Electron Microscopic Examination of Corynebacterium ovis

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Corynebacterium ovis (C. pseudotuberculosis) was examined by electron microscopy after being subjected to various methods of fixation. The organism exhibited a fine structure similar to other corynebacterial species in the appearance of its cell wall, plasma membrane, nuclear apparatus, cytoplasmic matrix, wealth and complexity of intracytoplasmic membrane systems, and polysphate granules. An outstanding structural feature was the existence of an electron-dense, floccular layer external to the cell wall which both ligroin and acetone-methanol extractions demonstrated to be the previously postulated surface lipid of this organism. The only variations in structure evident between virulent and attenuated strains was a quantitative difference in the thickness and appearance of the surface lipid. The observation of this layer provided a basis for explaining the surface properties of C. ovis, with particular respect to its clumping capacity in suspension, the waxiness of its growth on solid media, and its ability to grow as a pellicle on suitable liquid media. The variation in the visible amount of surface lipid between the virulent and avirulent strains adequately explained the divergence of these three surface properties between the strains.

In the past 8 years, the ultrastructural morphology of a number of bacterial species within the family Corynebacteriaceae has been described, including those of the human and animal pathogen Listeria monocytogenes (5, 15), the human disease agents Corynebacterium diphtheriae type gravis (9) and C. diptheriae type mitis (17), C. minutissimum, the causal agent of human erythrasma (13, 14), a plant pathogen C. fascians (6), and the nonpathogenic C. xerosis (21). Known pathogens of domestic animals belonging to the genus Corynebacterium have received little attention in ultrastructural studies however.

Corynebacterium ovis (C. pseudotuberculosis), the causal agent of caseous lymphadenitis in sheep and ulcerative lymphangitis in horses, displays certain unusual characteristics such as the marked propensity for organisms to adhere together closely as firm aggregates in suspension. This property is doubtless associated with the waxy integrity possessed by colonies grown on solid media and with the characteristic ability of C. ovis to grow as a surface pellicle in some liquid media and as submerged clumps or balls in others (3). It has been shown that the organism possesses a high percentage of easily extractable, and so presumably surface, lipid, which on removal with ligroin does not impair viability (2, 4). Jolly (8) has drawn attention to the possibility that such a large amount of surface lipid might provide the bacterium with a structural barrier against the antibacterial mechanisms of phagocytic host cells. An electron microscopic examination of C. ovis was undertaken to determine the existence of a structural basis for some of the physical and pathogenic properties of this organism.

MATERIALS AND METHODS

Organisms and culture. Three strains of C. ovis were investigated, one virulent and two attenuated, all initially recovered from sheep in New South Wales. The attenuated strains 137E and 133A were derived from virulent strains by repeated in vitro passage on solid media. They retained some degree of pathogenicity in comparatively high doses, however. The virulent strain 137C/2 was uniformly hemolytic on sheep blood-agar and strongly toxigenic in suitable media. Strain 133A was also uniformly hemolytic on sheep blood-agar and toxigenic, but less so than 137C/2. Strain 137E was neither hemolytic nor toxigenic. Colonies were freshly subcultured from lyophilized preparations onto sheep blood-agar with or without a 0.04% content of sodium tellurite, tryptose agar, or viande-
foie broth. The period of culture varied from 24 to 96 hr.

**Preparation of specimens for electron microscopy.** Small colonies cultured on solid media were removed in toto and prepared according to the procedure of Kellenberger's standard fixation (10). Samples of surface pellicle growth were handled in the same way, but other broth cultures were centrifuged to produce small pellets. The organism was also examined after fixation in 0.6% potassium permanganate in veronal acetate buffer (12) and after fixation in 6.25% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 hr followed by postosmification for 2 hr in 1% OsO₄ in veronal buffer. Some colonies were immersed in a sterile solution of ligroin and mechanically agitated for 5 hr prior to washing in Kellenberger's buffer and subsequent fixation. After post-fixation staining in 0.5% uranyl acetate, the specimens were dehydrated in a graded series of alcohols followed by propylene oxide, and thence into a 60-40 mixture of Araldite and propylene oxide for up to 12 hr. Infiltration of the samples was allowed to continue for 16 hr in Araldite before polymerization. Sections were cut on either an LKB ultramicrotome or Porter-Blum MT-2 microtome with glass knives. All sections were stained on the grid with Reynold's lead citrate (18).

Bacterial cell walls were prepared in a Mickle disintegrator by the method of Salton and Horne (19) from both a fresh culture of 137C/2 and a freeze-dried, acetone- and methanol-extracted strain of virulent 133. Cell walls were spun out at 10,000 × g, and a Gram stain on a smear of the final pellet showed 90% of the content to be cell wall ghosts. Samples of these preparations were negatively stained in 1% aqueous phosphotungstic acid, according to the method of Brenner and Horne (1).

