Electron Microscopy of a Strain of *Bordetella bronchiseptica*

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A strain of *Bordetella bronchiseptica* that had been isolated from a rat hepatoma cell culture was investigated by means of electron microscopy. Bacteria were examined after (i) negative staining with phosphotungstate or uranyl acetate, (ii) metal shadowing with platinum-palladium, and (iii) fixation with glutaraldehyde followed by embedding, sectioning, and staining. The multilayered bacterial cell walls appeared lobulated in negatively stained and in metal-shadowed specimens; the lobules were demarcated by grooves, 100 to 200 Å in width, but without interruption of continuity in any layer of the cell wall. Cross sections of fixed material revealed wrinkled cell walls in many—but not all—preparations. Bacterial cell membranes and cytoplasm were similar to those of other gram-negative bacilli (e.g., *Escherichia coli*). Bacteria fixed in 1.5% glutaraldehyde contained intertwined or whorled fibrils, down to about 20 Å in thickness. The flagella were peritrichous, measured about 200 Å in width, and were composed of braided strands, about 20 Å in width.

In the course of experiments with rat hepatoma cells cultured from a large, necrotic tumor in a Wistar-Sherman rat, a strain of *Bordetella bronchiseptica* was isolated as an unexpected contaminant. Since the hepatoma cells grew well in the presence of the bacteria and displayed considerable endocytic ("phagocytic") activity, the commensal system was characterized further (18), and the fine structure of the bacteria was investigated. To our knowledge, there have been no publications of studies on the fine structure of *B. bronchiseptica*, and we have, therefore, made a detailed study.

MATERIALS AND METHODS

Isolation and identification of the bacteria. The bacteria were isolated directly from cultures of rat hepatocarcinoma cells (strain R-21) that had been divided and subcultured three times in modified Eagle's medium. The organisms were routinely cultured on ordinary Nutrient Agar (Difco) at pH 6.8 and 37 C. They also grew well on blood-agar, in nutrient broth, and in media enriched with calf serum or horse serum. They are gram-negative, motile rods that most often grow in small, round, smooth, glistening, gray colonies. However, some colonies had rough and raised or heaped-up centers (Fig. 1). This was particularly striking when bacteria were obtained directly from tissue culture media containing penicillin (100 units/ml) and streptomycin (100 mg/ml).

To identify the organisms, fermentation and agglutination reactions were done with R-21 bacteria and with three certified strains of *B. bronchiseptica* (785, 4617, and 14065) provided by the American Type Culture Collection.

To obtain antisera to the various strains of bacteria, vaccines were prepared and injected into rabbits as follows. Cultures grown on Nutrient Agar slants for 2 days were washed off with sterile 0.9% aqueous NaCl solution to which Formalin had been added so as to give a final concentration of approximately 0.25% formaldehyde. The resulting suspensions were shaken, incubated at 56 C for 1 hr in sterile bottles, then stored at 4 C. After 2 days, the suspensions were tested for sterility and if sterile, were used for immunization of rabbits after appropriate dilution with physiological saline. A 2-ml amount of vaccine containing approximately 1×10^9 organisms per ml was twice injected subcutaneously into healthy white rabbits, with a 10-day interval between the two injections. Antisera were obtained 2 weeks after the second injection. Serum agglutination tests were done in conventional serial dilutions, with either the vaccines or suspensions of live bacteria in saline as antigens. Immobilization of live bacteria by antisera was checked microscopically.

Results of identification tests are summarized in Tables 1 and 2. R-21 bacteria had essential characteristics of the reference strains of *B. bronchiseptica*, and they gave appropriate serological cross reactions. Problems of identifying bacteria belonging to this or to related species have been analyzed by Proom (16), Beer (3), and others.

Preparation for electron microscopy. Samples of bacteria were obtained in several different ways. (i) Cultures were washed off agar slants with glutar-

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aldehyde (*see below*). (ii) Cultures were seeded in cooling, liquid agar by inoculation at approximately 40 C. When this was done, colonies usually grew into the agar. After 18 to 48 hr, colonies were cut out as blocks and were fixed as such. (iii) Bacteria grown in nutrient broth or in tissue culture media were pelleted by centrifugation, then suspended in fixative.

