Ultrastructure of Cell Envelopes of Bacteria of the Bovine Rumen

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Received for publication 27 January 1975

Most of the bacteria found in rumen fluid samples taken from cows fed hay, or a concentrate diet, had cell walls of the gram-negative type. Most were intact, with only a small proportion of lysed cells, and many of the cells contained electron-translucent cytoplasmic deposits similar to the carbohydrate reserve material described in pure cultures of rumen organisms. All of the bacteria observed in these samples had an external “coat” layer outside the outer membrane when fixed in glutaraldehyde and osmium, stained with uranyl acetate and lead citrate, and examined as sectioned material. These coat layers varied from thin (ca. 8 nm) structures to very extensive fibrous systems, sometimes including concentric arrangements and radial fibers extending up to 1,200 nm from the cell. The thin-coat layers sometimes exhibited a rough periodicity. In all, 10 different types of coat layers were distinguished on a morphological basis. It is proposed that these external coat layers have protective and adherence functions for the rumen bacteria in the environment.

Mixed microbial populations of certain specific environments have been described recently by direct observation with electron microscopy (2-4, 15). Fletcher and Floodgate (15) examined marine bacteria adherent to surfaces, and Casida’s group (3, 4) described bacteria immediately after their elution from soil. A common finding in these and other studies is that each of the gram-negative bacterial cells that make up most of these populations is enclosed by an extensive and complex capsular structure external to the outer membrane. The marine organisms adhere to their substrate by a fibrous polysaccharide (15), and the capsule surrounding the soil bacteria is often fibrous in nature (2, 3).

These findings suggest that bacteria living in natural, challenging environments may depend for their survival on the production of external structures on the cell wall that dictate their adhesion pattern and provide a measure of protection for the cells (12). Salmonella typhimurium shows little external polysaccharide in shaken laboratory culture but produces a very extensive (150 nm) lipopolysaccharide microcapsule in infected tissue (26), indicating that cells in laboratory cultures may differ from cells in their natural environment. In contrast, some bovine rumen bacteria, grown in the rumen (6) or in pure cultures that have been repeatedly transferred in the laboratory (11, 23), showed the presence of layers of fibrous polysaccharide outside the cell wall or of external patterns of globular units resembling the protein coats of Spirillum (5) and many marine bacteria (29). In one case (23), the fibrous polysaccharide coat of the cells of a rumen bacterium has been shown to mediate their attachment to cellulose fibers in pure culture.

In this study, we used direct transmission electron microscopy to determine the extent to which complex cell coats are formed by bacteria within the rumen.

MATERIALS AND METHODS

Rumen contents were collected from eight fistulated cows fed a daily ration of 5.4 kg of a pelleted all-concentrate diet, or 6.2 kg of alfalfa hay, in two equal feedings. Samples were collected before the morning feeding and 4 and 8 h after this feeding on days 21, 27, and 35 after initiation of each of these diets. Rumen contents were filtered through four layers of cheesecloth and centrifuged at 48,000 × g at 4 C for 20 min. The pellet from this centrifugation contained most of the bacteria in the sample, and this pellet was prefixed for 1 h by the addition of 0.5% glutaraldehyde in 0.067 M cacodylate buffer at pH 6.8. Fixation was carried out by resuspending the material in 5% glutaraldehyde in 0.067 M cacodylate buffer at pH 6.8 for 2 h at room temperature. The material was enrobed in agar by resuspension in 4% agar at about 40 C and expressed by Pasteur pipettes.
as a cylindrical core. The cores were washed five times in the cacodylate buffer, postfixed in 2% osmium in the buffer, washed five times in the buffer, and dehydrated through a graded acetone series before embedding in Vestopal (23). Thin sections were stained with uranyl acetate (2% aqueous) and then lead citrate (25) and were carbon coated before examination, using an A.E.I. 801 electron microscope. Glutaraldehyde and osmium were obtained as concentrated solutions, under argon, and the embedding materials were kept under freon to minimize oxidation and standardize block hardness.

