The Ultrastructural Morphologic Features of Pittsburgh Pneumonia Agent

Francis M. Gress, PhD, Richard L. Myerowitz, MD,
A. William Pasculle, ScD, Charles R. Rinaldo, Jr., PhD,
and John N. Dowling, MD

The fine structure of "Pittsburgh Pneumonia Agent" (PPA) was studied in infected human lung, guinea pig omentum, yolk sac membrane, Vero cell culture, and after cultivation of the organism on buffered charcoal yeast extract agar. The organism is a prokaryotic cell with the general features of a gram-negative bacillus. PPA is ultrastructurally distinctive because of an unusually thick, electron-dense band present within the periplasmic space adjacent to the outer membrane of the cell wall. This band, presumably a mucopeptide (peptidoglycan) layer, was seen in about 95% of organisms in human lung but less frequently under certain conditions of laboratory infection or cultivation. Future studies are required to determine whether this ultrastructural dimorphism of PPA is related to variation in other properties of this bacterium, eg, gram-variability, acid-fastness, colony morphology, and virulence. (Am J Pathol 1980, 101:63-78)

PITTSBURGH PNEUMONIA AGENT (PPA) is a newly recognized bacterium that causes purulent, often fatal pneumonia in immunocompromised patients.[1-3] The organism was originally isolated by inoculation of infected lung tissue into embryonated eggs and guinea pigs.[1] Recently, PPA was cultivated on artificial media [4,5] and was preliminarily characterized as a bacterium similar to *Legionella pneumophila,* but distinct from the latter by antigenic, biochemical, and genetic criteria. The name *Legionella pittsburgensis* has been proposed for PPA.[5] By light microscopy, PPA is a short bacillus that stains faintly gram-negative in smears and occasionally gram-variable in tissue sections. PPA is also weakly acid-fast; ie, a fraction of the organisms retain the carbol-fuchsin dye with the use of the regular (Kinyoun) acid-fast stain for tissue or the modified acid-fast stain for tissues (Fite) or smears.[1,2] When infected tissue is cultured on artificial medium, prototype colonies (Type 1) are observed, which are white and have a cut-glass appearance. After several days of further incubation, Type 1 colonies give off a second colony type (Type 2), which has a ground-glass texture and appears slightly yellow-green. Type 2 colonies are then stable on subculture.[5] Preliminary evidence (Myerowitz RL et al, unpublished observations) suggests that organisms derived

From the Departments of Pathology and Medicine, Presbyterian-University Hospital and the University of Pittsburgh School of Medicine, and the Department of Microbiology, University of Pittsburgh Graduate School of Public Health, Pittsburgh, Pennsylvania.

Supported by a grant from the Samuel and Emma Winters Foundation.

Accepted for publication April 7, 1980.

Address reprint requests to Richard L. Myerowitz, MD, Department of Pathology, Presbyterian-University Hospital, DeSoto at O'Hara Streets, Pittsburgh, PA 15213.

0002-9440/80/1008-0063$01.00

from this second colony type are less virulent for guinea pigs than those derived from Type 1 colonies.

Preliminary ultrastructural study of the organism as seen in infected human lung tissue indicated that PPA is a prokaryote and that its cell wall/membrane complex is similar to that of many other gram-negative bacilli.[2,3] Distinctive for PPA, however, was a thick electron-dense band present in the periplasmic space. This report extends these preliminary observations and describes the fine structure of the PPA under a variety of natural and artificial conditions. We present evidence that PPA is ultrastructurally dimorphic and discuss this finding with respect to the observed histochemical and microbiologic variation of PPA.

Materials and Methods

Samples of human lung tissue from patients infected with PPA were obtained at biopsy or autopsy and fixed as described.[1] Inoculation and culture of embryonated eggs were also performed as described.[1] Homogenates of infected human lung and aliquots of infected egg yolk were stored at −70C and were the sources of the infecting inocula for most subsequent cultures. Yolk harvested from embryonated eggs inoculated 3, 4, or 5 days previously with PPA was washed three times in phosphate-buffered saline (PBS, 0.01 M, pH 7.2), then fixed for electron microscopy. Excised yolk sac membranes were rinsed in PBS prior to fixation. Female Hartley guinea pigs weighing about 200 g were inoculated intraperitoneally with 0.5 ml of infected egg yolk and inflamed omentum tissue was excised and fixed 3 and 4 days after inoculation. Continuous cultures of African green monkey kidney (Vero) cells were maintained in plastic flasks with Eagles' minimal essential medium supplemented with 10% heat-inactivated fetal calf serum, twice the standard concentration of vitamins and essential amino acids, 25 U/ml nystatin, and 2 mg/ml sodium bicarbonate. Subcultures in plastic Petri dishes were inoculated with infected egg yolk, incubated at 37C in 5% CO_2 with humidity, and fixed 4 or 9 days after-inoculation. Organisms grown in artificial medium were obtained by inoculating buffered charcoal yeast extract medium [5] with a stock strain of PPA that had been previously passaged on agar medium several times (Type 2 colonies). Single colonies were harvested at 3, 4, 5, and 6 days after-inoculation and fixed for electron microscopy. All preparations used in these studies were screened for contaminating bacteria by culture of samples on sheep blood agar at 37C for 24 hours. Any preparation found to be contaminated was discarded.

