Fine Structure of *Thermus aquaticus*, an Extreme Thermophile

THOMAS D. BROCK AND MERCEDES R. EDWARDS

Department of Microbiology, Indiana University, Bloomington, Indiana 47401, and Division of Laboratories and Research, New York State Department of Health, Albany, New York 12201

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Electron microscopic studies using thin sections revealed that *Thermus aquaticus* has a structure similar to that of most other gram-negative bacteria. The cell envelope is tripartite: plasma membrane, thin middle layer, and a thicker and irregular outer layer. The outer layer appears to be joined to the plasma membrane by a series of connections and, when seen in tangential section, the outer layer appears as a series of parallel bands. The cell division mechanism resembles that of typical gram-negative bacteria. Large spherical bodies designated "rotund bodies" are formed as a result of the association of a number of separate cells. In this association the outer envelope layers of the cells fuse and pull away from the middle layer. The rotund body thus appears as a series of rods, usually lying in parallel around the periphery of the sphere, completely connected by means of the fused outer layer.

Brock and Freeze (6) recently described the characteristics of a new thermophilic bacterium, *Thermus aquaticus*, which grows at temperatures somewhat higher than do previously reported thermophiles. This organism is yellow-pigmented, gram-negative, and grows either as a long rod or short filament. Although it has some morphological resemblance to the flexibacteria, gliding or other forms of motility were not observed. Spores or cysts were never seen. Frequently, the rod-shaped forms showed a tendency to aggregate, either as linear arrays or as rosettes, and this aggregation was attributed to the presence of slime. A number of strains were isolated from hot springs and other aquatic thermal sources (6), and it was suggested that this organism is widespread but that it had been missed because the isolation procedures usually used for thermophiles were not effective for *T. aquaticus*.

The purpose of this paper is to present electron microscopic studies of the fine structure of this organism. In many respects the organism has a fine structure and cell division mechanism similar to other gram-negative bacteria. However, in one respect *T. aquaticus* is unique. One of the most distinctive characteristics of the organism is the formation of large spherical bodies, considerably larger than spheroplasts, which are often seen in older cultures. Although Brock and Freeze (6) suggested that the large spheres might be formed from a single filament in the manner of spheroplasts, the present paper shows this interpretation may be erroneous. The large spheres seem to be formed as a result of the association and structural alteration of a number of separate rods. Since our electron microscopic studies show that these structures are distinct microformations with unique cell arrangement, the structures will be called "rotund bodies."

MATERIALS AND METHODS

Organism and growth conditions. All studies were performed with *T. aquaticus* strain YT-1 (ATCC 25104). The organism was grown in the mineral salts medium D of Castenholz (7) with 0.3% tryptone and 0.3% yeast extract. The culture was grown aerobically at 70 to 75 C into mid-logarithmic phase, at which time it was fixed for electron microscopy without centrifugation or cooling.

Fixation. Three fixation procedures were used. Method 1 was modified from Ryter and Kellenberger (15). Equal amounts of culture and 2% osmic acid in Veronal acetate buffer containing 0.1 m CaCl₂ at pH 6.1 were mixed and shaken gently at room temperature for 30 min, at which time the cells were centrifuged and suspended in 1% osmic acid in the same buffer. After 3 hr of shaking at room temperature, the cells were centrifuged and suspended in 1.5% water-agar at 45 to 50 C. The mixture of cells and agar was then pipetted onto warm microscope slides; the agar was allowed to cool and was cut into 2-mm cubes. The agar blocks were placed for 15 min in 0.5% uranyl acetate in the same Veronal acetate buffer, whose pH then dropped to 5.0. This treatment with fresh uranyl acetate was repeated twice at 15-min
intervals. In method 2, modified from Glauert and Thornley (10), a cell suspension was mixed with equal parts of 5% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.3) containing 2 mg of CaCl₂ per ml. The mixture was shaken at 10 to 15 C for 30 min and centrifuged; the cells were then suspended in 0.1 M cacodylate-CaCl₂ buffer at 4 C. After 20 min of shaking in the cold, the suspension was centrifuged, resuspended in the same buffer, and shaken for 30 min in the cold; the suspension was then centrifuged and the cells were suspended in 1% osmic acid in Veronal acetate. Thereafter, the samples were processed as in method 1. Method 3 was the paraformaldehyde-glutaraldehyde method of Karnovsky (11). Method 1 gave the most satisfactory fixation, and all the figures except Fig. 5 and 9 were obtained with material fixed in this way. These two figures were derived from material fixed with method 2.

Dehydration and embedding. The agar blocks were dehydrated through a graded series of water-ethanol mixtures followed by acetone. They were infiltrated with an acetone-Epon-Araldite mixture and embedded in Epon-Araldite by conventional methods (12). Thin sections were cut on a Porter Blum or LKB ultramicrotome and stained with a saturated aqueous uranyl acetate solution followed by lead citrate, as described by Reynolds (14). Electron microscopy was done by using a Siemens Elmiskop I or IA electron microscope and Kodak Electron Image plates.

