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Ultrastructure of *Butyrivibrio fibrisolvens*: a Gram-Positive Bacterium?

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The cells of bacteria of the genus *Butyrivibrio* are universally described as being gram negative, and they produce an unequivocal gram-negative reaction in the standard staining procedure. However, their cell walls contain derivatives of teichoic acid, which are characteristic of gram-positive cells. In this study, the cell walls of two representative strains of *Butyrivibrio* were of the gram-positive morphological type, as seen by electron microscopy, but they were very thin (12 to 18 nm). The thinness of these cell walls may account for the tendency of these cells to stain gram negatively in the standard staining procedure. Ruthenium red staining revealed an extracellular structure surrounding cells of *Butyrivibrio* sp. (strain C_3). This structure was composed of individual "knobs" that sometimes mediated cell-to-cell adhesion in the culture.

Butyrivibrio fibrisolvens, one of the most common rumen bacteria (2, 3, 18), is gram negative when stained by the standard technique. However, Hewett et al. (15) and Sharpe et al. (25) recently isolated lipoteichoic acid and glycerol teichoic acid, respectively, from *B. fibrisolvens*, even though these compounds are typical components of gram-positive bacterial cell walls (26). Sharpe et al. (25) reported that the ultrastructure of the cell wall was typical of neither gram-positive nor gram-negative bacteria. Hewett et al. (15) conclude that, though *B. fibrisolvens* stains gram negatively, its cell envelope contains teichoic acids, and thus it is an enigmatic and unique organism with respect to its cell envelope structure.

The ultrastructure of the bacterial cell envelope reflects the fundamentally different chemical and physical structures of gram-negative and gram-positive cell walls. The outer membrane is clearly defined in the former but is a more amorphous, fibrous structure in the latter (11, 14).

This paper describes an ultrastructural examination of *B. fibrisolvens* (strain D1) and *Butyrivibrio* sp. (strain C_3) to determine which morphological type the cell walls of these organisms resemble most closely.

MATERIALS AND METHODS

B. fibrisolvens D1 (6) was generously provided by M. P. Bryant, University of Illinois, Urbana. *Butyrivibrio* sp. (strain C_3) was isolated from bovine rumen contents and was capable of degrading rutin, quercetin, and naringin (10).

The anaerobic culture technique used was that of Hungate (17) as modified by Bryant and Burkey (4). The cells were grown in rumen fluid medium (5), except in cases where a chemically defined medium (24) was used to demonstrate that any extracellular material present was synthesized by the bacteria.

When the outer carbohydrate coat was to be demonstrated, a 20-ml sample of each culture (from various stages of growth) was centrifuged (15,000 × *g*, 10 min), and the residue was suspended in a 0.15% aqueous solution of ruthenium red (19) (BDH Chemicals, Toronto, Ontario) and held at room temperature for 30 min. The sample was then centrifuged (18,000 × *g*, 10 min), and the sediment was suspended and fixed for 1 h in 1.2% glutaraldehyde (purchased as a 70% solution under argon from Ladd Industries, Burlington, Vt.) in a 0.067 M cacodylate buffer (pH 6.5) with 0.05% ruthenium red. The sample was then washed three times (10 min each) in ruthenium red and cacodylate buffer and postfixed (3 h) at room temperature (22°C) in 1.33% osmium tetroxide (purchased as a 4% solution under argon from Polysciences Inc., Rydall, Pa.) in ruthenium red and cacodylate buffer. After osmium fixation, the sample was washed three times (10 min each) in ruthenium red and cacodylate buffer and then was subjected to an acetone dehydration series of 30 min in each of 30, 50, 70, 90, and 100% acetone by volume. Freshly distilled acetone was diluted to 30, 50, and 70% with ruthenium red and cacodylate buffer, but the 90% acetone was made by dilution of 100% acetone with distilled water. The sample was then washed twice (20 min each) in 100% propylene oxide (Polysciences Inc.) and embedded in Vestopal (Polysciences Inc.). Morphological control preparations for embedding were prefixed by adding 0.1 volume of 5% glutaraldehyde in cacodylate buffer to a sample from the culture medium. After 20 min, the sample was centrifuged (15,000 × *g*, 10 min), and the resi-

due was suspended and fixed (2 h, 22°C) in 5% glutaraldehyde in cacodylate buffer. After fixation, the sample was enrobed (21) in 4% agar and washed five times (15 min) in cacodylate buffer before postfixation (2 h) in 1.33% osmium tetroxide in cacodylate buffer. After five washes (15 min each) in the cacocylate buffer, the sample was dehydrated in an acetone series (see above). The agar cores were then washed twice (20 min each) in 100% propylene oxide and embedded in Vestopal W.