**Electron microscopy.** Specimens were examined in a Philips EM 200 microscope at 80 kv with a 50-μm objective aperture. Micrographs were made on Kodak projector slide plates (contrast grade) and enlarged photographically.

**RESULTS**

The bacterial shape most commonly observed was squatly ovoid (Fig. 1), but elongate forms including cigar-shaped and club-shaped individuals were also frequent (Fig. 2). Viewed in cross section, bacteria were round or oval. Dimensions varied from 0.55 by 0.40 μm in the shorter forms to 1.2 by 0.50 μm in the longest organisms.

An outstanding characteristic of the ultrastructural appearance of *C. ovis* was the existence of an electron-dense layer, external to the cell wall, surrounding each organism. The layer con-

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**FIG. 1.** Cell (in early division) of the virulent *C. ovis* strain 137C/2 from a 24-hr culture on tellurite-blood-agar. A thick, electron-dense layer of lipid (arrows) surrounds the cell wall. Denser areas of cytoplasm border the nuclear apparatus. *T* indicates crystals of tellurite reduction product. × 74,500.

**FIG. 2.** Dividing cell of the attenuated strain 137E cultured in viande-foie broth for 48 hr. An external lipid layer is present (arrows) but is not as prominent as that of the virulent organism. × 74,000.
sisted of floccular material, usually separated from the cell wall itself by a narrow clear space of about 4 to 8 nm. There was considerable variation in the thickness of this layer within individual organisms, but particularly between strains. The zone was widest and most dense in the virulent strain 137C/2, ranging from 7 to 37 nm with an approximate mean of 18 nm (Fig. 1). In the attenuated strains, the range of width was 2.5 to 25 nm (approximate mean width, 10 nm) in 137E (Fig. 2) and 3 to 18 nm (approximate mean width, 9 nm) in 133A. Adjacent organisms were aggregated together by the enveloping contiguity of this layer, which was common to all bacteria within the clump. In colonies of strain 137C/2 washed in ligroin for 5 hr, the external dense layer in a great majority of organisms was either very difficult to see or had disappeared altogether (Fig. 3). In bacteria still retaining remnants of the layer, its density and thickness were markedly diminished. Negatively stained preparations of the pure cell wall samples, from which the presumably surface lipid had been extracted with acetone and methanol prior to disintegration, demonstrated the existence of an intricately etched pattern over the entire exterior. Convoluted and intertwining bands of low electron density (about 5 nm wide) separated by similar bands of higher density made up the overall "crazed" surface pattern (Fig. 4a). Cell walls prepared from fresh, unextracted organisms were clumped together by an enveloping material which had the appearance of lipid and which obliterated the surface characteristics of the wall itself (Fig. 4b).

The detailed structure of cell wall, plasma membrane, polyphosphate granules, intracytoplasmic membrane systems, and nuclear apparatus were identical with those described previously for C. minutissimum (13, 14) and C. diphtheriae (9), including the appearance of an irregular, dense, granular perinucleoid band of cytoplasm, as noted by Kawata (9) in the latter species. The basic pattern of the mesosome consisted of two closely apposed unit membranes representing an invagination of the complete tripartite structure of the plasmalemma; thus, it conformed in detail to the diagrammatic representation of these organelles provided by Pate and Ordal (16) in their ultrastructural study of Chondrococcus columnaris (Fig. 5). The elaborate structure of membrane systems and their common association with invaginating septa of cells in division were consistent with previous descriptions of a number of gram-positive bacteria (7).

Cell division normally occurred across the center of the bacterial cell, but in a small proportion of dividing bacteria a second or third invaginating septum was observed. The line of division was not always in one plane but often assumed a haphazard pattern. Figure 6 shows a more extreme example with multiple septa. It appeared that as division occurred, daughter cells adhered together by virtue of the cohesive properties of the surface material. Thus, large numbers of organisms of the strain 137C/2 were often involved in the formation of individual clumps, whereas aggregations of 137E contained fewer cells, and only 2 to 4 neighboring bacteria of strain 133A were usually clumped together within a common surface envelope.

In bacteria cultured on blood agar containing sodium tellurite, small black crystals appeared in the cytoplasm subjacent to the plasma membrane (Fig. 1). The demonstration of large crystals occupying a major proportion of the cytoplasm of apparently unaffected organisms after culture for 3 days substantiated Winkler's comment on the ability of Corynebacterium to store and tolerate a considerable quantity of tellurite (24).