RESULTS

Most of the bacteria seen in about 100 rumen samples showed the gram-negative pattern of cell wall structure, and very few were seen to have a thick peptidoglycan layer similar to that seen in pure cultures of *Megaspheara elsdenii* (11) and *Ruminococcus albus* (23). All of the cells examined had outer membranes of the usual dimensions (about 8.5 nm), and all had some structure external to this double-track layer (Fig. 1–5). Ten different morphological variants of this external structure were discerned.

One of the simplest of the extracellular coats was a single, thin, electron-dense layer, separated from the outer membrane by a regular space (Fig. 1 and 2a, F). An irregular periodicity (Fig. 1 and 2a, arrows) similar to that seen in cells of pure cultures of *Bacteroides ruminicola* (11, 12) and short, irregularly spaced connectives between the coat layer and the outer membrane (Fig. 1b, C) were apparent in high-magnification electron micrographs of this structure. The absence of a fibrous coat on these cells is an important observation because it suggests that the fibrous coats seen on other cells are not the result of the nonspecific adhesion of fibers produced elsewhere in the rumen.

A relatively simple coat structure was seen in the diffuse coat of particles and fibers that appear to be anchored directly to the outer membrane of some cells (Fig. 1b, 2, 3, 4a, G). The particles were intensely electron dense and the fibers moderately electron dense, and the possibility must be considered that the particles are cross-sections of the fibers. These thin fibers extended up to 1.2 μm from the cell surface and, where similar cells were clustered, produced small areas of continuous fibrous material (Fig. 2a, P).

In a few cells, the extracellular coat consisted of a thin deposit of electron-dense material in the outer aspect of the outer membrane (Fig. 2b, H). More often, cells showed a thicker (50 nm) layer of electron-dense material (Fig. 4a, I). In both cases, this intensely stained material appeared to be composed of fine granules that were aggregated into clusters in the thicker structure.

One of the most common forms of the extracellular coat in these rumen organisms was a discrete mat of fibrous material (80 to 200 nm thick) with a distinct outer boundary (Fig. 1 and 4c, J). This fibrous coat often served to connect the cell to a piece of detritus, to a different cell (Fig. 1), to a similar cell or, rarely, to a series of similar cells (Fig. 4c).

Other types of extracellular coats, which were only rarely seen, were a highly convoluted, double-track structure outside the outer membrane with adherent bleblike structures (Fig. 2b, K), a homogeneous electron-dense mass maintained at a constant distance from the outer membrane by radial connective structures (Fig. 4b, L), and a thick electron-dense layer with thick and irregular radiating fibers (Fig. 5, M). Small numbers of cells in these rumen samples were encased by a single, thin, electron-dense layer, with apparent periodicity in tangential section. This layer was maintained.
Fig. 3
FIG. 4
Fig. 5
at about 75 nm from the outer membrane by radial fibers that extended to it and beyond it into the meninstrum (Fig. 3, N). A few cells were enclosed by two concentric electron-dense layers maintained at considerable distance from the outer membrane, and from each other, by radial fibers (Fig. 4a and 5, O).

Sectioned material is not ideal for the study of adhesion, but the fibrous extracellular coats of bacteria often appeared to mediate an adhesion of these rumen bacteria to food particles.

In the bacterial rumen populations, we always found some cells that contained electron-transparent masses (Fig. 2b and 4b) within their cytoplasm. Each of these masses, like the α-1,4 glucan deposits (9) seen in cells of a pure culture of a rumen organism (M. elsdenii), were delimited by a single electron-dense layer. Because cells of all physiological ages were present in these samples, the appearance of their cytoplasm and the degree of condensation of their nucleoids was highly variable, but very few lysed cells were seen.