Ultrathin sections of material embedded in plastic were cut with a Reichert model OM U2 ultramicrotome.

To ensure that increased contrast in the outer carbohydrate coat was due to ruthenium red staining and not to secondary staining of sections with uranyl acetate (used as a 1% aqueous solution at pH 5) and lead citrate (23), unstained sections were examined in the electron microscope after mounting on clean 400-mesh copper grids, while other sections were stained with uranyl acetate and lead citrate.

All preparations for electron microscopy were examined at an accelerating voltage of 60 kV with an AEI 801 electron microscope equipped with a 30° tilt stage.

All preparations for light microscopy were examined with a Zeiss microscope. Phase contrast optics were used to observe living cells, and nonphase apochromatic oil-immersion objectives were used to examine stained preparations.

RESULTS

Phase microscopy of living cells showed that the cells of both *B. fibrisolvens* (strain D1) and *Butyrivibrio* sp. (strain C_3) were short, curved rods that were between 1.5 and 6.0 μm in length and 0.4 and 0.8 μm in diameter. Short chains of two to three cells were occasionally seen, and the cells were generally motile in wet mounts. Gram staining consistently produced stained cells that were unequivocally gram negative.

Electron microscopy of sections of the cells of *B. fibrisolvens* (strain D1) showed that the cell wall of this organism was of the gram-positive (11) morphological type (Fig. 1 and 2). The cell wall showed an even electron density, except for some enhanced density at both surfaces (Fig. 1, inset), and tilting of individual cells through 30° on both x and y axes failed to reveal a trilamellar outer membrane structure. The unique characteristic of the gram-positive cell wall of this organism was its exceptional thinness. Cells with morphologically intact membranes and cytoplasm (Fig. 1) had cell walls only ca. 12 nm thick, whereas older cells with damaged membranes and diffuse cytoplasm (Fig. 2) had cell walls ca. 16 nm thick. The cell envelopes of cells of *B. fibrisolvens* (strain D1) stained with ruthenium red were indistinguishable from those of unstained cells.

Electron micrographs of cell sections of *Butyrivibrio* sp. (strain C_3) showed that the cell wall of this organism was also of the gram-positive type (Fig. 3 and 4). The cell wall had a very thin "rim" of enhanced electron density at each surface, but no trace of trilamellar outer membrane structure could be seen with specimen tilting or by examining a large number of photographs. As in *B. fibrisolvens* (strain D1), long, morphologically intact cells had cell walls ca. 17 nm thick, whereas shorter and thicker cells had cell walls ca. 18 nm thick.

Cells of *Butyrivibrio* sp. (strain C_3) stained with ruthenium red showed a remarkable extracellular structure outside the gram-positive cell wall (Fig. 5 and 6). The cell surface was covered by electron-dense material that formed a pattern of "knobs." This pattern could be seen in an almost topographical view in areas where the section was tangential to the cell surface (Fig. 5, T), and the "knobs" occupied a significant proportion of the cell surface. In areas where the cell envelope was perpendicular to the plane of the section, the close relationship between the "knobs" and the gram-positive cell wall was clear (Fig. 5, P). The inner and outer electron-dense "rims" of the ca. 16-nm-thick cell wall were also clear (Fig. 5, W), and this cell wall structure resembles that seen by Sharpe et al. (25) and contrasts effectively with the trilamellar pattern of the ca. 9-nm cytoplasmic membrane (Fig. 5, M). In many instances, the "knobs" of extracellular material appeared to mediate an attachment between adjacent cells (Fig. 6). This extracellular material was produced by *Butyrivibrio* sp. (strain C_3) in both chemically defined and rumen fluid media.