Primary fixation for electron microscopy was achieved with 3% glutaraldehyde buffered to pH 7.4 with either 0.1 M sodium cacodylate containing 0.02 M calcium chloride or with 0.12 M sodium phosphate. Cells were incubated in the primary fixative for 4–24 hours at 4C. Vero cells were fixed with glutaraldehyde while still attached to their culture dishes. Thereafter, cells were scraped from their dishes with a rubber policeman and handled as cell pellets. Bacteria derived from infected yolk or harvested from agar medium were handled as centrifuged pellets after fixation. Following brief washing in the appropriate buffer containing 10% sucrose, specimens were transferred to 1% osmium tetroxide in either cacodylate or phosphate buffer. Specimens were then rinsed in 0.1 M veronal acetate buffer, pH 7.4, and stained *en bloc* in 2% uranyl acetate in the same buffer prior to dehydration in ethanol and embedding in Epon or Spurr epoxy resin. Thin sections were stained with 2% aqueous uranyl acetate and lead citrate prior to examination in a Philips EM 200 or EM 300 electron microscope operated at 60 kV.

Results

General Characteristics

Bacterial cells varied from 1.1 μ to 1.5 μ in length and from 0.3 μ to 0.5 μ in width. The fine structure of organisms examined in sections cut from pelleted egg yolk, yolk sac membranes, Vero cell cultures, infected guinea pig omentum, human lung tissues, and of organisms harvested from agar medium was typically prokaryotic. The cells contained free ribosomes, a variable number of cytoplasmic vacuoles, and occasional internal membrane configurations. There was no evidence of nuclear membranes, mitochondria, and other organelles, which are virtually ubiquitous among eukaryotic life forms. Multiplying forms in all preparations demonstrated pinching, nonseptate division.

Dimorphism of PPA

Two distinctive forms of PPA, herein designated "B" (for banded) and "U" (for unbanded) were recognized morphologically. These two ultrastructural variants were best distinguished by differences in cell wall construction, cytoplasmic constituents, and staining properties. The cell surface of form B was delimited by a wall/membrane complex interpreted as consisting of three parts: 1) an outer membrane; 2) an electron-dense band in the periplasmic space; and 3) an inner or cytoplasmic membrane. The outer membrane of form B organisms was probably of trilaminar construction, but a tripartite structure was not visualized in electron micrographs. Instead, only a single electron-opaque leaflet and underlying lucent zone were evident (Figures 1–5). In many preparations a layer of particulate elements that appeared attached to the outer dense leaflet invested the entire cell surface (Figures 1, 2, and 5). An electron-dense band was the second and most conspicuous component of the cell wall/membrane complex of form B (Figures 1–5). This band varied in thickness from 12 nm to 19 nm. The inner membrane was a trilaminar structure and was generally poorly visualized in form B organisms (Figures 2, 3, and 4).

By comparison, the cell wall/membrane complex of form U organisms was morphologically similar to that of many gram-negative organisms, consisting of an outer and an inner membrane, which were both of trilaminar construction (Figure 6) and which were separated by an electron-lucent periplasmic space. Whereas the outer membrane of form B organisms was typically closely adherent to the contours of the bacterial cell surface, the outer membrane of form U organisms either followed similar contours (Figure 6) or displayed elaborate surface plications (Figure 7).

As evidenced by their staining density, form B organisms had a greater affinity for uranyl and lead salts than did form U cells (Figure 19). Likewise, form B organisms typically contained numerous electron-lucent cytoplasmic vacuoles in a single section plane, whereas form U cells usually had no more than one or two vacuoles per cell in a single thin section (Figures 1–3, 6, and 20). Cytoplasmic membrane configurations were evident in many form U cells (Figure 8) but were seldom seen in form B cells.