As a general rule, agar blocks or pellets of bacteria were fixed for 3 hr in 1.5% redistilled glutaraldehyde in Millonig's phosphate buffer at pH 7.2 (15). The fixed material was rinsed four times with the buffer, at intervals of 15 min, and was centrifuged after each rinse; afterwards, it was kept in the buffer overnight. Then the samples were kept in 1% OsO₄ in Millonig's

FIG. 1. Colonies of rough variants of *Bordetella bronchiseptica* R-21. Note heaped-up centers. Bacteria in these centers often showed disintegration of cell walls. $\times 13$.

TABLE 1. Comparison of *Bordetella bronchiseptica* R-21 and of three other strains of *B. bronchiseptica*^a

Test	R-21	785 ^b	4617 ^b	14065 ^b
Colonial morphology	Small, round convex, smooth ^c , glistening, gray	Similar to R-21	Similar to R-21	Similar to R-21
Motility	+	+	+	+
Flagella	Peritrichous ^d	Peritrichous ^d	Peritrichous ^d	Peritrichous ^d
Blood-agar	No hemolysis	No hemolysis	No hemolysis	No hemolysis
Urea	+	+	+	+
Lactose	—	—	—	—
Litmus milk	Alkaline	Alkaline	Alkaline	Alkaline
Dextrose	— ^c	—	—	—
Maltose	— ^c	—	—	—
Gelatin	Not liquefied	Not liquefied	Not liquefied	Not liquefied
Catalase	+	+	+	+
Indole	—	—	—	—
Phenylalanine deaminase	—	—	—	—
Niacin-free media	No growth	No growth	No growth	No growth

^a Reproduced in part from Richter and Kress (18).
^b Obtained from the American Type Culture Collection.
^c Rough variants have been isolated, which fermented dextrose and maltose.
^d As determined by electron microscopy of specimen replicas.

TABLE 2. Agglutination titers of R-21 bacteria and of certified strains of *Bordetella bronchiseptica* and *B. pertussis*

Antigens	Titers of rabbit antiserum					
	R-21	4617	<i>B. pertussis</i> (Parke-Davis)	<i>Pseudomonas diminuta</i> , strain 2284-3	<i>Alcaligenes faecalis</i> , strain 337	Two control sera
R-21 bacteria Formolized vaccine	1:4,096	1:128	Negative	Negative	Negative	Negative
<i>B. bronchiseptica</i> Formolized vaccines						
Strain 785	1:4,096	1:4,096	1:4,096	Negative	Negative	Negative
Strain 4617	1:4,096	1:4,096	1:4,096	Negative	Negative	Negative
Strain 14065	Negative	1:128	Negative	Negative	Negative	Negative
<i>B. pertussis</i> Formolized vaccine (Parke-Davis)	1:128	Negative	1:4,096	Negative	1:256	Negative

buffer at pH 7.2 for 1 hr. After another centrifugation, the blocks or pellets were dehydrated in graded concentrations of ethyl alcohol, then in propylene oxide. This was followed by embedding in Epon 812 epoxy resin, essentially according to methods of Luft (14). After polymerization, sections were cut, mounted on carbon substrates, and stained with 7% aqueous uranyl acetate solution for 30 min and with Reynold's lead citrate stain (17) for 15 min. Unstained sections and sections treated only with the uranyl or only with the lead stain were also examined.

For negative contrast staining, either one of two procedures was followed. (i) Bacteria were washed from colonies as desired, and suspended in 1% aqueous sodium phosphotungstate solution at pH 7. Drop-lets of the suspensions were allowed to dry on grids coated with Formvar. (ii) Suspensions of bacteria were dried on specimen grids coated with Formvar. These specimens were stained with 2% aqueous uranyl acetate solution (pH 5.0) by floating the inverted grids on the surface of the stain for 15 to 30 min. The grids were then rinsed briefly with distilled water.

Bacteria were also examined after evaporating a thin film of metal upon them. For this purpose, platinum-palladium or platinum-carbon was evaporated at various angles onto bacteria that had been dried on Formvar-coated specimen grids.