RESULTS

General appearance and cell envelope structure. The general appearance of a _T. aquaticus_ rod is shown in Fig. 1. The cell envelope structure is of the complex _gram-negative_ type, although the plasma membrane, peptidoglycan, and outer layer are more clearly defined than in many other _gram-negative_ bacteria. In examining electron micrographs of several extreme thermophilic bacteria, our experience has been that the plasma membranes of these organisms are usually quite well delineated. The nuclear material appears similar to that of other bacteria and the ribosomes are well defined and of an appropriate size (about 15 nm). The ribosomes of this organism have a normal sedimentation constant of 70S (16).

In some thin sections, we have seen simple invaginations of the plasma membrane (Fig. 9) but not complex membranous structures such as typical mesosomes (i.e., vesicular or lamellar systems). The outer cell wall layer is electron dense, often quite regular in thickness, with alternating depressions, invaginations, and protrusions (Fig. 2 and 3). In tangential section, this regular structure is seen as a series of parallel bands (Fig. 2). We suggest that this outer layer is not the cell wall (as generally delineated) of _gram-negative_ bacteria but a definite membranous structure, portions of which are connected with the underlying innermost layer, leading to the appearance seen in Fig. 3 and 7. Connections between the outer wall layer and the underlying innermost layer have also been seen in _Escherichia coli_ (2) and in _Thiobacillus thiooxidans_ (13), although less distinctly.

Brock and Freeze (6) noted that _T. aquaticus_ produces a considerable amount of slime under some conditions. In some of our electron microscopic preparations, large amounts of such slime were found, as shown in Fig. 8; note especially the distinctly fibrous nature of this slime.

Cell division. The cell division mechanism in _T. aquaticus_ resembles that of typical _gram-negative_ bacteria such as _E. coli_ rather than that of bacteria related to the blue-green algae, such as _Leucothrix mucor_ (5). Figures 13 to 15 illustrate the cell division process in _T. aquaticus_. At the initial stages, an invagination of the whole cell envelope (cell wall and plasma membrane) is seen in the zone of cell separation (Fig. 1 and 13). The invagination is a furrowing process which, as it progresses to the center of the cell, produces a deep narrow infolding of the cell envelope. As seen in Fig. 14, the layers or membranes of the two opposite sides of the infolding are very close to one another and are fairly well outlined. This is not a true septum in the sense generally applied to _gram-positive_ bacteria (8) or fungi, because in _T. aquaticus_, as in most _gram-negative_ organisms, the outer wall layers infold as a whole, all the way to the center of the cell, following the plasma membrane. In the slightly oblique longitudinal section shown in Fig. 15, the dense material (peptidoglycan) of the inner wall is seen as a dark zone between the sister cells, but the outer wall is not clear. Serial sections are necessary to demonstrate the details of this process. In this figure, the plasma membrane was in part pulled away from the inner wall (arrow side), thus enhancing its appearance. No “true septum” was found in the numerous micrographs examined.

Formation of rotund bodies. Rotund bodies, characteristic of all strains of _T. aquaticus_ so far isolated (6), were originally interpreted as unusually large spheroplasts. Our electron micrographs, however, reveal that they are more complex structures formed as a result of the association of a number of separate rods or filaments. The general appearance of one of these bodies as seen by Nomarski interference contrast is shown in the through-focus series of photographs presented in Fig. 16. These photographs demonstrate that a rotund body consists of more than one _T. aquaticus_ rod. The manner in which these
FIG. 1. Longitudinal, transversal, and oblique views of cells, revealing nucleoplasm (n) with dense thin deoxyribonucleic acid fibrils, surrounded by cytoplasm containing numerous ribosomes (ri). Cell envelope comprises plasma membrane (pm) and wall exhibiting outer dense layers (ow), middle light zone (mw), and inner dense layer (iw). Note cell division by furrowing (f). Where two cells are in contact, the external wall layer (ow) has separated from the inner wall which remains adherent to the plasma membrane. × 60,000; bar indicates 0.5 μm.
Fig. 2. Part of a twisted rod exhibiting grooves of outer wall in tangential view (arrows), as well as in profile. \( \times 50,000 \); bar indicates 0.5 \( \mu m \).

Fig. 3. Portions of three rods displaying outer wall in grazing section (center filament) and in middle longitudinal section (lower right-hand). The latter shows sharp indentations of the outer wall reaching to the inner wall which is apposed to the plasma membrane. \( \times 60,000 \); bar indicates 0.25 \( \mu m \).

Fig. 4. Portions of two rods, upper one shown in transversal and lower in oblique views. Note outer wall (arrow) peeled off from the inner part of the cell envelope. \( \times 50,000 \); bar indicates 0.5 \( \mu m \).

Fig. 5. Portions of outer walls in two opposing cells. Note filamentous material in the region where outer walls are fused (arrow). \( \times 80,000 \); bar indicates 0.25 \( \mu m \).
rods associate to form a rotund body is revealed by the electron microscope studies. Transverse or slightly oblique sections through two of these bodies are seen in Fig. 10 and 11. The rotund body consists of a number of cells surrounded by a membranous structure. As seen in the enlargement of one portion of a rotund body in Fig. 12, this membranous structure is composed of the outer cell envelope layer which has partly peeled away from the cell surface. The connections between the cells by way of this outer cell envelope layer are easily traced in Fig. 10.
FIG. 9. Portions of a rod displaying infoldings of the plasma membrane (arrows). × 80,000; bar indicates 0.5 μm.