Bacterial forms transitional in appearance between form B and form U were observed in numerous preparations. Principally, these intermediate forms displayed increasing degrees of electron opacity in the periplasmic space; otherwise, they resembled form U cells (Figure 9).

This ultrastructural dimorphism of PPA was observed in yolk sac membranes, guinea pig omentum, human lung tissue, Vero cell cultures, and in preparations of organisms grown on artificial medium, but not in parallel egg yolk preparations. In the latter case only form B organisms, including a few possibly dividing forms (Figure 10), were observed.

PPA in Various Conditions of Growth

The approximate proportions of form B and for U PPA organisms seen under the various growth conditions studied are shown in Table 1. Infected human lung tissue from 3 patients contained numerous bacilli, most of which were intracellular within the cytoplasm of polymorphonuclear leukocytes and alveolar macrophages. Single or multiple bacterial cells were usually within well-defined membrane-delimited phagosomes. Most of the organisms in human lung tissue were densely banded form B cells (Figure 11). However, careful examination of thin sections revealed several organisms lacking a conspicuous dense band, which probably represented the U form (Figure 12). The yolk sac membranes of eggs inoculated with homogenates prepared for the same infected human lung tissue contained both form B and form U organisms (Figures 13 and 14) in roughly equal numbers. These bacilli were also predominantly intracellular within the cytoplasm of epithelial and macrophagelike cells.

Form U bacilli were the predominant organisms seen in infected guinea pig omentum at 3 and 4 days following intraperitoneal inoculation (Figures 15 and 16). Form B organisms constituted less than 30% of all bacteria in omentum tissue samples examined. Presumptive dividing forms of the form U cell were commonly seen (Figure 15), but dividing forms of the form B variant were not detected. Again, most organisms were within the cytoplasm of macrophages, usually in groups engulfed by membrane-delimited phagosomes (Figure 17). Form U organisms were almost always observed to be enclosed within phagosomes, whereas form B organisms occurred in both intracellular and extracellular locations.

Table 1—Proportion of PPA as B Forms and U Forms in a Variety of Growth Conditions

Growth condition	% Form B	% Form U
Infected human lung	95	5
Infected guinea pig omentum	20	80
Yolk sac membrane	50	50
Cultured Vero cells (4 days)	20	80
Cultured Vero cells (9 days)	60	40
Agar medium	10	90

In Vero cell culture, PPA was again predominantly seen within intracellular cytoplasmic phagosomes. Cultures examined 4 days after-inoculation contained predominantly U forms (Figure 18). Dividing organisms were frequently seen at this time and appeared to be exclusively U forms. Cultures examined 9 days after-inoculation showed predominantly B forms (Figure 20). PPA harvested from agar medium showed predominantly (~90%) U forms regardless of the duration of incubation. A distinguishing feature of the B forms grown on artificial medium was the presence of cell surface plications similar to those of U forms (Figure 19) but not seen on B forms under any other conditions.

Discussion

The results of this study indicate that PPA is a prokaryotic cell whose cell-wall structure is similar to that of most other gram-negative bacilli by virtue of having an outer membrane, an inner membrane, and an intervening periplasmic space. However, this organism is unusual among gram-negative bacilli in that under certain circumstances an electron-dense band occupies most of the periplasmic space. The chemical nature of this electron-dense band remains to be elucidated. However, it is likely that this band represents a rigid mucopeptide (peptidoglycan) layer analogous to the much thicker outer dense band seen by transmission electron microscopy in most gram-positive bacilli. Such a mucopeptide layer can be demonstrated in other gram-negative bacilli such as *Escherichia coli*, depending upon the strain and/or the conditions of fixation.[6,7] This layer is adjacent to the inner leaflet of the outer membrane and is usually demonstrable only by special techniques, eg, papain digestion.[8] The inability to demonstrate an inner leaflet of the outer membrane of PPA was probably due to its intimate association with the electron-dense band.

Several studies of the ultrastructure of *L pneumophila* [9-15] have shown that it also has the general features of a gram-negative bacillus. *L pneumophila* cells grown *in vivo* or *in vitro* and not otherwise manipulated failed to demonstrate any recognizable mucopeptide layer in the periplasmic space.[9-14] However, Keel et al [14] showed that the chemical constituents of a peptidoglycan coat were present, and Flesher et al,[15] us-

ing plasmolysis and papain digestion of bacterial cells, were able to clearly demonstrate a thin mucopeptide layer that was separable from the inner leaflet of the outer membrane. Under certain circumstances as defined in this report, PPA, in contrast to *L pneumophila,* appears capable of synthesizing a much thicker mucopeptide layer.

