CHARACTERISTIC CELL WALL STRUCTURE
OF A FUSOBACTERIUM

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Electron microscopic observations of the genus Fusobacterium were reported by rather a small number of investigators(1-4) Up to now, there are no referable studies, which were made especially by means of ultrathin sectioning technique, except for the report of Miyazaki(5) However, detailed fine structures could not be examined in his work because of the indistinctness of his electron micrographs. The present authors examined the fine structure of a strain of Fusobacterium polymorphum employing both ultrathin sectioning and carbon replica techniques which enabled the observation of characteristic cell wall structure.

MATERIALS AND METHODS

Strain used: Fusobacterium polymorphum, strain F 409, which was supplied by Dr. Onishi, Department of Oral Hygiene, Tokyo Medical and Dental University, Tokyo, Japan, was used throughout this study. The strain was maintained on Miyazaki's No. 11a media(5) (peptone 1%, meat extract 1%, yeast extract 1%, glucose 0.5%, sodium thioglycollate 0.05%, agar 0.1%, NaCl 0.25%, pH 7.4). The organism subcultured on media 11a was transferred to Miyazaki's No. 11c media (same composition as No. 11a except that no agar was added), and employed for experiments after incubation at 37°C for 6 to 96 hours.

Ultrathin sectioning: Ultrathin sectioning was performed according to the method of Kellenberger et al.(6) for the most part. Ten volumes of liquid culture were mixed with one volume of a 1% osmic acid solution in acetate veronal buffer (pH 6.1). The mixture were centrifuged at 3,000 r.p.m. for 10 minutes. The sediment was suspended in about 1 ml of a 1% osmic acid solution in acetate veronal buffer and fixed at 4°C for 14~17 hours. After washing once with the buffer, the sediment was embedded in 2% agar in the buffer, and then small agar blocks (about 2×2×2 mm) were cut off. These blocks were treated with 0.5% uranyl acetate in the buffer at room temperature for 2.5 to 3 hours. After dehydration in a series of acetone solutions (25, 50, 75, 90, and 100%),
the agar blocks were embedded in a mixture of n-butyl- and methylmethacrylate. Polymerization of methacrylates were carried out at 60°C for 12 to 24 hours. Sections were cut with a JUM-3 type ultramicrotome (Japan Electron Optics Laboratory Co.) fitted with a glass knife. Observations were made with an Hitachi electron microscope, type HU-11, with an accelerating voltage of 75 kv.

**Carbon replica technique**: In order to observe the surface structure of the organisms in detail, carbon replica technique, as employed by Suganuma et al.\(^\text{(7)}\), was applied. The liquid culture was centrifuged, and the sediment was washed twice without fixation, or after fixing with 0.5% osmic acid in acetate veronal buffer for 10 minutes, or with a 1% solution of the same fixative for 15 hours. To the buffer which was used in the preparation of ultrathin sections and carbon replicas, CaCl\(_2\) and NaCl were added according to that described by Kellenberger et al.\(^\text{(6)}\). The sediment thus obtained was smeared on glass slides and dried. Onto these slides carbon was evaporated under vacuum at the inclination whose tan\(^{-1}\) equaled 1/2\(^\text{(8)}\). Then the glass slides were immersed gently from their ends into a Petri dish containing a 10% NaOH solution. By this procedure, carbon films with most of the cells floated off on the surface of the solution. After 30 minutes the films were transferred onto the surface of distilled water and held for 10 minutes with gentle agitation. Then the films were picked up on 150 mesh copper grids, dried, and examined under the electron microscope.

**RESULTS**

The surface structure as observed by carbon replica technique. The replicas of non-fixed cells showed, in general, irregularly waved furrows on the cell surface, their average width being about 300 Å. Granular protuberances were observed at places. Moreover, several depressions, which were demonstrated in case of enteric bacteria by Suganuma et al.\(^\text{(7, 8)}\) and thought to coincide with the nuclear sites, were also recognizable in this organism (Fig. 1).

In the replicas of the cells fixed with 0.5% osmic acid for 10 minutes, the granular protuberances became more conspicuous in contrast to those of unfixed cells (Fig. 2). Furthermore, in the replicas fixed by 1% osmic acid for 15 hours, a continuity of relatively regularly arranged granular protuberances was observed (Fig. 3). Such characteristic figures were not seen in the replicas of *Escherichia coli* fixed in a similar manner (Fig. 4).

The surface structure as observed by ultrathin sectioning. As shown in Fig. 5, a characteristic cell wall structure is distinctly recognizable. A sketch attached to the figure will provide a better understanding of the fine structure. The cell wall, the entire width being about 250 Å, consisted of two layers. The first outer layer, 80–100 Å in width, shows a three-layered structure; the outer and inner dense layers are separated by a less dense intermediate zone, simulating an unit membrane. This first layer undulated in periodicity of 200–600 Å wave
length, and in wave height of about 60 Å. The infoldings of the waves into the interior were mostly deep and sharp. It was noteworthy that the period of waves correspond roughly to the diameters of the surface granules observed in carbon replica preparations. The second inner layer was homogeneous and less dense. However, its innermost edge was seen as a dense line about 20 Å in width, being frequently adjacent to the tips of the infoldings of the first layer like a tangent line.