Electron microscopy was done with a Siemens Elmiskop 1, operated at 80 kv, or with an RCA EMU-3B, operated at 50 kv. Magnifications were calibrated by means of ferritin (5).

RESULTS

General observations. The bacteria vary considerably in length, less in width. For example, some of the diameters measured in two dimensions are 3 by 0.5 μ , 1 by 0.5 μ , and 0.5 by 0.4 μ . Most are roughly ellipsoidal and, in general, possess peritrichous flagella (Fig. 2 and 3). Our observations were made mainly on organisms that had been grown on solid media. With the

exception of bacteria from senescent colonies or from colonies with rough centers, the organisms displayed little pleomorphism when grown on solid media.

Bacterial cell wall. Figure 2a shows bacteria covered with evaporated platinum-palladium. The bacterial cell surface appears wrinkled (Fig. 2b), with lobules, up to several hundred Angstroms in width, that are separated by furrows (about 100 A to 200+ A in width). This appearance may be an exaggeration of the true state owing to dehydration or to collapse of bacteria in the vacuum evaporator. However, a similar structure is revealed in preparations stained with phosphotungstate (Fig. 4). In cross sections of bacteria fixed in glutaraldehyde and processed as described, the cell wall has a multilaminar structure: three osmiophilic layers, alternating with less opaque layers (Fig. 5-7).

As seen in perpendicular cross sections of fixed, embedded bacteria, the total thickness of the cell wall is 100 to 120 A. Although the cell walls are furrowed (Fig. 5 and 6), they appear to be continuous in all layers. Additional evidence was obtained by examination of bacterial cells freshly isolated from tissue culture medium and grown on Nutrient Agar for 5 to 7 days. Grossly, these cultures often displayed furry (rough) centers (Fig. 1).

Cell membranes. In cross sections of bacteria fixed either in glutaraldehyde or in buffered solutions of OsO₄, the cell membranes have the usual trilaminar structure with the following dimensions: thickness of outer and inner opaque layers, 20 to 30 A each; thickness of nonopaque intermediate layer, about 15 A. Structures resembling chondrioids (mesosomes) of other

FIG. 2. (a) *Bordetella bronchiseptica* R-21 bacteria in this electron micrograph are coated with platinum-palladium. Note lobular and furrowed surface structure which is shown at higher magnification in the inset (b). Grooves measure about 100 Å in width. (a) $\times 25,000$; (b) $\times 75,000$.

FIG. 3. (a) Negative contrast preparation of *Bordetella bronchiseptica* R-21 indicating peritrichous arrangement of flagella. Note furrows in bacterial surface (f). Some of these are shown at higher magnification in b. Stain: 2% sodium phosphotungstate at pH 7.0. (a) $\times 20,000$; (b) $\times 75,000$.

bacterial species were encountered very infrequently.

Bacterial cytoplasm and nucleoid. The appearance of the cytoplasm of various bacterial species varies considerably with the mode of fixation and subsequent processing (10). Our description is based on electron micrographs of *B. bronchiseptica* after fixation with glutaraldehyde, postfixing with OsO_4 , and staining with either or both uranyl acetate and lead citrate. In such preparations, ribosomes are abundant and

are distributed throughout the cytoplasm, but are absent from the nucleoid—whether compact or fibrillar (Fig. 5 and 6). When the uranyl stain is omitted, the particles identified as ribosomes appear indistinct, as is true of ribosomes in other species. The cytoplasmic matrix was poorly defined in the material examined.

The nuclear zones of *B. bronchiseptica* R-21 are quite distinct and consist of whorls or of a network of fibrils (deoxyribonucleic acid?)

FIG. 4. Surface furrows of *Bordetella bronchiseptica* R-21. Note that parts of cell walls are seen not only en face but also edgewise, in various profiles, some of which (arrow) suggest partial collapse of bacteria on carbon substrate. Sodium phosphotungstate stain, pH 7.0. $\times 25,000$.

FIG. 5. Longitudinal cross section of *Bordetella bronchiseptica* R-21. Note furrowed cell wall. Ribosomes are disposed in a well-demarcated zone all around the nucleoid. The latter appears as a coarsely fibrillar mesh that contains some granular condensations. Fixed in glutaraldehyde, postfixing with osmium tetroxide, and further processed as described in text. $\times 40,000$.