The evidence presented suggests that synthesis of the electron-dense band may be related to the phase of growth of the organism as well as the milieu in which the bacterium is residing. Form U bacilli predominated under conditions of early infection, ie, shortly after experimental infection of guinea pigs or Vero cell cultures. Form B organisms were predominant in established infections, eg, in human lung tissue from patients with pneumonia or Vero cell cultures incubated for a prolonged interval. In addition, dividing bacilli in all the material studied were almost always of the U form. Thus, the synthesis of the electron-dense (mucopeptide) band may occur only after the organism has reached a "resting," nondividing, phase. The milieu provided by intracellular growth may also be important for mucopeptide synthesis, since almost all agar-grown PPA failed to show an electron-dense band.

The relationship of this ultrastructural dimorphism to other variations noted in PPA requires further study. The presence of a mucopeptide band may be responsible for the occasional gram-variability of this organism in tissue sections stained with the Brown and Brenn stain.[1,2] The fact that only a fraction of the bacilli retain the carbol fuchsin dye in standard or modified acid-fast stains may also be related to the presence or absence of the mucopeptide band. Finally, it will be important to study the possible relationship of the presence of this band to colony morphology and to virulence. The latter factor will be especially crucial for the development of an animal model in which to study pathogenesis and treatment of PPA disease.

References

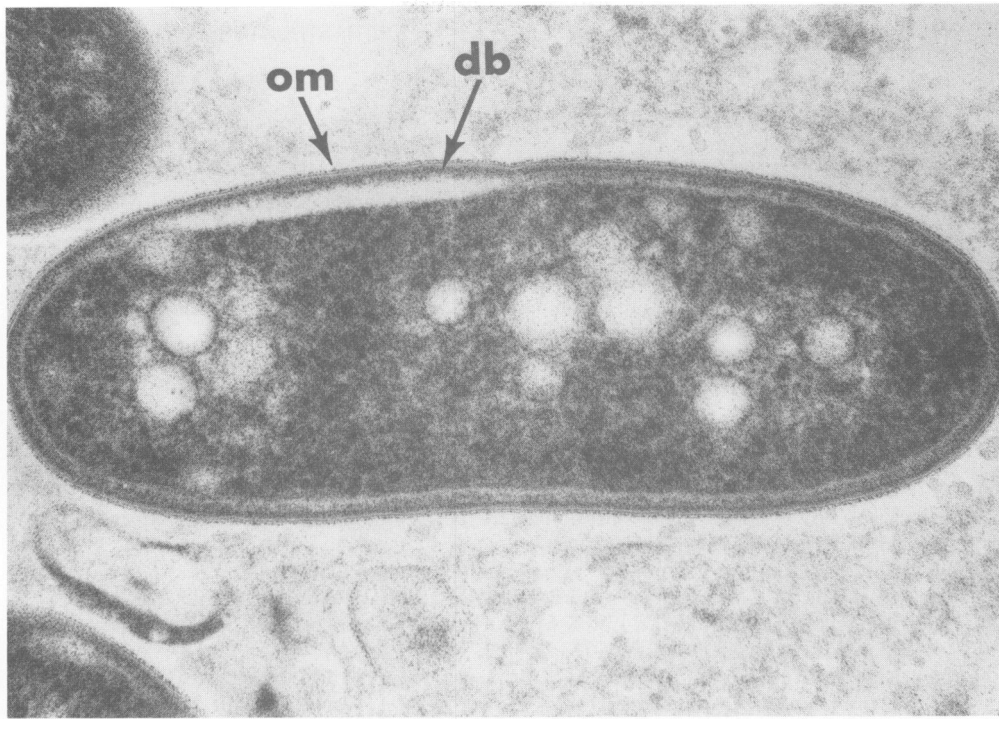
1. Pasculle AW, Myerowitz RL, Rinaldo CR, Jr: New bacterial agent of pneumonia isolated from renal-transplant recipients. Lancet 1979, 2:58–61
2. Myerowitz RL, Pasculle AW, Dowling JN, Pazin GJ, Puerzer M, Yee RB, Rinaldo CR Jr, Hakala TR: Opportunistic lung infection due to "Pittsburgh Pneumonia Agent." N Engl J Med 1979, 301:953–958
3. Rogers BH, Donowitz GR, Walker GK, Harding SA, Sande MA: Opportunistic pneumonia: A clinicopathologic study of five cases caused by an unidentified acid-fast bacterium. N Engl J Med 1979, 301:959–961
4. Hébert GA, Thomason BM, Harris PP, Hicklin MD, McKinney RM: Pittsburgh Pneumonia Agent": A bacterium phenotypically similar to *Legionella pneumophila* and identical to the Tatlock bacterium. Ann Intern Med 1980, 92:53–54
5. Pasculle AW, Feeley JC, Gibson RJ, Cordes LG, Myerowitz RL, Patton CM, Gorman

