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An Electron Microscope Study of the Spores of some Species of the Genus *Bacillus* using Carbon Replicas

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SUMMARY: A method for the preparation of carbon replicas of *Bacillus* spores is described. Micrographs of these replicas reveal much more of the surface detail than direct electron micrographs. Rib formations were found on five different species and the micrographs obtained suggest that the sculpturing varies according to the species.

Hitherto, little work has been carried out on the study of spore surfaces, partly because of lack of suitable techniques, and partly because the likelihood of any surface structure being present seemed remote. The electron microscope provides the necessary resolution required to reveal submicro-structure, and spores can be examined by thin sectioning, by replica techniques, or by direct examination. Thin sections of *Bacillus megaterium* and some other spores (Robinow, 1953; Van den Hoof & Aninga, 1956) show what may be seen by this method. The internal structure of the spore is clearly shown, but there is no indication of any surface detail, and the technique seems unsuitable. Because of the high density of the spores to electrons, direct examination (Knaysi, Baker & Hillier, 1947; and see Pl. 4, fig. 11) provides only an outline of the spore and little information about the nature of the surface. The most obvious approach is the use of a replica method, but few of the existing techniques can be used for the examination of biological materials. Nevertheless, a variation of the carbon replica technique (Bradley, 1954) was successfully used in a study of yeast bud scars (Bradley, 1956), and a modification of this method has provided a simple means of studying spore surfaces. The results are both striking and unexpected. Instead of the smooth surface which was expected, deep sculpturing was found. Moreover, this sculpturing differs widely from species to species.

METHODS

Preparation of spore suspension

A medium of the following composition was used: Peptone (Oxoid), 6 g.; Bacto tryptone, 3 g.; Yeastrel, 3 g.; Lab. Lemco (Oxoid), 1.5 g.; agar, 25 g.; Mn^{++} , 1 p./m. (as sulphate or chloride) in 1 l. distilled water; pH 7.0; sterilized at 121° for 20 min.

Nutrient agar slope cultures of the organism (24 hr. at 37°) were emulsified with sterile distilled water and approximately 1 ml. inoculated into Roux

bottles (1 l.) containing 140 ml. of the above medium. After incubation for 1 week at 37°, spores were harvested in distilled water and shaken for 1 hr. in 4 oz. screw-cap bottles containing glass beads. Suspensions were washed by centrifuging four times with distilled water, heated in a water bath at 80° for 20 min. to kill vegetative organisms, and finally stored in a refrigerator (c. 5°) until required. In some cases this method of preparation did not provide suspensions free from vegetative organisms. This was particularly so with *Bacillus sphaericus*.

Organisms used

Bacillus subtilis 4, raw tanker milk;* 18, NCIB 8057; 41, Knight & Proom (1950), CN 2745; 22, raw tanker milk.*

B. licheniformis 92, NIRD stock culture; 93, NCIB 6816; 94, NCIB 7224.

B. circulans bulk tanker milk.*

B. polymyxa bulk tanker milk.*

B. brevis bulk tanker milk.*

B. sphaericus from J. Wolf, University of Leeds.

The replica technique

The replica technique is very simple. The spores are coated with a thin film of evaporated carbon, and then dissolved, leaving a thin carbon shell which conforms exactly to the original surface (see fig. 1). An electron microscope specimen support grid is first slightly bent, mounted on a $\frac{1}{8}$ in. diameter metal peg and coated with a thick Formvar film. One drop of an aqueous suspension of unfixed spores is then allowed to dry on the film (Fig. 1a). The grid, still on

Fig. 1. Diagrammatic representation of the method used for making replicas of spores.

the peg, is next transferred to a vacuum plant where a film of carbon about 100Å. thick is deposited on it (Fig. 1b). This is done by passing about 40 A. a.c. through the points of two carbon rods lightly pressed together. The intense local heating which occurs at the points causes the carbon to evaporate, and the required deposit is obtained in a few seconds. This carbon film constitutes

* Franklin, Williams & Clegg, 1956.

the final replica. The grid and the peg are removed from the plant and the Formvar substrate is washed away by allowing about 1 ml. of chloroform to flow over the grid from a burette with the nozzle held about 2 mm. above the grid (Fig. 1c.). The spores can now be dissolved away from the carbon film. This is achieved by placing the grid in an acid mixture which contains 1.5 g. potassium permanganate, 1.5 g. potassium dichromate and 15 ml. of concentrated sulphuric acid. (This mixture is dangerous and should be treated with great care. It should be kept covered at all times as it deteriorates after a few hours; it should therefore be used freshly prepared.) A few drops of the mixture are placed in a small crucible, and the grid is placed, carbon film upwards, on the surface. The spores dissolve in a few minutes, but the carbon film and the copper grid are not attacked (Fig. 1d.). The grid is removed from the mixture by a pair of forceps and then dipped into water where it is moved about until the acid is washed away. It is then dipped into concentrated hydrochloric acid for a few moments to remove any manganese dioxide formed by the decomposition of the acid mixture. The grid is finally washed in water, dried and shadowed with 60/40 gold-palladium alloy before examination in the electron microscope.

It may be found that the carbon film detaches itself from the grid on immersion in the hydrochloric acid. It will generally remain floating on the surface. A fresh grid or lifter can then be used to transfer it to the water bath, where it is left floating for a short time, before being finally picked up on a grid and dried.

