Ultrastructure of an Anaerobic Filamentous Oral Micro-organism

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SUMMARY

Thin sections of an anaerobic filamentous oral organism possibly related to *Leptotrichia buccalis* have been examined in the electron microscope. The organism exhibited a cell wall profile characteristic of Gram-negative bacteria, viz. a solid membrane and an outer double-layered membrane. The outer membrane could be removed by treating intact cells with trypsin or phenol-water. The organism contained numerous intracytoplasmic vacuoles.

INTRODUCTION

Morphological and physiological properties of an anaerobic filamentous micro-organism isolated from the human oral cavity have been reported (Hofstad, 1967*a*). This micro-organism, which proved to be identical to a filamentous bacterium described by Theilade & Gilmour (1961), appeared to be related to *Leptotrichia buccalis* (Gilmour, Howell & Bibby, 1961), but differed from that species serologically and in showing a heterofermentative type of sugar metabolism. Like *L. buccalis*, very young cells of the atypical organism were Gram-positive, while most cells in cultures in the stationary phase of growth were Gram-negative.

In the electron microscope, *Leptotrichia buccalis* exhibits a cell wall profile characteristic of Gram-negative bacteria (Hofstad & Selvig, 1969). The fine structure of the related atypical filamentous micro-organism has been reported by Theilade, Theilade & Scott (1962). These authors did not, however, study the cell wall structure of this organism.

The present report concerns the ultrastructure of the filamentous micro-organism as revealed by electron microscopy of intact cells and cells treated with trypsin or phenol-water, with particular attention to the cell wall profile.

METHODS

The isolation of the filamentous organism, strains L44 and L49, has been described earlier (Hofstad 1967*a*). Cultivation was performed in enriched nutrient broth (Hofstad 1967*a*), and the bacteria were harvested by centrifugation when in exponential phase of growth (1 or 2 days at 37°).

In preliminary experiments the harvested bacteria were washed with saline or phosphate buffer at neutral pH. However, the washings caused many organisms to break, and the remainder appeared fuzzy and with poorly resolved cell wall layers

when examined in the electron microscope. Therefore, the washing was omitted and subsequent preparations were carried directly to fixative.

Trypsin digestion was done on washed bacteria with crystalline trypsin (Trypure Novo, Novo Industri A/S, Copenhagen), freshly prepared in 0.02 M-tris buffer of pH 7.8, using 10 mg. trypsin/g. packed wet bacteria. The digestion was performed at 37° for 20 hr. The digested bacteria were centrifuged and washed twice in saline.

Treatment with phenol-water was carried out by homogenizing equal volumes of an aqueous suspension of 200 mg. wet packed bacteria and 90 % phenol for 15 min. at 4°.

Following centrifugation the water and phenol phases were pipetted off and the treated bacteria washed once in distilled water.

Pellets of intact or treated bacteria were resuspended in acetate+veronal buffered osmium tetroxide fixative, transferred to agar and embedded in polyester resin according to the procedure suggested by Kellenberger, Ryter & Séchaud (1958). Thin sections for electron microscopy were cut with glass knives, collected on carbon-coated formvar membranes, and stained with 2 % aqueous phosphotungstic acid for 30 min., or with lead citrate (Reynolds, 1963) for 5 to 15 min. Some bacteria were resuspended in distilled water. Drops of the suspension were placed on specimen support grids coated with carbon and formvar membranes, dried and shadowed with palladium from an angle of 20 degrees. Specimens were examined in the electron microscope and photographed at plate magnifications of 5,000 to 20,000 diameters.

RESULTS

Intact organisms

The anaerobic filamentous organism, strains L44 and L49, appeared in thin sections as elongated structures whose shorter diameter ranged from 0.4 to 0.6 μ (Pl. I, fig. 1). The bacteria had rounded ends and were often adhering end to end. A moderate amount of floccular substance was seen adhering to the surface and in the surrounding medium.

The concentric membranous structures surrounding the cytoplasm will be referred to as the plasma membrane, solid membrane and the outer membrane. The plasma membrane appeared as a double structure, 70 to 80 Å in width, which followed a straight or slightly wavy course (Pl. I, fig. 2).

The solid membrane appeared as a distinct single electron-dense line, less than 50 Å in width (Pl. I, fig. 2). It followed the course of the plasma membrane, separated from it by a space of 50 to 150 Å. This space contained varying amounts of granular electron-dense material.

The outer membrane was separated from the solid membrane by a less electron-dense layer, around 40 Å in width. Where the multi-layered cell wall was best resolved, the outer membrane could be seen as a double-tracked structure. In other organisms the outer membrane appeared as a less distinct single line. The outer membrane was regularly coated by a more or less dense crust of granular or floccular material (Pl. I, fig. 2).