GW, Carmack CL, Ezzell JW, Dowling JN: Pittsburgh Pneumonia Agent: Direct isolation from lung tissue. J Infect Dis 1980, 141:727–732

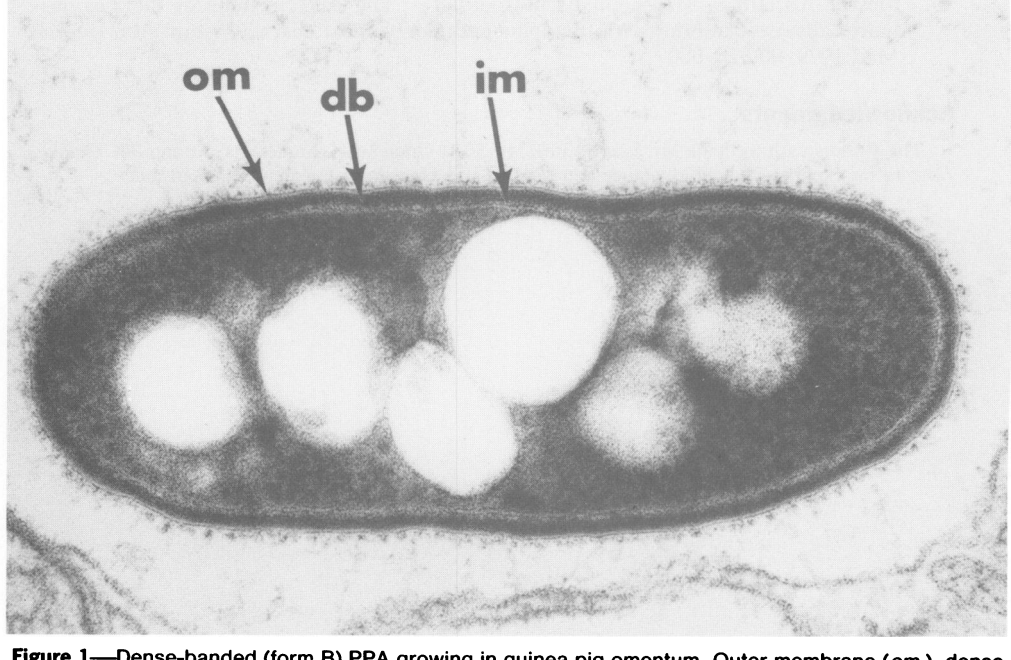
6. Murray RGE, Steed P, Elson HE: The location of the mucopeptide in sections of the cell wall of *Escherichia coli* and other gram-negative bacteria. Can J Microbiol 1965, 11:547–560
7. Steed P, Murray RGE: The cell wall and cell division of gram-negative bacteria. Can J Microbiol 1966, 12:263–270
8. DePetris S: Ultrastructure of the cell wall of *Escherichia coli* and chemical nature of its constituent layers. J Ultrastruct Res 1967, 19:45–83
9. Chandler FW, Blackmon JA, Hicklin MD, Cole RM, Callaway CS: Ultrastructure of the agent of Legionnaires' disease in the human lung. Am J Clin Pathol 1979, 71:43–50
10. Chandler FW, Cole RM, Hicklin MD, Blackmon JA, Callaway CS: Ultrastructure of the Legionnaires' disease bacterium: A study using transmission electron microscopy. Ann Intern Med 1979, 90:642–647
11. Katz SM, Nash P: The morphology of the Legionnaires' disease organism. Am J Pathol 1978, 90:701–722
12. Katz SM, Brodsky I, Kahn SB: Legionnaires' disease: Ultrastructural appearance of the agent in a lung biopsy specimen. Arch Pathol Lab Med 1979, 103:261–264
13. Rodgers FG, Macrae AD, Lewis MJ: Electron microscopy of the organism of Legionnaires' disease. Nature 1978, 272:825–826
14. Keel JA, Finnerty WR, Feeley JC: Fine structure of the Legionnaires' disease bacterium. In-vitro and in-vivo studies of four isolates. Ann Intern Med 1979, 90:652–655
15. Flesher AR, Ito S, Mansheim BJ, Kasper DL: The cell envelope of the Legionnaires' disease bacterium: Morphologic and biochemical characteristics. Ann Intern Med 1979, 90:628–630

Acknowledgments

The authors wish to thank Mr. Larry Estes, Mr. Scott Graner, Ms. Annie McCoy, and Ms. Donna Ziesmer for expert technical assistance and Mrs. Cheryl Myerowitz for secretarial assistance.