DISCUSSION

Anaerobic cellulolytic rumen bacteria similar to *Butyrivibrio* were first described by Hungate (17), and the genus was established with *B. fibrisolvens* (strain D1) as the type species by Bryant and Small in 1956 (3, 6). Organisms of this genus are universally described as being gram negative (2, 3, 6, 10, 18), and we have found that *B. fibrisolvens* (strain D1) and *Butyrivibrio* sp. (strain C_3) are also gram negative. Therefore, when Sharpe et al. (25) found glycerol teichoic acids and Hewett et al. (15) found lipoteichoic acids in the cell walls of strain D1, they correctly attached considerable importance to the presence of these gram-positive cell wall components (26) in a "gram-negative" organism.

A very extensive body of literature has been generated to document the fundamentally different chemical composition and molecular ar-

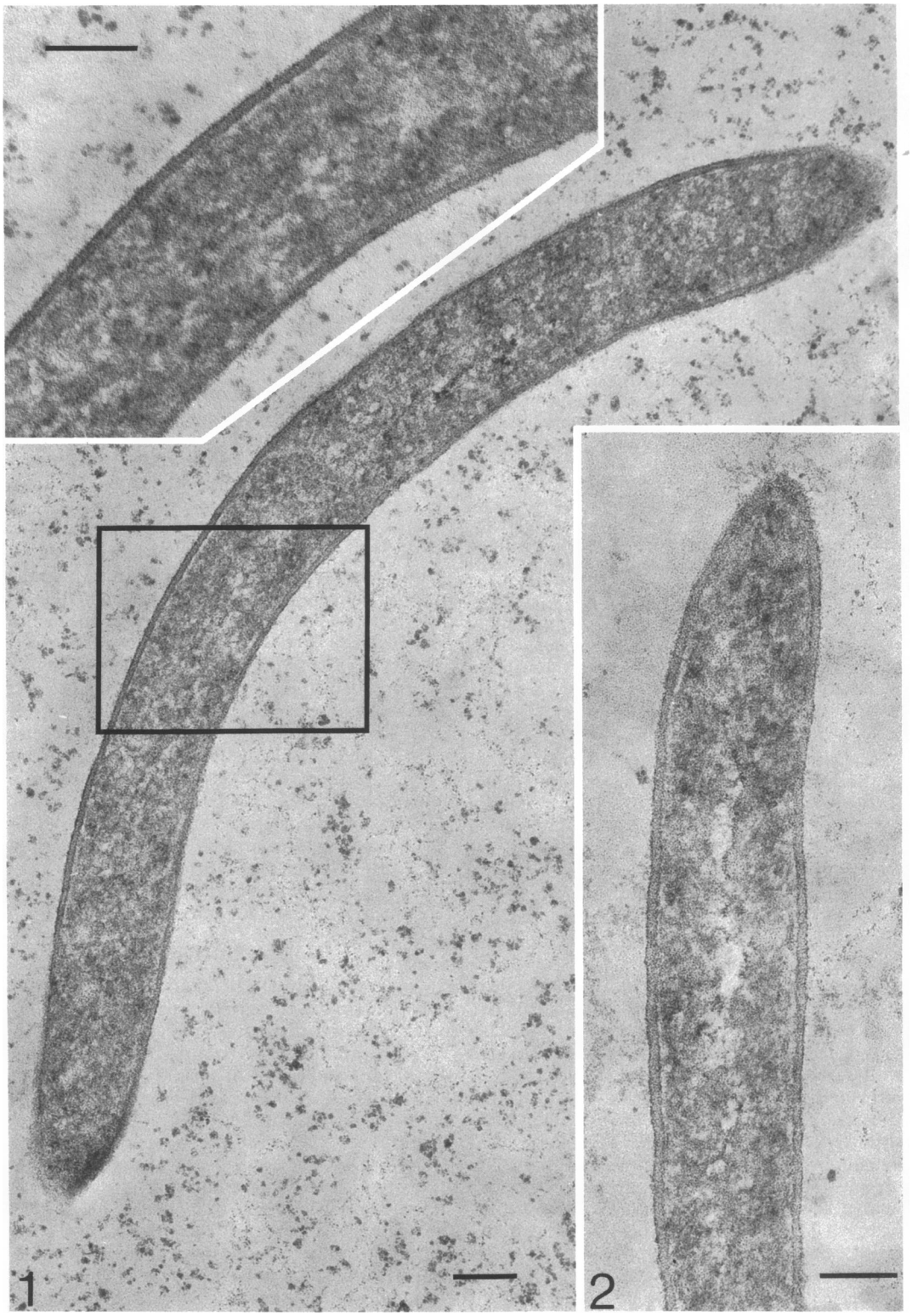

FIG. 1. *Electron micrograph of a section of a long curved cell of Butyrivibrio fibrisolvens (strain D1) showing the thin (ca. 12 nm) gram-positive cell wall of this organism. The area delineated by the box is magnified further in the inset. The bar in this and subsequent electron micrographs indicates 0.1 μm.*