FIG. 6. In this electron micrograph of a section of *Bordetella bronchiseptica*, retraction (shrinkage?) of the bacterial cell membrane, *m*, and cytoplasm from the cell wall, *c*, is evident in several bacteria. Profile, *L*, at top of picture may represent an L-form or "spheroplast." Note that this body possesses a trilaminar membrane and ribosomes. Bacterium, *B3*, at lower right corner contains an intracytoplasmic structure resembling a membrane. Appearance of nucleoid of bacterium, *B2*, suggests cross section of a fibrillar whorl. Prepared as for Fig. 5. $\times 80,000$.

FIG. 7. Detail of bacterial envelopes. *m*: trilaminar cell membrane; *a*, *b*, *c*: three most clearly visible layers of cell wall. Prepared as for Fig. 5. $\times 400,000$.

and of dense bodies (Fig. 5 and 6). The thinnest fibrils measured about 20 to 30 Å in width, but the width of most fibrils ranged between 50 and 70 Å. The mesh of fibrils seen in many cross sections may represent disorder due to fixation, since more regular arrangements of fibrils, suggesting sectioned whorls or spirals, were also encountered. The large, dense bodies in many nucleoids were found to take the uranyl stain. In electron micro-

graphs of whole, unsectioned bacteria, dense bodies were also visible, when contrast conditions were favorable (Fig. 4). That these dense bodies were situated underneath the bacterial surface, was indicated by the finding that surface furrows of the cell wall, outlined by phosphotungstate, could be seen to traverse the profiles of dense bodies (Fig. 4).

Flagella. The general arrangement of flagella around the bacteria is shown in Fig. 2 and 3. The flagella are distributed peritrichously about bacterial bodies, though perhaps they are sparser at the poles than elsewhere. The fine structure of these flagella resembles that of flagella of some other gram-negative bacilli, e.g., *Pseudomonas aeruginosa*, *Salmonella* sp., *Proteus vulgaris* (11-13). In negative-contrast preparations, the thickness of flagella was generally between 180 and 220 Å, and these flagella were multistranded (Fig. 9 and 10). Images of metal-coated flagella indicate a "braided" structure (Fig. 8). In negative contrast preparations, we observed profiles

FIG. 8. Flagella coated with platinum-palladium. They appear as braids; this indicates the presence of interlacing strands. $\times 125,000$.

FIG. 9. Profile of part of flagellum of *Bordetella bronchiseptica* R-21, negatively contrasted with uranyl acetate at pH 5. Note interlaced strands which measure approximately 20 Å as seen in this projection. $\times 500,000$.

of five to six strands per flagellum. This variation is probably due to differences in orientation on the specimen grids. The exact number of strands present cannot be determined from the electron micrographs, but, from all appearances, it is reasonable to conclude that the strands are interlaced. Measurements from negative contrast specimens (Fig. 9 and 10) indicate strand widths of about 20 Å.

DISCUSSION

The fine structure of *B. bronchiseptica* R-21, as revealed by our studies, closely resembles that of other gram-negative bacteria. Briefly stated, the findings indicate that the organism possesses: (i) a cell wall composed of five or more layers, of which at least the outer three often give the surface contours a lobulated appearance; (ii) a trilaminar cell membrane; (iii)

abundant ribosomes embedded in (as yet poorly defined) cytoplasmic matrix; (iv) nucleoid material consisting of fibrils and of dense bodies; and (v) peritrichous flagella composed of interlacing strands.

Hatasa described the fine structure of a Japanese strain of *B. pertussis* (7, 8). He stated that in sectioned bacteria the cell wall possesses three "electron-dense" layers, outer, median, and inner. The outer and inner layers each measured 15 to 20 Å in thickness, and the median layer, 20 to 30 Å. Evidently, he did not note intermediate, less dense layers. The plasma membrane possessed the usual unit membrane structure. Flagella were not found. About 1% of the organisms examined possessed intracellular crystals of undetermined nature. Intracytoplasmic membranes (mesosomes?) were also observed. The cytoplasm appeared dense, with

FIG. 10. In this negative contrast image, a selected part of a flagellum (which was attached to a bacterium elsewhere in the preparation) is seen over a hole in the supporting carbon film. Profiles of five strands run in parallel, but since they are notched, they probably represent projections of interlaced strands. Uranyl acetate, pH 5. $\times 500,000$.

a "microgranular structure." The nucleoid presented no striking or unusual features.

