig. 3a). Again, surface morphology cannot be seen by phase-contrast optics (Fig. 3a, b, inserts). The sporangium of *B. alvei* covers the spore in such a way that it is somewhat difficult to distinguish intact from free spores by scanning electron microscopy (Fig. 3c, d). However, through phase-contrast optics, the sporangium can be seen surrounding the spore (Fig. 3c, insert). As expected, it cannot be seen after sonic treatment (Fig. 3d, insert). The surface of both the intact and free spores appears dimpled.

No differences are apparent between intact and free spores of *B. licheniformis* (Fig. 4a, b); however, there may be folding of the sporangium near the ends of the intact spore (Fig. 4a). Sur-

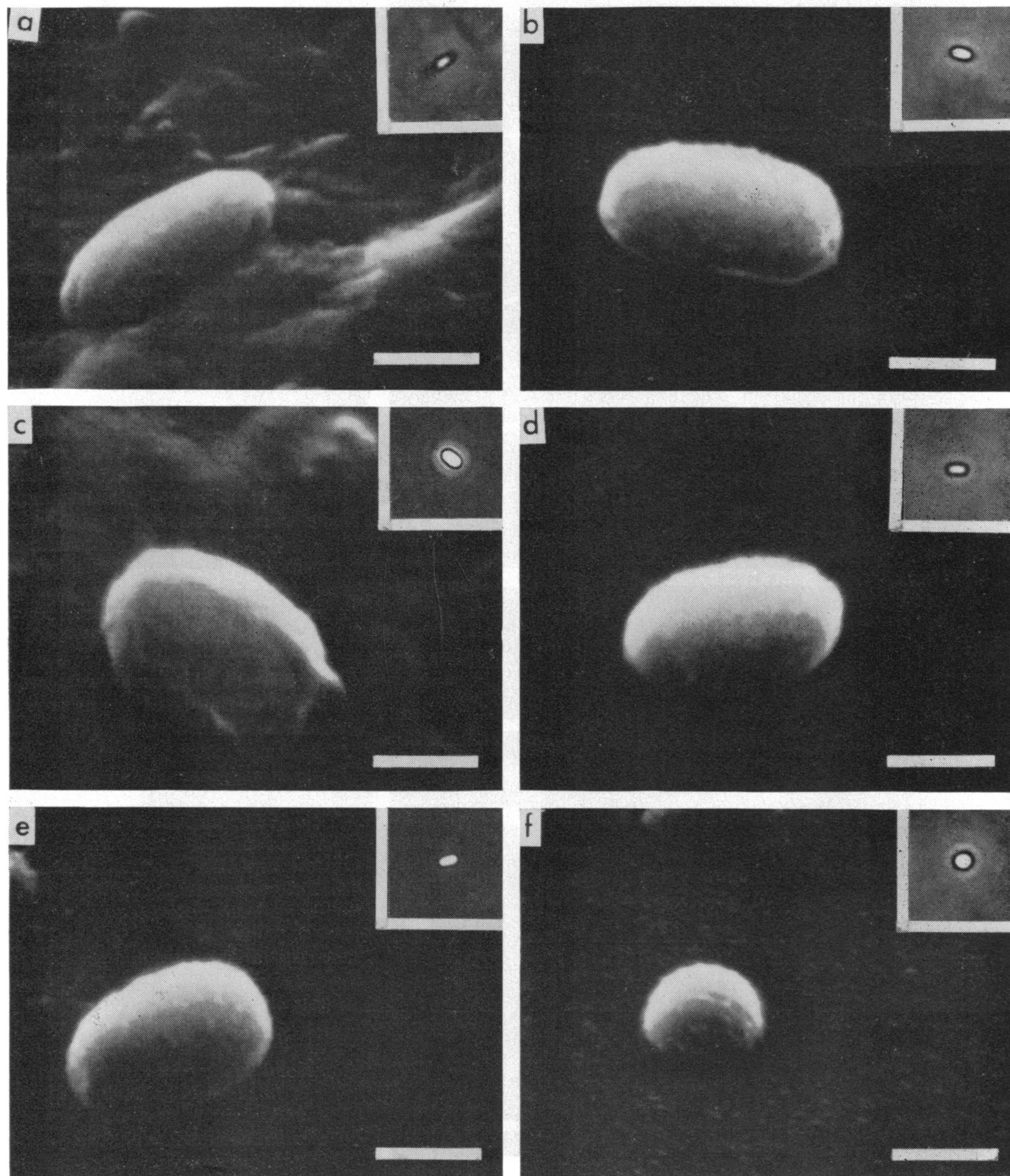

FIG. 4. *Scanning electron micrographs of an intact spore (a) of B. licheniformis and of free spores; (b) B. licheniformis; (c) B. cereus; (d) B. subtilis; (e) B. larvae; and (f) Sarcina ureae.* ×*30,000. Corresponding phase micrographs inserted.* ×*1,250. Markers represent 0.5 μm.*

Downloaded from http://aem.asm.org/ on June 4, 2013 by guest

face irregularities are absent. Phase-contrast photomicroscopy of an intact spore of *B. licheniformis* (Fig. 4a, insert) discloses the sporangium as filmy material loosely enveloping the spore. This material was not observed in preparations of sonically treated spores (Fig. 4b, insert).

Free spores of *B. cereus*, *B. subtilis*, *B. larvae*, and *S. ureae* also are shown in Fig. 4. The surface of a free spore of *B. cereus* (Fig. 4c) possesses slightly elevated ridges, which appear to fuse at the ends of the spore. Free spores of *B. subtilis* (Fig. 4d) and *B. larvae* (Fig. 4e) have no surface irregularities. The free spore of *S. ureae* (Fig. 4f) has continuous parallel ridges. Small particles on the surface of these spores and in the background are probably grains of evaporated aluminum because such particles do not appear in scanning electron micrographs of uncoated spores.