FIG. 2. *Electron micrograph of a section of a cell of Butyrivibrio fibrisolvens (strain D1) in which the cytoplasm is uneven in electron density and the cytoplasmic membrane is apparently damaged. The gram-positive morphological pattern of this marginally thicker (ca. 16 nm) cell wall is clear, as is the absence of trilamellar structure.*

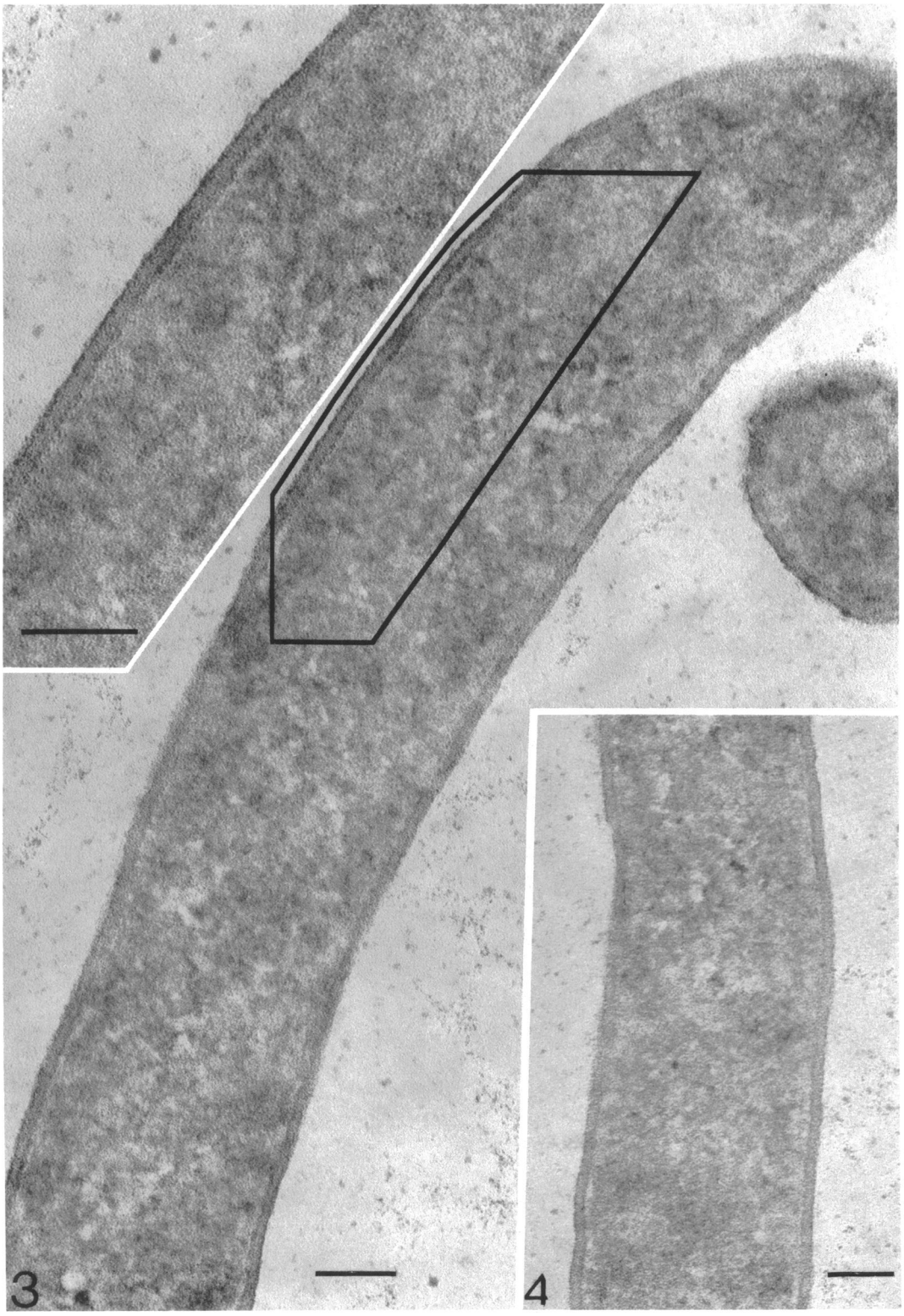

FIG. 3. *Electron micrograph of a section of a long, curved cell of Butyrivibrio sp. (strain C_3) showing the thin (ca. 17 nm) gram-positive cell wall of this organism. The area delineated by the box is magnified further in the inset.*

FIG. 4. *Electron micrograph of a section of a short, wide cell of Butyrivibrio sp. (strain C_3) showing the marginally thicker (ca. 18 nm) cell wall.*