Apart from the details pertaining to the surface lipid layer, no other structural differences were observed between the virulent and attenuated strains of C. ovis. The only morphological changes
Fig. 4. Negatively stained cell wall preparations. (a) Cell wall of virulent *C. ovis* prepared from organisms which had been subjected to extraction with methanol chloroform. The spheroplast presents an intricate surface pattern from which the lipid coating is absent. Only clumps of cytoplasmic debris are adherent (arrow). × 58,000. (b) In contrast, cell walls prepared from unextracted virulent organisms are covered with a lipidlike material which obscures the surface pattern and is responsible for the clumping together of the spheroplasts. × 58,000.

Fig. 5. Bacterium of strain 137C/2 containing a mesosome which arises as an invagination of the tripartite plasma membrane. × 156,000.
noted with an increasing period of culture were an increase in polyphosphate granule content and a decrease in mesosomes.

DISCUSSION

The outstanding feature of this study was the observation of an electron-dense layer around each organism, which was responsible for the adherence of many bacteria into clumps. That this layer represented the surface lipid, the existence of which had been postulated on the basis of extraction studies (2, 4), was verified by its partial or total removal by lignoin or acetone and methanol treatment. Lacave et al. (11) recently characterized the lignoin extract of C. ovis and found that the bulk consisted mainly of sodium corynomycolate together with sodium salts of C16, C18, and C19 fatty acids. Some triglycerides and free fatty acids were also detected. Jolly (8) has shown that quantitative differences exist between the surface lipids of virulent and attenuated strains, which supports the hypothesis that they are associated with pathogenicity and virulence. He found strain 137E to contain 28% less extractable lipid than 137C/2, although there was no strain difference in the toxic quality of the material. Morphological substantiation of Jolly's work was demonstrated by the quantitative difference in thickness and appearance of the surface layer in the three strains, with a decreasing sequence of 137C/2 through 137E to 133A.

A further manifestation of the variation in surface lipid was observed as a greater clumping effect in 137C/2, involving more organisms in each aggregate than were present in the smaller clumps of the attenuated strains. Such an observation explains some of the differences in surface properties of the strains. For example, the more hydrophilic growth of the attenuated varieties on solid media, in comparison with the waxy integrity of virulent colonies, is doubtless a reflection of the quantitative difference in surface lipid and consequent clump-size. The same observations have application to the variation between strains in ability to grow as a pellicle in some liquid media. The fact that light extraction does not impair the viability of the organisms (4) further suggests that only the surface layer is removed. The observation of this material also provides a basis for the hypothesis that the bacterium may possess a barrier which protects it from post-phagocytic degradation by the host cell's lysosomal enzyme complement.

The existence of material in some form or other, external to the cell wall, has been recorded in most other taxonomically related bacteria so far studied. Material of unknown composition at the periphery of the cell wall of L. monocytogenes has been reported (5, 6), Glaeuer (6) described a "thick capsular layer" composed of an extensive network of fibrous material with little organized structure around the cell wall of C. fascians. A moderate amount of floccular material was found adherent to the external surface of C. diphtheriae (17), whereas extremely electron-dense material surrounded C. minutissimum and was usually closely adherent to the cell wall (13, 14). Stanley and Rose (21) observed that C. xerosis exhibited a clumping capacity when it was grown at 30°C and promptly cooled to 15°C with rapid stirring. Electron microscopy showed that electron-dense material distributed over the bacterial surface was responsible for adherence of these organisms into clumps. Further study indicated that the material responsible for the clumping of C. xerosis was a cell-surface protein. Different types of cell-surface component have been associated with the manifestation of clumping in other microorganisms. Such surface material includes carbohydrate in Escherichia coli (20), protein in the yeast Hansenula wingei (22), and a hyaluronidase-sensitive polysaccharide in Pseudomonas aeruginosa (23). The demonstration of a lipid component responsible for the surface properties of C. ovis
adds a further example to the list and contrasts this organism with \textit{C. xerosis}. In contrast to most related bacteria which possess a morphologically demonstrable surface coat, the external lipid layer of \textit{C. ovis} was not usually adherent to the cell wall; in fact, it appeared to be separated from that structure by a clear space. That this zone of separation might have constituted an artifactual change cannot be discounted, but it is noteworthy that not only was this characteristic a consistent finding in most bacteria examined but also its presence was a feature of viable intracellular organisms some hours after ingestion by mouse phagocytes (unpublished data).

The frequently irregular disposition of the dividing septa of \textit{C. ovis}, together with the surface properties of the external lipid layer, are doubtless responsible for the palisade and Chinese-letter groupings which characterize these organisms in smears and histological sections. Similar oblique and wavy septa have been described in \textit{C. diphtheriae} (17), and they most probably constitute a corynebacterial characteristic.

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LITERATURE CITED