RESULTS

Replicas of thin-walled oval spores of two species, *Bacillus subtilis* and *B. licheniformis*, were examined. The spores of *B. subtilis* were found to be ribbed, the ribbing being in two rather different forms. These are illustrated in Pl. 1, fig. 1, which is of strain 4. In Pl. 1, fig. 1(a), a form of spore is shown in which the ribs run longitudinally down the cell; they appear, however, to be joined at the ends. Another form of spore is shown in Pl. 1, fig. 1(b). Here, the ribs are in the form of a network on the spore surface. In both cases, the average width of the ribs is about 800 Å, and they appear to be raised from the spore surface by about half this amount. The spores of three other strains of *B. subtilis* (strains 18, 41, 22) were also examined to see whether any difference in the ribbing could be detected. These are illustrated in Pl. 1, figs. 2-4, respectively. It is quite clear that the ribbing in strain 18 is much less marked than in the other two strains. In studying the degree of ribbing, it is important that a large number of spores be examined, since individuals are not always representative. The spores shown are typical of each strain examined.

Spores of three strains of *Bacillus licheniformis* exhibited a different structure (Pl. 2, figs. 5, 6; Pl. 3, figs. 7, 8). The characteristic marking in this species is a longitudinal groove in addition to some ribbing. The groove may perhaps mark a line of weakness on the spore as is suggested in Pl. 3, fig. 7, where one of the spores is broken along it. Pl. 3, fig. 8, shows the rib formation of this

strain (93) more clearly. The ribs run lengthwise down the spore and are about 1000 Å. in width, but are not raised as high as those on most of the strains of *B. subtilis*. The spores of the other two strains of *B. licheniformis* (strains 92 and 94) are quite distinct from one another. In the case of strain 92 (Pl. 2, fig. 5) the groove is very shallow, and the ribbing sharply defined, but strain 94 (Pl. 2, fig. 6) has virtually no ribs but a much deeper groove.

The spores of one strain each of *Bacillus brevis*, *B. circulans* and *B. polymyxa* (which form thick-walled oval spores) were examined. Those of *B. brevis* have very little sculpturing on the surface. A single longitudinal rib is shown in several spores in Pl. 3, fig. 9. In some cases it appears to terminate about half way down the spore. Its dimensions are approximately the same as those of the *B. subtilis* spores.

Bacillus circulans spores are covered with a less marked, but rather more complicated, structure. The whole spore has many longitudinal ribs (Pl. 3, fig. 10). The end of one of the spores, visible at the point marked *A* in the figure, is covered with reticulate markings. The width of the ribs is again about 800 Å, although their lateral limits are less clearly defined than in former cases because of the lower angle of slope of the sides.

Bacillus polymyxa spores are perhaps the most interesting of those described here. Pl. 4, fig. 11, gives some idea of the appearance of a direct micrograph of the spores. There appear, in some cases, to be a number of terminal spikes (Van den Hoof & Aninga, 1956). An examination of Pl. 4, figs. 12 and 13, which are replicas, shows, however, that this interpretation is false and that the spikes are in fact pronounced ribs seen in profile. These ribs again run lengthwise down the spore and curve round the ends. They are about 1300 Å. wide and raised about the same amount from the spore surface.

The results obtained with thick-walled round spores (*Bacillus sphaericus*), which did not readily separate from the vegetative cell wall are shown in Pl. 4, fig. 14. The cell wall completely masks any surface structure which may be present.

DISCUSSION

The presence of sculpturing on the surface of bacillus spores was quite unexpected. We were not aware of the observations of Meyer (1897, 1908) and those of Van den Hoof & Aninga (1956) appeared after this work was carried out. It is possible that the differences between the spores of the various species so far examined may be sufficiently well defined to be useful in identification. There is little doubt that a set of cultures of these species, whose individual identities were unknown to the operator, could be correctly classified by studying replicas of the spores in the electron microscope.

The difference between strains of two of the species examined here was not so well marked. The basic form of the sculpturing remains, the chief variation being in the number of ribs present and also in the amount by which they are raised above the spore surface. The structural significance of the rib formations is not yet known. It is possible that thin sections may provide some information by comparing the results with micrographs of replicas (Van den Hoof & Aninga, 1956).

D. E. BRADLEY AND D. J. WILLIAMS—MORPHOLOGY OF BACILLUS SPORES. PLATE 1

(Facing p. 78)

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EXPLANATION OF PLATES

PLATE 1

- Fig. 1. *Bacillus subtilis*, strain 4. $\times 12,000$. (First published in the *Proc. of 1st European Conference on Electron Microscopy, Stockholm, 1956*).
- Fig. 2. *B. subtilis*, strain 18. $\times 7500$.
- Fig. 3. *B. subtilis*, strain 41. $\times 4000$.
- Fig. 4. *B. subtilis*, strain 22. $\times 4000$.

PLATE 2

- Fig. 5. *B. licheniformis*, strain 92. $\times 5250$.
- Fig. 6. *B. licheniformis*, strain 94. $\times 5250$.

PLATE 3

- Fig. 7. *B. licheniformis*, strain 93. $\times 10,000$.
- Fig. 8. *B. licheniformis*, strain 93. $\times 10,000$.
- Fig. 9. *B. brevis*. $\times 9000$. (First published in the *Proc. of 1st European Conference on Electron Microscopy, Stockholm, 1956*).
- Fig. 10. *B. circulans*. $\times 5000$.

PLATE 4

- Fig. 11. *B. polymyxa*, direct electron micrograph of shadowed spores. $\times 5000$.
- Fig. 12. *B. polymyxa*, carbon replica of spores. $\times 5000$.
- Fig. 13. *B. polymyxa*. $\times 5000$ (By permission of Research.)
- Fig. 14. *B. sphaericus*. $\times 5250$.

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