Ultrastructure of the Obligate Halophilic Bacterium

*Halobacterium halobium*

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The fine structure of *Halobacterium halobium* was studied by means of a modified double-fixation technique. The cell envelope is shown to consist of both a "wall" and a plasma membrane. Some electron-dense strands were seen inside the cytoplasm running parallel to the cell envelope. An unusual organelle (or organelles) appeared inside the cytoplasm in the form of parallel striated strands.

Bacterial cell envelopes, both chemically and morphologically, can generally be divided into two main groups by means of Gram staining (18). Gram-positive bacteria possess a thick cell wall homogeneous in electron density, and the bacterial cytoplasm is bounded by a triple-layered plasma membrane. Gram-negative bacteria, on the other hand, possess a multilayered cell wall, sometimes referred to as a compound membrane or an external membrane (1, 18). Intracytoplasmic membranes often known as mesosomes (6, 10) so far have been found mainly in gram-positive bacteria and have been shown to be the site of respiratory enzymes (11, 19). They may also play a role in cell wall synthesis and septum formation (3, 6, 7, 9, 10, 14, 17).

*Halobacterium halobium*, a gram-negative obligate halophilic bacterium requires a high salt concentration for growth and for maintaining its morphological integrity. Chemically its cell envelope is characterized by the high content of acidic amino acids, the high ratio of nitrogen to carbon, and the absence of diaminopimelic acid, muramic acid, and esterified fatty acids (2, 4). The fine structure of *H. halobium* had not been extensively studied previously, because this organism is difficult to fix with osmium tetroxide solutions alone (2). Earlier work of Houwink (8) with shadow casting shows the presence of spherical macromolecules in the cell envelope of intact cells. Brown and Shorey (2) fixed cells by using potassium permanganate solution buffered with salts required to maintain morphological integrity. They reported that *H. halobium* was bounded only by a triple layered plasma membrane. Some unknown electron opaque particles within the bacterial cytoplasm were also noted.

In our studies of *H. halobium*, we have developed a method which fixes whole cells and isolated cell envelopes without lysis or fragmentation being detected at the electron microscopic level. The present paper describes the fine structure of *H. halobium* fixed by this method.

**MATERIALS AND METHODS**

*Source of organism and medium used.* The strain of *H. halobium* was kindly provided by A. D. Brown. The growth medium consisted of: peptone (Oxoid), 40 g; sodium citrate-2H2O, 8 g; KCl, 12 g; MgSO4·7H2O, 80 g; NaCl, 1 kg; and distilled water to give a final volume of 4 liters. The medium was filtered after thorough stirring; its pH was adjusted to 7.2 and it was then autoclaved before use.

*Growth conditions.* Cells were grown in batches of 500 ml in 2-liter Erlenmeyer flasks shaken vigorously in a New Brunswick gyrotary shaker at an oscillation rate of 60 times per min and at an incubation temperature of 37 °C. They were harvested after 48 hr when they were close to the stationary phase of growth.

Cells were washed once with 5 M sodium chloride before fixation.

*Fixation and embedding.* The washing solution used after glutaraldehyde fixation was prepared by omitting the peptone and citrate from the growth medium, adding sodium cacodylate to give a molarity of 0.05, and adjusting the pH to 7.0 with 0.1 N HCl. The glutaraldehyde fixative was prepared by adding glutaraldehyde (stored in the cold in the presence of barium carbonate) to the washing solution to a final concentration of 2% (v/v). A white precipitate always formed on the addition of glutaraldehyde. Before use, the fixative was centrifuged and filtered to remove this precipitate.

Specimens were fixed with the freshly prepared fixative for 2 hr at room temperature (20 to 25 °C), centrifuged, and then washed by suspending the dispersed specimens for 5 min. The washing was repeated six times to remove excess glutaraldehyde. Specimens thus fixed were still sensitive to lysis when suspended in 0.1 M sodium phosphate buffer (pH 7.0) without having the salts normally required for main-
aining their morphological integrity. They were, however, quite stable when postfixed for 4 hr with 1% osmium tetroxide in acetate-veronal buffer (pH 7.0) supplemented with 0.01 M calcium chloride. After postfixation, they were treated with 1% uranyl acetate for 2 hr. Dehydration of specimens was carried out in a graded series of mixtures of ethyl alcohol and water. The specimens were infiltrated with araldite and acetone (1:1, v/v) for 2 hr, with araldite for 24 hr, and with freshly prepared araldite at 60 C for 1 hr. Embeddings were done in freshly prepared araldite and were polymerized at 60 C for 2 days.

We tried to substitute formaldehyde in place of glutaraldehyde or to fix specimens directly with 1% osmium tetroxide in acetate-veronal buffer supplemented with sodium chloride (5 M). The first fixative did not give good preservation of cytoplasmic fine structure, particularly the bacterial nucleoplasm, and the second resulted in considerable lysis.

Electron microscopy. Sections were cut with glass knives on either a LKB ultratome or Reichert ultramicrotome. Sections were collected on unsupported 400-mesh grids and were double-stained by floating grids on saturated uranyl acetate in 50% ethyl alcohol (v/v) for 1 hr and then on lead citrate for 30 min (16). Electron micrographs were taken with a Siemens Elmiskop electron microscope with a double condenser system operating at an accelerating voltage of 80 kV, with a condenser aperture of 100 μ and an objective aperture of 50 μ. Instrumental magnifications were from 10,000 to 40,000. Further enlargements were obtained photographically.