**DISCUSSION**

The relationship between a microbial population and its environment is mediated by the cell envelope of the bacterial cells. The cell envelopes of bacteria growing in the normal bovine rumen are predominantly of the gram-negative type, and all have additional cell coats outside the outer membrane. The bacteria of freshwater environments are also predominantly gram negative (M. Franklin, “Hotpack” lecture of Canadian Society of Microbiologists, Montreal, 1974), as are those of marine environments (17), and many of these bacteria have been shown to possess extracellular coats of fibrous carbohydrate (15, 18) or of globular protein (5, 29).

Many enteric pathogens have been shown to produce externally located carbohydrate materials (16), and lipopolysaccharide, which is a component of the outer membrane, is actively shed into the medium (19, 30) in shaken batch culture or accumulated around the bacterial cells in a capsular form in infected tissue (26). Similarly, bacteria eluted directly from the soil are often surrounded by a mat of fibrous material (2–4) that forms an enclosing capsule, and gliding bacteria exude a slime (22) that is important in their motility (13).

Thus, it is clear that many bacteria can produce and assemble complex and often extensive coat layers on the outer surface of their already complex gram-negative cell wall (12). These gram-negative cell walls by themselves confer protection from antibodies (24), antibiot-

ics (20), and other hazards of microbial life (12) and also maintain a molecular environment so that cell wall-associated enzymes are conditioned (28) and protected (8). Part of this protection is provided by the limited penetrability of the outer membrane, but the Donnan effect exerted by ions bound within the structural molecules that constitute the cell wall is also important in conditioning the molecular environment within the cell wall and in limiting the access of extraneous molecules and ions to the cytoplasmic membrane (12). Coat layers have been observed to confer protection from attack by predatory bacteria (Bedellovibrio) (F. L. A. Buckmire, Bacteriol. Proc., p. 43, 1971) and to inhibit phagocytosis (14). Whether coat layers are composed of carbohydrate or of protein, they must be expected to contain bound ions that would act in the manner of a complex ion exchange resin to further condition the molecular environment of the cell envelope and to limit its penetrability (12). Cell coats are also sometimes important in the adhesion of bacteria to surfaces in their environment (10, 18). At least one species of rumen bacteria adheres to cellulose fibers by means of its polysaccharide coat layer (1, 23), and the secondary and irreversible attachment of aquatic bacteria to surfaces is a function of their production of a carbohydrate material (10, 15, 21). That this attachment may be of physiological and ecological significance is indicated by the finding that Myxobacteria must adhere to the surface of blue-green algae for the enzymes associated with their cell wall to digest the cell walls of the algae (27).

The predominance of gram-negative bacteria with extracellular coat layers in these environments may also result, in part, from their content of wall-associated enzymes. These enzymes have been shown to be located in the periplasmic space and at the cell surface of gram-negative cells (12), and some rough strains of S. typhimurium release an alkaline phosphatase-lipopolysaccharide complex into their environment (19). Studies of pure cultures of rumen organisms have shown that one “marker” enzyme (alkaline phosphatase) for the wall-associated group of enzymes is tenaciously bound to structural elements in the periplasmic space (7). The retention of degradative enzymes within the gram-negative cell wall and at its surface allows the enzymes access to external “food” molecules, even if these are insoluble polymers, and prevents the loss of these enzymes into the meninstrum. The activity of these enzymes provides products that are spatially very close to the permeases that will
transport them into the cell and that are vital to cellular growth.

Thus we find that the predominant bacteria of the bovine rumen have a gram-negative cell wall with an additional external cell coat. This cell coat, which may be composed of protein or of carbohydrate, may function in adhesion of the cells to surfaces, and the whole cell envelope probably functions in the protection of the cell and the retention of cell wall-associated enzymes. This external coat layer takes 10 morphological forms in the material we examined and, although there is a possibility that capsules may change as the cells age (9), further studies indicate that there is an even greater variety of distinct capsular types among rumen bacteria.

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