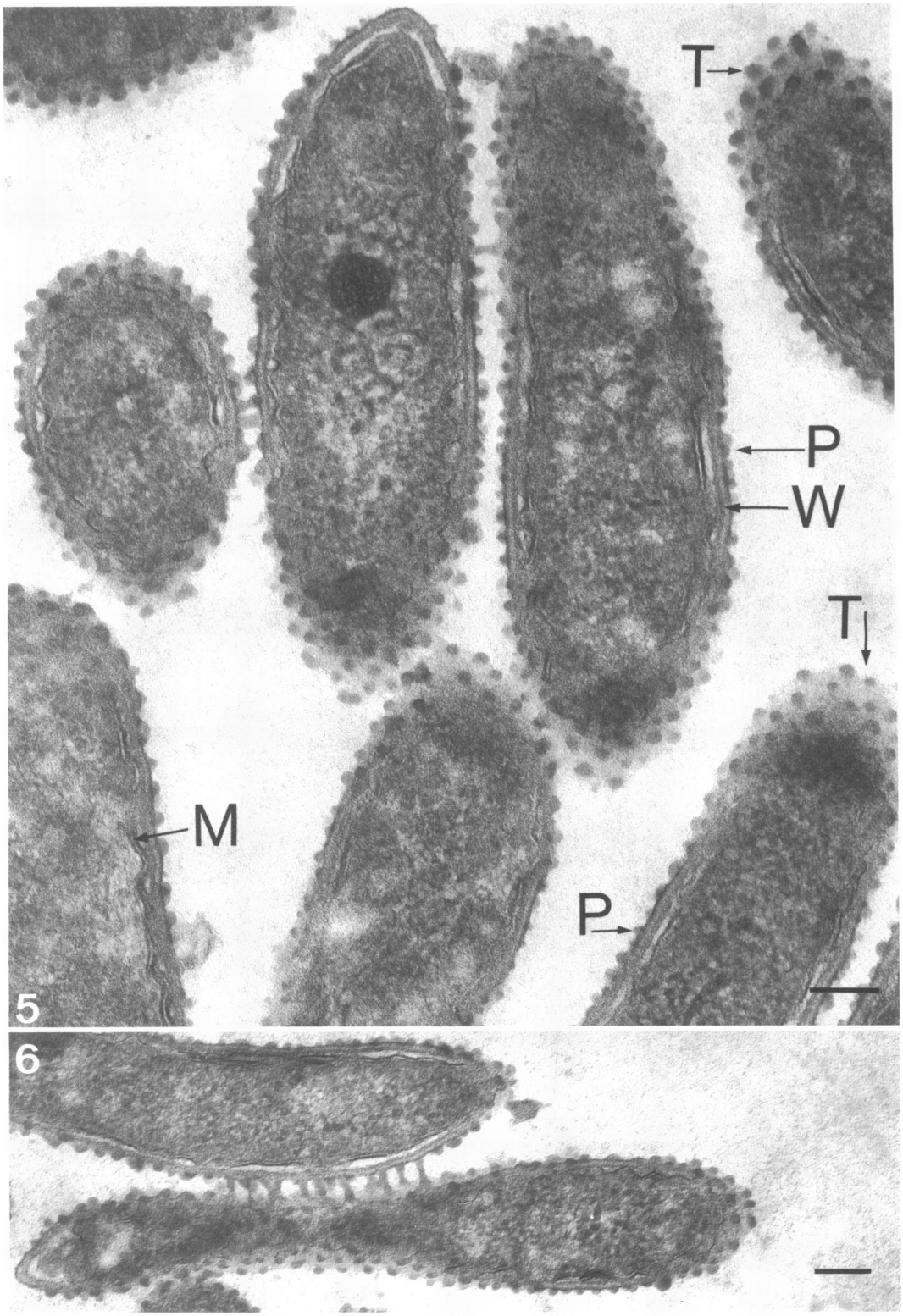

FIG. 5. *Electron micrograph of a section of a cell of Butyrivibrio sp. (strain C_3) fixed and dehydrated in the presence of ruthenium red. Note the extracellular material that forms "knobs" on the cell surface. The distribution of these "knobs" is well seen in areas of tangential section (T), whereas the relation of the "knobs" to the cell wall is best seen in areas (P) where the section plane is perpendicular to the plane of the cell wall. The morphology of the cell wall (W) is especially clearly seen, and it contrasts with the clearly trilamellar structure of the cytoplasmic membrane (M).*

FIG. 6. *Electron micrograph of a section of ruthenium red-treated cells of Butyrivibrio sp. (strain C_3) showing cell-to-cell adhesion mediated by the "knobs" formed by the extracellular material.*

chitecture of the gram-negative and gram-positive bacterial cell walls (11, 12, 14), and no truly intermediate forms have been described. Moreover, certain physiological corollaries of these different cell wall structures are widely recognized (12) (e.g., antibiotic sensitivity and periplasmic enzyme retention). These important differences in molecular architecture are reflected in morphological differences, since the phospholipids, lipopolysaccharide, and hydrophobic proteins of the gram-negative cell wall are arranged in an outer membrane (12, 14) that is very important physiologically, morphologically distinct, and absent in the gram-positive cell wall. While the distinctly trilamellar outer membrane of the gram-negative cell wall is 8.5 nm thick, the less structured fibrous mass of peptidoglycan and teichoic acid, which comprise the gram-positive cell wall, is between 30 nm (9, 16) and 50 nm (11, 20) thick. In these two strains of *Butyrivibrio*, the thickness of the cell wall varies between 12 and 18 nm, and these structures do not contain the trilamellar "membrane profile" characteristic of gram-negative cell walls, even when the specimen is tilted to alter sectional geometry or when the trilamellar pattern of the cytoplasmic membrane is clearly revealed (Fig. 5). Sharpe et al. (25) have reported a similar thickness (ca. 16 nm) for the cell wall of *Butyrivibrio fibrisolvens* strain NOR 37, and their illustration shows electron-dense inner and outer rims of the cell wall, very similar to those seen in our Fig. 5, that probably result from the incomplete penetration of the heavy-metal fixatives into the cell wall in these preparations.