RESULTS

General morphology. A general field of cells of *Halobacterium* fixed by the method described above is shown in Fig. 1. Negligible lysis was observed. There was no cell swelling, and the fine structure was well preserved. The cell envelope was more complex than a typical plasma membrane (Fig. 2 and 3). Organelles were seen in parts of the cytoplasm, usually close to the plasma membrane and to the nucleoplasm. As with some other bacteria, the nucleoplasm occupied much of the cell. It was not membrane bounded, and it contained numerous fibrils (20 to 30 A thick). Membranes internal to the plasma membrane and possibly an extension of it were occasionally observed (Fig. 2). An electron-dense strand (average thickness, 80 to 90 A) running parallel to the plasma membrane is also seen in Fig. 2.

Cell envelope. The cell envelope consisted of at least five layers, three of them electron-dense and two electron-light (Fig. 2 and 3). Each of the dense layers was 25 to 30 A thick, and two of these together with the inner electron-light layer (25 to 30 A) can be regarded as a plasma membrane (75 to 90 A). The dense layer bounding the cell was usually spiky in appearance and was separated from the plasma membrane by an electron-light layer (50 to 60 A). Together these layers can be regarded as a cell wall (75 to 90 A). The overall thickness of the cell envelope was therefore from 150 to 180 A, although in a few cells the overall thickness was 200 to 220 A thick, such as that shown in Fig. 3. The electron-dense layers were not of uniform electron density throughout, but consisted of repeating electron-dense units.

Organelles. At low magnification, one or more organelles were often seen, usually as a group of somewhat wavy alternating electron-dense and electron-light bands (Fig. 1). These were frequently in close association with the plasma membrane but not enclosed by a unit membrane.

At higher magnification, a greater degree of differentiation was noted (Fig. 4 and 5). In Fig. 4, a structure similar to that already described in Fig. 1 is resolved further. The wavy electron-dense bands of Fig. 4 are cut at right angles by less electron-dense strands. The dense bands may not be strictly continuous but divided by the transverse strands into smaller units. These smaller units are 120 to 130 A by 75 to 100 A with an electron-light gap (45 to 50 A) between them. The close association of the organelle with the envelope can be seen.

The organelle in Fig. 5 is morphologically different from that in Fig. 4. However, at this stage it is not certain whether they are sections of different organelles or whether the observable morphological difference is due to the same organelle cut in a different plane. A close association with the plasma membrane is again evident. The electron-dense particles are separated by about 120 to 130 A. This does not correspond strictly to any dimension from Fig. 4. However, we have noted a considerable variation in parameters, such as the dimensions of the plasma membrane and envelope. This may indicate variation in effectiveness of fixation.

DISCUSSION

Although *Halobacterium* is classified as a gram-negative bacterium, previous electron microscopic studies led to the conclusion that its envelope consisted only of a plasma membrane (1). In contrast, the envelope of *Escherichia coli*, a typical gram-negative bacterium is multilayered and variously described as a wall and a plasma membrane, or as a compound membrane and a plasma membrane (18). The present work shows that in fact the envelope of *Halobacterium* is more complex than a single unit membrane. The simplest interpretation is that it consists of a plasma membrane and a wall, with the latter composed of an electron-dense and electron-light layer. In *E. coli*, the portion corresponding to the electron-dense...
FIG. 1. General field of sections of *Halobacterium halobium*. Cells are well fixed, and fine structure is well preserved. Cell envelope is not well resolved at this magnification. Some organelles (ORG) appear in a few cells. The nucleoplasm (N) consists of fine fibrils. × 30,000.
FIG. 2. Section of Halobacterium halobium, showing the presence of both the cell wall (CW) and plasma membrane (PM). Internal membranes (IM) and electron-dense strands (S) also appear inside the cytoplasm. × 60,000.

FIG. 3. Portions of the cell envelope showing the electron-dense layers of both the cell wall and plasma membrane which are made of electron-dense repeating units. The outer electron-dense layer of the cell envelope forms protrusions in many places. × 200,000.
Fig. 4 and 5. Sections of Halobacterium halobium showing an unusual organelle (ORG). It is not certain whether the difference in appearance between the figures reflects that the cell is cut at different planes or whether there are morphologically different organelles. Fig. 4, × 160,000; Fig. 5, × 90,000.
part of the wall is much thicker and has recently been resolved into electron-dense and electron-light layers (5, 12).

It is known that the envelopes of *E. coli* and *H. halobium* are different. This is because muramic acid, a characteristic constituent of the muropeptide, is absent from *H. halobium*, as are esterified fatty acids. We have found (unpublished results) that the growth and morphology of *H. halobium* are not affected by either penicillin or β-glutamic acid, both of which can cause spheroplast formation in *E. coli*. These observations are also consistent with the absence of muropeptide.

Although some gram-negative bacteria possess intracytoplasmic membranes (13, 15), none has yet been found to possess an organelle like that now described for *H. halobium*. The function of this organelle is at present unknown, and indeed it is not certain that only one kind of organelle is present. Stoeckelius and Rowen (Abstr., Intern. Congr. Electron Microscopy, 6th Kyoto, vol. 2, p. 273–274, 1966) have recently described briefly a different method for fixing *H. halobium*. They report the presence of internal membranes which they describe as typical triple-layered structures, sheet-like in appearance, and probably attached to the cell membrane. These structures have been isolated, and they have concluded from negative staining that two morphologically different components are present. They may correspond to the structures shown in Fig. 4 and 5, but this is not certain. At present, we would not describe the organelles as typical triple-layered structures.

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**Literature Cited**