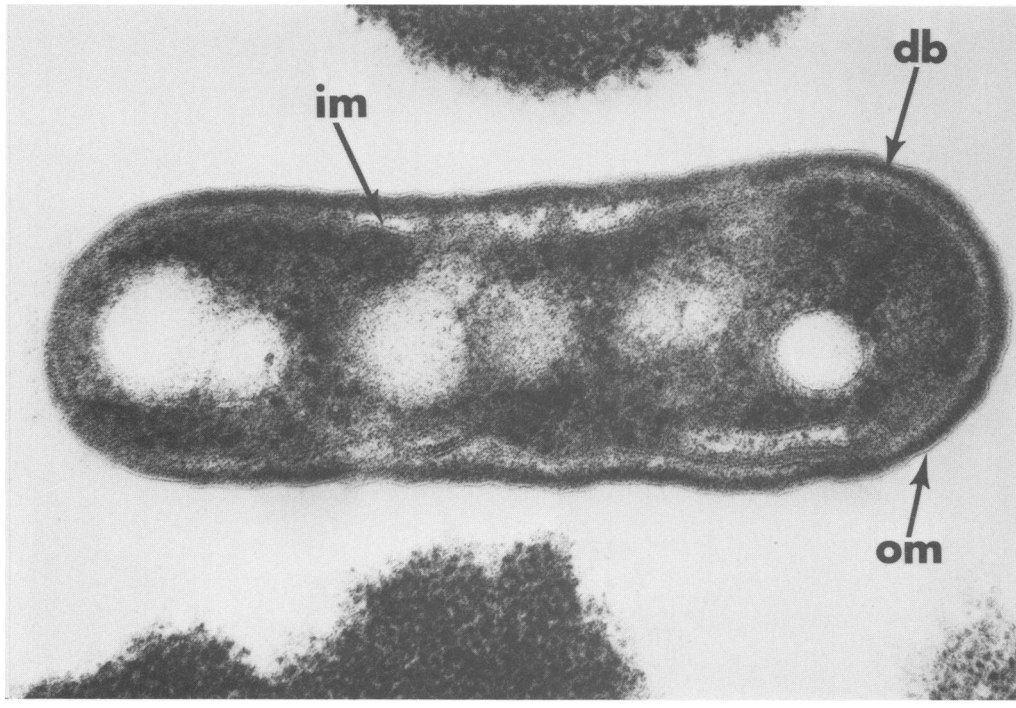


1

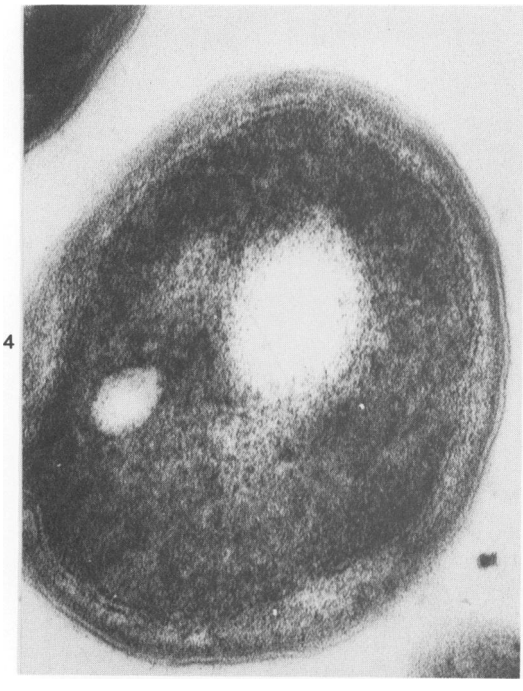


2

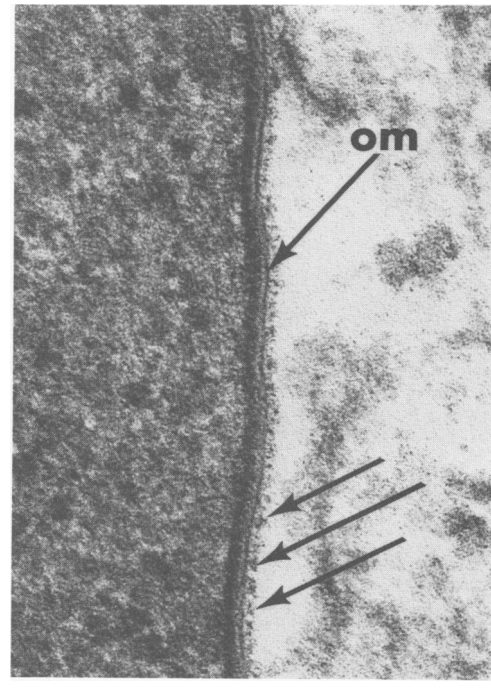
Figure 1—Dense-banded (form B) PPA growing in guinea pig omentum. Outer membrane (*om*), dense band (*db*), and numerous cytoplasmic vacuoles are visualized, but the inner cytoplasmic membrane is not resolved. (×95,300) The outer membrane appears to be tripartite because of an outer layer of adherent particulate elements, which are better visualized in Figure 5. **Figure 2**—Form B PPA growing in yolk sac membrane. Outer membrane (*om*), dense band (*ob*), inner membrane (*im*), and numerous intracytoplasmic vacuoles are visualized. Note particulate elements attached to exterior surface of outer membrane. (×115,400)