The cytoplasm consisted mainly of an evenly dispersed electron-dense granular material. Nuclear regions appeared as less electron-dense regions filled with fibrillar substance (Pl. I, fig. 1). Many cells contained oval or circular vacuoles (Pl. I, fig. 1). The contents of the vacuoles were less electron-dense than the surrounding cytoplasm.

The filamentous shape of the micro-organism was confirmed in shadowed preparations. Some of the organisms were collapsed; these showed numerous rigid bodies which were interpreted as corresponding to the intracytoplasmic vacuoles (Pl. 1, fig. 3).

Organisms treated with trypsin or phenol-water

Following digestion with trypsin, most bacteria maintained their original shape, but the wall structure was considerably modified (Pl. 1, fig. 4). Generally, the cell wall consisted of a single electron-dense layer separated from the plasma membrane by a space of varying width. This space did not contain appreciable amounts of stainable material.

After treatment with phenol-water the organisms were extensively altered (Pl. 1, fig. 5). The cell content appeared as a homogeneous mass retracted from the wall. The cell wall appeared as a single line, frequently with attached outer cell wall material.

DISCUSSION

The cell wall profile of the filamentous oral organism conforms to the general description of the Gram-negative cell wall type (Murray, Steed & Elson, 1965; de Petris, 1965), and is characteristically different from the cell wall profile of Gram-positive bacteria (Glauert, 1962; Murray, 1962). The various layers were not as clearly differentiated, however, as in the possibly related species *Leptotrichia buccalis* (Hofstad & Selvig, 1969).

The finding of a Gram-negative cell wall type is in agreement with previous chemical studies of isolated walls (Hofstad, 1967*b*). Cell walls of the filamentous organism contained a wide range of amino acids, and protein and lipid accounted for approximately 50 and 20 % of the dry weight, respectively. In contrast, the hexosamine content did not exceed 5 %. Furthermore, the cell wall preparations contained an aldohexose, a component previously demonstrated in Gram-negative bacteria only.

The disappearance of the outer membrane from the trypsin-treated organisms indicates that this membrane of the filamentous organism is made up mainly of protein. This hypothesis is supported by chemical analysis of the material extracted from washed whole bacteria or cell wall preparations by digestion with trypsin. The extracted material contained 55 % by weight protein, 25 % lipid and 20 % neutral sugars (T. Hofstad, unpublished results).

The removal of the outer membrane by trypsin digestion is somewhat at variance with the results of de Petris (1967), who reported on the cell wall structure of *Escherichia coli*. Digestion of intact or heat-treated organisms with proteolytic enzymes caused no removal of the outer membrane in this species. Rather, the double-layered outer membrane was sharply outlined and apparently unaffected by the treatment. On the other hand, this membrane could be removed by treatment with phenol-water, which in the present study was less effective than trypsin treatment in this respect.

Trypsin-digested cell walls of the filamentous organism contain approximately 25 % amino acids, 38 % neutral sugars and 4 % fatty acid esters (Hofstad, 1967*b*). This indicates that the solid membrane comprises structures other than pure mucopeptide.

The removal of the stainable material between the plasma membrane and the solid membrane by digestion with trypsin shows that protein is interposed between these

membranes. Analogous findings have been made in *Escherichia coli* (Weidel, Frank & Martin, 1960; de Petris, 1967).

The nature of the intracytoplasmic vacuoles is not clear. However, similar structures, interpreted as intracellular polysaccharide, have been found in cariogenic streptococci (Guggenheim & Schroeder, 1967) and in coccoidal, rodshaped and filamentous bacteria in human dental plaques overlying various and, to a lesser degree, normal enamel (Frank & Brendel, 1966). The finding of rigid bodies within collapsed bacteria supports the assumption of a consolidated content of these vacuoles.

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EXPLANATION OF PLATE

Fig. 1. Thin section of the anaerobic filamentous oral organism (strain L44). The cytoplasm is surrounded by three concentric structures: the double-tracked plasma membrane, the solid membrane and the double-tracked outer membrane. Numerous intracytoplasmic vacuoles are present, especially in the bacterium to the right. Small amounts of floccular material adhere to the cell surface. N—nuclear regions. Magnification $\times 40,000$.

Fig. 2. Detail from Fig. 1. PM—plasma membrane, SM—solid membrane, OM—outer membrane. $\times 100,000$.

Fig. 3. Shadowed specimen (strain L 49). The collapsed bacterium contains intracytoplasmic rigid structures. $\times 10,000$.

Fig. 4. Result of trypsin digestion (strain L 44). The bacteria have maintained their shape, but the outer membrane is missing. PM—plasma membrane, SM—solid membrane. $\times 40,000$.

Fig. 5. Result of treatment with phenol-water (strain L 44). The cell wall appears in general as a single electron dense line which corresponds to the solid membrane (SM). $\times 40,000$.