FIG. 10. Cross sections of eight rods, seven of which are connected by their outer wall to form a ring-like structure. × 40,000; bar indicates 0.5 μm.

FIG. 11. Rotund body showing its component rods in oblique and longitudinal sections. Note (arrows) the continuation between the outer wall of the filamentous cell (longitudinal section) and neighboring cells (oblique sections), each of which is, in turn, connected to other neighbors. × 20,000; bar indicates 1.0 μm.
Fig. 12. Portion of a rotund body, showing a cross section through one component rod. The outer wall (ow) with a striated appearance is separated by a light zone from the inner wall (iw) which remains apposed to the plasma membrane (pm). × 120,000; bar indicates 0.25 μm.

Fig. 13. Middle longitudinal section of a rod at early phase of division. Note beginning of furrow (center). × 60,000; marker = 0.25 μm.

Fig. 14. Advanced phase of cell division. Note approximation of cell envelope (arrows). × 80,000; bar indicates 0.25 μm.

Fig. 15. Portions of two recently divided cells. Note plasma membrane and wall layers in this area (center). × 80,000; bar indicates 0.25 μm.

Fig. 16. Through-focus series (left to right) by Nomarski interference contrast microscopy of a rotund body. × 1,400.
How are rotund bodies formed? It seems clear that formation involves an association and interaction by way of the outer cell envelope of a number of separate rods or filaments. Brock and Freeze (6) noted that rods and filaments frequently formed aggregates or linear arrays. Figures 1, 4, and 5 show views in which two cells are in contact by means of their outer cell envelope layer, and the outer layer has partially or completely fused. In some cases, apparently as a consequence of fusion, the outer cell envelope layer is pulled loose (Fig. 1 and 4). We assume that partial loosening followed by fusion of the outer layer between adjacent cells results in the formation of the boundary layer of the rotund body. The cells in the rotund body are not arranged in parallel in every case. Figure 11 shows a section through a rotund body in which one of the filaments lies at right angles to the other cells. This picture shows clearly that the boundary layer of the rotund body is continuous from cell to cell.

**Pleomorphic cells.** Brock and Freeze (6) noted that *T. aquaticus* occasionally formed swollen and rather pleomorphic cells. Figure 6 shows a section through a filament which has a distinctly bifid morphology.

**DISCUSSION**

*T. aquaticus* has most of the structural features of the gram-negative bacterial cell, although the cell envelope structure is more clearly seen than in most gram-negative bacteria studied. The typical double track of the plasma membrane, often difficult to see clearly in electron micrographs, is especially clear in *T. aquaticus*, as it is in the other extreme thermophiles studied (unpublished observations). The great stability of the plasma membrane of extremely thermophilic bacteria was discussed previously (3, 4). This stability may be a factor in permitting successful fixation of the bacterium for electron microscopy. The fatty acids of the plasma membrane of *T. aquaticus* were studied by Paul Ray (Ph.D. Thesis, Indiana Univ., 1970). Unsaturated fatty acids are virtually absent, and a high content of branched fatty acids is present. A similar pattern was also seen by Bauman and Simmonds (1) in analyses of naturally collected extremely thermophilic bacteria, some of which we also observed (unpublished observations).

The outer cell envelope layer (usually considered to be lipopolysaccharide in gram-negative bacteria) is especially well defined in *T. aquaticus* and is noteworthy for the banded appearance seen in profile. We attribute this appearance to the presence of regular connections between the outer layer and the underlying peptidoglycan layer. Such connections lead to the formation of periplasmic pouches running transversely around the cell. Conceivably, a “Bauplan” of this sort makes possible a more secure retention of periplasmic constituents by the cell, a property which may be of considerable significance in permitting the organism to grow and survive at high temperatures. Similar invaginations have also been seen in the extreme acidophilic bacterium *T. thiooxidans* (13).

The most intriguing aspect of the present study relates to the structure and mode of formation of the rotund bodies. In these bodies, a number of cells are connected, probably as a result of fusion of their outer cell envelope layers. The average number of cells per body is around 14. Very similar structures (which were called round bodies) were reported by Felter et al. (9) for *Vibrio marinus*, although in this case only two or three cells were found in association. In addition, all of the round bodies observed by these workers may not have had the same origin. Felter et al. (9) concluded that their bodies arose as a result of an aberrant division process, presumably from several adjacent cells which had not completely separated. The rotund bodies of *T. aquaticus* probably did not arise in this way because of the large number of rods involved. Knowledge of their fate would be of value in interpreting the nature of these structures. Rotund bodies have never occurred at concentrations of more than 1% rods and filaments, and usually considerably less than that. To determine whether the individual units of these bodies are able to reform viable bacterial cells will require the use of micromanipulation techniques, coupled with slide cultures.

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**LITERATURE CITED**