Mounting spores for electron scanning. The rough, uneven background for some of the spores (Fig. 3b–d; 4a) was created by the double-coated Scotch tape, on which they were mounted, becoming folded and gnarled during microscopy. At times, this folding partially enveloped the spores. In contrast, spores mounted on glass squares have a smooth, even background (Fig. 2; 3a; 4b, d-f). Therefore, it is recommended that glass mounts be used for scanning electron microscopy of bacterial spores.

DISCUSSION

Transmission electron microscopy has shown that the coat of mature spores of *B. popilliae*, *B. lentimorbus*, and *B. cereus* is ridged (2, 3, 5). However, the overall arrangement of these ridges is not clear. Franklin and Bradley (6) studied spore surfaces of *B. alvei*, *B. subtilis*, and *B. licheniformis* by carbon replication. The spores of *B. alvei* and *B. subtilis* were also described as ridged, and those of *B. licheniformis* were described as having a deep groove caused by thinning of the spore coat.

In this study, spores of *B. popilliae*, *B. lentimorbus*, *B. thuringiensis*, *B. alvei*, *B. cereus*, and *S. ureae* were found to possess surface ridges with various patterns of reticulation. Conversely, *B. licheniformis*, *B. subtilis*, and *B. larvae* spores are relatively smooth. Spores of *B. popilliae* and *B. lentimorbus* are similar to each other but unlike those of any other organism studied.

Resolution with the instrument used in this investigation is 15 to 30 nm, much inferior to that of transmission electron microscopy. Consequently, no fine surface detail was elucidated like that with direct carbon replication and freeze-etching. At its present stage of development, the scanning electron microscope alone cannot be used to study fine structure and surface detail of bacteria, as has been done with procaryotic organisms. However, when used to its full advantage it is a valuable adjunct to the already existing techniques of surface observation.