Thus, it is clear that the cell walls of these two strains of *Butyrivibrio* are of the gram-positive morphological pattern, and that the cell walls of other strains of *Butyrivibrio* contain teichoic acid derivatives, which are characteristic of a gram-positive cell wall.

The only characteristic of these cell walls that may explain the definite gram-negative staining reaction of these cells is their extreme thinness. We have noted (unpublished observations) that older cells of many gram-positive bacteria stain gram negatively, and we speculate that a minimum thickness of intact gram-positive cell wall may be required to retain the Gram stain complex during decoloration. We suggest, therefore, that the cell walls of *Butyrivibrio* are of the "gram-positive" pattern in both their chemistry and their molecular architecture, as shown by morphology, but that these structures are too thin to retain the Gram stain complex and to yield a gram-positive reaction upon staining.

The ruthenium red-positive material that forms a pattern of knobs at the surface of cells of *Butyrivibrio* sp. (strain C_3) is another example of the diverse and unique array of extracellular structures formed by rumen bacteria (7–9, 13, 22). Cells morphologically similar to *Butyrivibrio* sp. (strain C_3), with the same pattern of knobs at the surface of cells, have also been observed in natural populations of rumen bacteria attached to forage cell walls (1). We have speculated (8) that these extracellular structures are involved in adhesion and protection. These knobs do appear to mediate cell-to-cell adhesion (Fig. 6), and they may mediate adhesion to cellulose fibers (1, 22), but the proposed protection of cells from deleterious agents in the environment is difficult to equate with the obvious discontinuity of the extracellular structure in this instance.

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