3

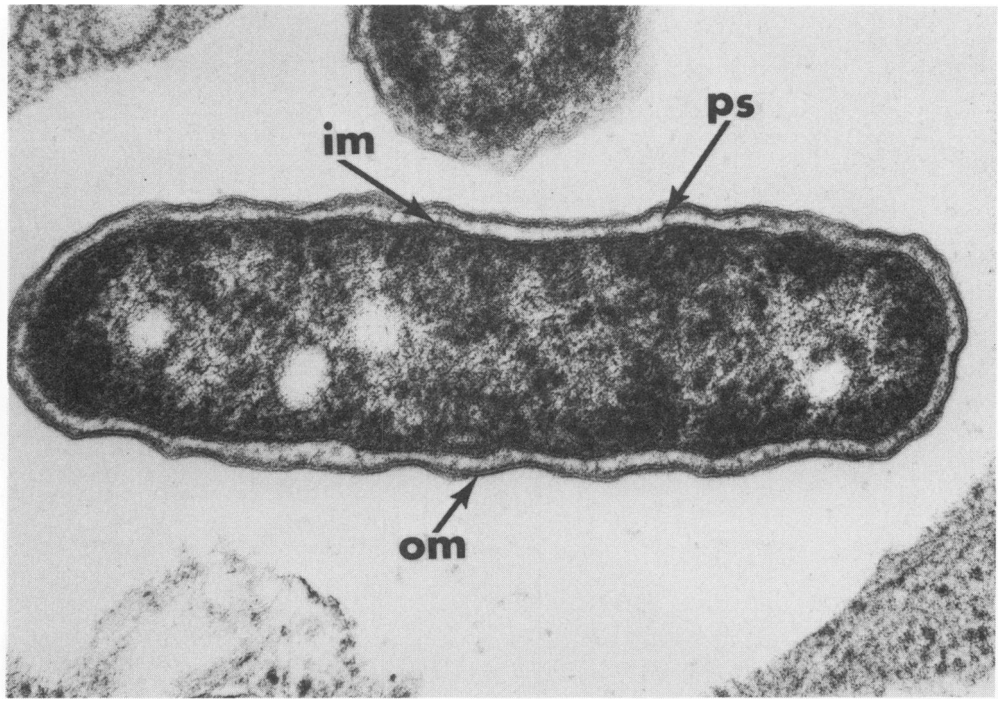


4

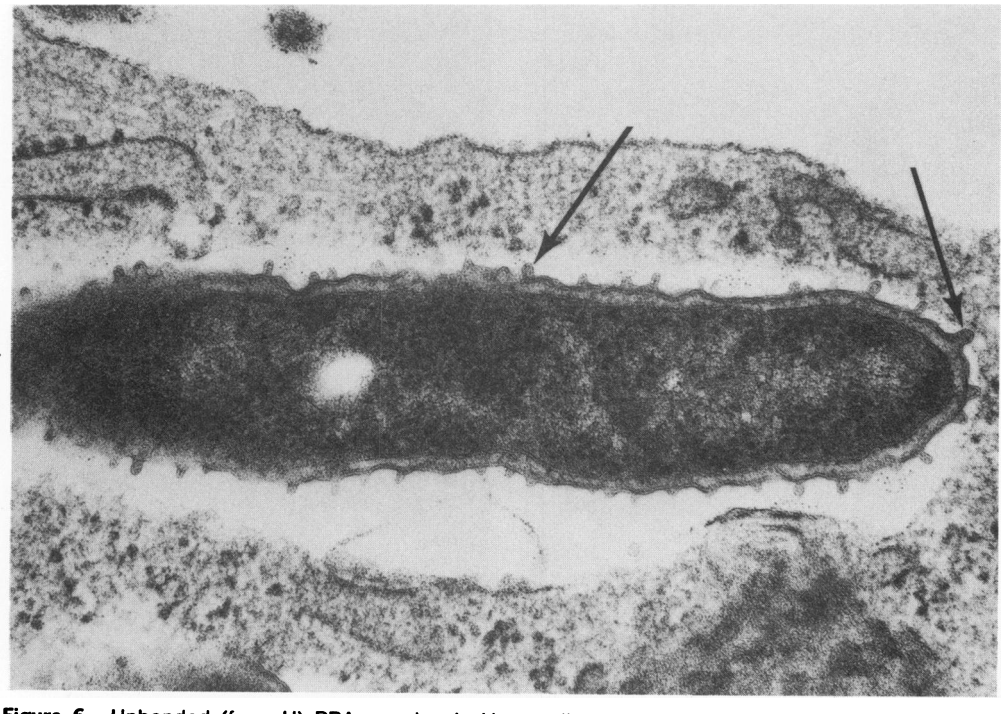


5

Figure 3—Form B PPA from egg yolk pellet illustrating outer membrane (*om*), dense band (*db*), inner membrane (*im*), and cytoplasmic vacuoles. (×117,900) **Figure 4**—Form B PPA from egg yolk pellet illustrating outer membrane, dense band, and tripartite inner membrane. Only a single dense leaflet of the presumably tripartite outer