For purposes of comparison, the best model at the present time is still *Escherichia coli*, mainly because its fine structure has been elucidated in great detail and by a variety of methods. In spite of the considerable biological variations in strains of *E. coli*, the fine structure of organisms of this species can serve as a paradigm.

The cell wall of *B. bronchiseptica* R-21 is similar in fine structure to that of various strains of *E. coli*, as reported, for example, by dePetrís (4) and by Bayer and Anderson (2). dePetrís found a multilaminar structure in thin sections of cell walls of *E. coli*, but the contours of the cell walls appeared relatively smooth (4). However, using negative-contrast methods and quick-freezing techniques, Bayer and Anderson (2) were able to demonstrate a lobulated structure of the cell wall in several strains of *E. coli* and, in addition, the presence of channels between the lobules. Although the methods of preparation employed by Bayer and Anderson differed significantly from ours, the organization of cell wall structure revealed by them for *E. coli* closely resembles that of *B. bronchiseptica* R-21. Bayer and Anderson have proposed that the outermost cell wall layer of *E. coli* is composed of lipoprotein in an array of patches or "sausagelike structures," interrupted by grooves or channels that extend into an underlying lipopolysaccharide layer, which is thus exposed to the environment about the bacteria. However, such discontinuities are not visible in fixed, sectioned *E. coli*. Our observations on cell walls of *B. bronchiseptica* R-21 are similar to those reported for *E. coli*, but we found no discontinuities in the cell walls. The cell wall lobulations in *B. bronchiseptica* R-21 are irregular, and it might be thought that they resulted entirely from shrinkage of bacteria during processing, even in negative-contrast preparations. If the grooves seen in negative contrast

preparations are a result of drying, their location and pattern may nevertheless be determined by fundamental properties of the cell wall.

The electron micrographs of cell walls of *B. bronchiseptica* sectioned after fixation, dehydration, and embedding clearly indicate continuity of all layers, and this observation is hard to fit into the structure proposed by Bayer and Anderson (2) for *E. coli*, according to which there are discontinuities in the outermost "lipoprotein" layer of the cell wall.

Our findings on the cell membrane of *B. bronchiseptica* R-21 are in agreement with the prevailing concept that bacterial cell membranes are trilaminar structures, about 50 to 70 Å in total thickness. As we observed, the cell membrane is separable from the covering cell wall (Fig. 6).

Cytoplasmic constituents and nucleoid of *B. bronchiseptica* R-21 display fine structure similar to that of other gram-negative bacteria (6, 9, 19).

The basic organization of flagella of *B. bronchiseptica* is that observed in other bacterial species: strandedness and helical configuration. Lowy and Hanson (13) have described two general types of flagellar structure, as revealed by negative-contrast staining. "Type A" consists of "helically connected globules, aligned in longitudinal rows." "Type B" gives images of longitudinal strands running in parallel to each other. A sheath consisting of additional strands may be wrapped helically about the longitudinal strands. However, Lowy and Hanson have observed transitions from the Type A to the Type B structure in *P. rhodos*, and they have also noted unsheathed segments of flagella in this species. Differences between the appearance of *B. bronchiseptica* R-21 in shadowed preparations and in negatively contrasted specimens may be due to such transitions. The images of strands in electron micrographs represent projections of a three-dimensional object, and thus far it has not been possible for us to deduce the actual

number of strands in a flagellum from these projections. The possible number of strands and their arrangement is limited by stereochemical principles of complementarity consistent with the arrangement of atoms in each strand.

Several features we have observed suggest conversion of bacteria to L-type forms. Thus, as shown in one instance in Fig. 6, samples from bacterial colonies may contain organisms without cell walls but with a cell membrane and intact cytoplasm. Also, retraction of cell wall from the subjacent cell membrane (Fig. 6) may not always be artifact: it might indicate impending disintegration of the cell wall, which could leave a "spheroplast" or L-type form.

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