ACKNOWLEDGMENT

We thank J. A. Brown of Alpha Research and Development, Inc., Blue Island, Ill., for his kind cooperation and skillful assistance in obtaining scanning electron micrographs and G. L. Adams of our laboratory for technical assistance.

LITERATURE CITED

1. Angus, T. A. 1965. Symposium on microbial insecticides. I. Bacterial pathogens of insects as microbial insecticides. Bacteriol. Rev. 29:364–372.
2. Black, S. H. 1968. Cytology of milky disease bacteria. I. Morphogenesis of *Bacillus popilliae* in vivo. J. Invertebr. Pathol. 12:148–157.
3. Black, S. H. 1968. Cytology of milky disease bacteria. II. Morphogenesis of *Bacillus popilliae* in vitro. J. Invertebr. Pathol. 12:158–167.
4. Campbell, R., R. W. Lainer, and M. F. Madelin. 1968. Notes on an albino mutant of *Alternaria brassicicola*. Mycologia 60:1122–1125.
5. Ellar, D. J., and D. G. Lundgren. 1966. Fine structure of sporulation in *Bacillus cereus* grown in a chemically defined medium. J. Bacteriol. 92:1748–1764.
6. Franklin, J. G., and D. E. Bradley. 1957. A further study of the spores of species of the genus *Bacillus* in the electron microscope using carbon replicas and some observations on *Clostridium welchii*. J. Appl. Bacteriol. 20:467–472.
7. Hannay, C. L. 1956. Inclusions in bacteria, p. 318–340. *In* Bacterial anatomy. Symp. Soc. Gen. Microbiol., 6th. Cambridge University Press, London.
8. Hawker, L. E. 1968. Wall ornamentation of ascospores of species of *Elaphomyces* as shown by the scanning electron microscope. Trans. Brit. Mycol. Soc. 51:493–498.
9. Hawker, L. E., and M. A. Gooday. 1968. Development of the zygospore wall in *Rhizopus sexualis* (Smith) Callen. J. Gen. Microbiol. 54:13–20.
10. Heimpel, A. M., and T. A. Angus. 1960. Bacterial insecticides. Bacteriol. Rev. 24:266–288.
11. Hoeniger, J. F. M., P. F. Stuart, and S. C. Holt. 1968. Cytology of spore formation in *Clostridium perfringens*. J. Bacteriol. 96:1818–1834.
12. Jones, D. 1968. Surface features of fungal spores as revealed in a scanning electron microscope. Trans. Brit. Mycol. Soc. 51:608–610.
13. Murphy, J. A., and L. L. Campbell. 1969. Surface features of *Bacillus polymyxa* spores as revealed by scanning electron microscopy. J. Bacteriol. 98:737–743.
14. Murrell, W. G. 1967. The biochemistry of the bacterial endospore, p. 133–251. *In* A. H. Rose and J. F. Wilkinson (ed.), Advances in microbiological physiology, vol. 1. Academic Press Inc., New York.
15. Remsen, C. C. 1966. The fine structure of frozen-etched *Bacillus cereus* spores. Arch. Mikrobiol. 54:266–275.
16. Rhodes, R. A. 1965. Symposium on microbial insecticides. II. Milky disease of the Japanese beetle. Bacteriol. Rev. 29:373–381.
17. Robinow, C. F. 1960. Morphology of bacterial spores, their development and germination, p. 207–248. *In* A. C. Gunsalus and R. Y. Stainer (ed.), The bacteria, vol. 1. Academic Press Inc., New York.
18. St. Julian, G., T. G. Pridham, and H. H. Hall. 1967. Preparation and characterization of intact and free spores of *Bacillus popilliae* Dutky. Can. J. Microbiol. 13:279–285.
19. Sussman, A. S., and H. O. Halvorson. 1966. The structure and formation of dormant cells, p. 7–44. *In* Spores: their dormancy and germination. Harper and Row, New York.

Downloaded from http://aem.asm.org/ on June 4, 2013 by guest