About 100 Å apart from the second layer of the cell wall to the inside, this may be an artefact, the cytoplasmic membrane was apparent. It consisted of three layers and appeared as an unit membrane, that is, two dense layers which measure 20~30 Å in width divided by a less dense interspace which measures 30 Å in width. The entire width was 100 Å (Fig. 5). The cytoplasmic membrane waved lightly along the cell wall. At sites where infoldings of the cytoplasmic membrane into the interior of the cytoplasm were observed (Fig. 6, arrows), septum formation during cell division was assumed. Besides, the complex intracytoplasmic membrane system, which was connected to the cytoplasmic membrane, was observable in the cytoplasm (Fig. 7).

**DISCUSSION**

The existence of a cell wall in the genus *Fusobacterium* had already been reported by Mudd *et al.*(1), Hampp *et al.*(2), Shiratsuchi *et al.*(3), and Yamaoka(4) on non-sectioned preparations, and by Miyazaki(5) on sectioned preparations. However, the irregularity of the surface structure was demonstrated only by Shiratsuchi *et al.* and Hampp *et al.*. The present authors recognized in the carbon replicas and ultrathin sections, a succession of granular protuberances or furrows on the surface of cells of a strain of *Fusobacterium polymorphum*. This finding differed entirely from the regularly arranged hexagonal surface patterns observed by Houwink(10) in *Spirillum* spec., by Salton and Williams(11) in *Rhodospirillum rubrum*, and by Tawara(12) in an unclassified bacteria isolated from the oral cavity. The separation of the cell wall into two layers is characteristic of this organism. The first layer in a three layered structure was distinctively wavy with marked infoldings, and the inner edge of the second layer showed a distinct dense line waving lightly without paralleling to the first layer. Although the photographs of the cell wall taken from ultrathin sections of *Escherichia coli* by Ryter *et al.*(13) and Kellenberger *et al.*, and of *Bacillus cereus* by Chapman and Hillier(14), resembled those of the present authors, they were different in view of the shallowness of the waves of the first layer and the lack of the dense line in the inner edge of the second layer. In *Fusobacterium*, Miyazaki(5) described the double nature of the cell wall. However, the present authors cannot accept this fact because of the indistinctness of his electron micrographs. Furthermore, his cell wall structure did not show the waved
Carbon replica technique was applied to the study of the cell division of *Saccharomyces cerevisiae* for the first time in 1956 by Bradley. Suganuma employed this technique to observe the structures of *Staphylococcus*, and Suganuma et al. to enteric bacteria. However, their micrographs did not show the successive granular protuberances observed on surface structure of the *Fusobacterium*. Since the diameters of the granules in carbon replicas of the cells treated in the same manner as the samples for ultrathin sectioning coincided, for the most part, with the wave period of the first layer of the cell wall, it can be concluded that the replicas reproduced the surface structure of the *Fusobacterium* faithfully.

**SUMMARY**

The surface structure of a strain of *Fusobacterium polymorphum* was studied electron microscopically employing both carbon replica and ultrathin sectioning techniques. The surface of the cell wall as revealed by the carbon replica technique showed a succession of granular protuberances. In ultrathin sectioned preparations, the cell wall consisted of two layers. The first layer consisted of a three layered structure, simulating the appearance of an unit membrane, and waved rather sharply. The innermost edge of the second layer waved lightly, and tended to be adjacent to the tips of the infoldings of the first. The diameters of the granular protuberances in carbon replicas coincided, on the whole, with the wave periods of the first layer in ultrathin sections. The cytoplasmic membrane was recognized as an unit membrane and infolded into the interior of the cytoplasm at certain places. The intracytoplasmic membrane system which bore a connection with the cytoplasmic membrane and showed diverse profiles was also observable.

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**REFERENCES**

EXPLANATION OF FIGURES

Fig. 1-4. Carbon replica preparations.

Fig. 1. Unfixed. Irregularly waved furrows are observable on cell surface, appearing granularly at places. Several depressions, which seem to correspond to nuclear sites, are seen.

Fig. 2. Fixed with 0.5% osmic acid for 10 minutes. Granular protuberances on the surface became conspicuous.

Fig. 3. Fixed with 1% osmic acid for 15 hours. A succession of granular protuberances is recognized rather regularly.

Fig. 4. Escherichia coli fixed with 1% osmic acid for 15 hours. Granular protuberances on the cell surface are not visible.

Figs. 5-7. Ultrathin sectioned preparations. Fixed with 1% osmic acid for 14 to 17 hours.

Fig. 5. The cell wall consists of two layers. The first layer shows an extremely waved appearance. However, the inner edge of the second layer does not run parallel to the first layer. Underneath the cell wall, is visible the cytoplasmic membrane, which appears as an unit membrane. The cytoplasm is filled with fine granules. The nuclear region in which lays reticulate fibrils is also observable in the cytoplasm.

Fig. 6. The infoldings of the cytoplasmic membrane into the cytoplasm are seen (arrows). This figure seems to present the initial stage of septum formation as related to cell division.

Fig. 7. The intracytoplasmic membrane system in complex profile is connected with the cytoplasmic membrane.

Abbreviations used in the figures:

CW, cell wall; CM, cytoplasmic membrane; N, nuclear region; M, intracytoplasmic membrane system.
1: The first layer
2: The second layer
3: Cytoplasmic membrane
4: Infolding of the cytoplasmic membrane into the cytoplasm