membrane is visualized. (×194,900) **Figure 5**—Outer membrane (*om*) and dense band of form B PPA growing in guinea pig omentum. Particulate elements (*arrows*) attached to the single dense leaflet of the outer membrane are visualized here. At lower magnification (see Figure 1) particulate elements appear continuous and the visualized outer membrane appears tripartite. (×205,200)

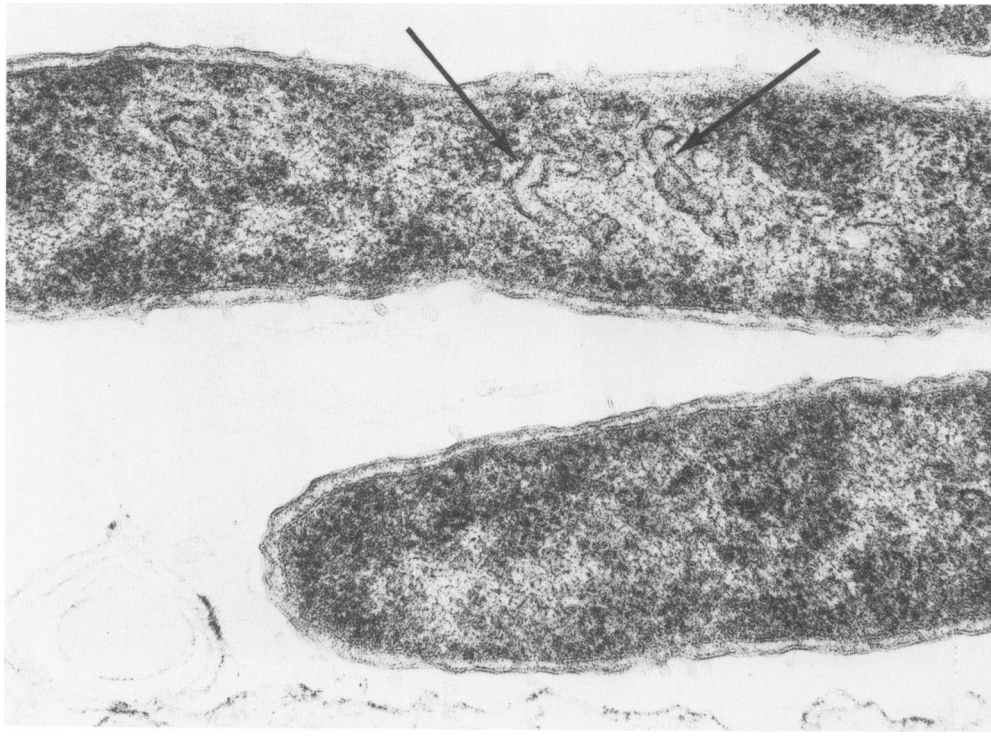


6

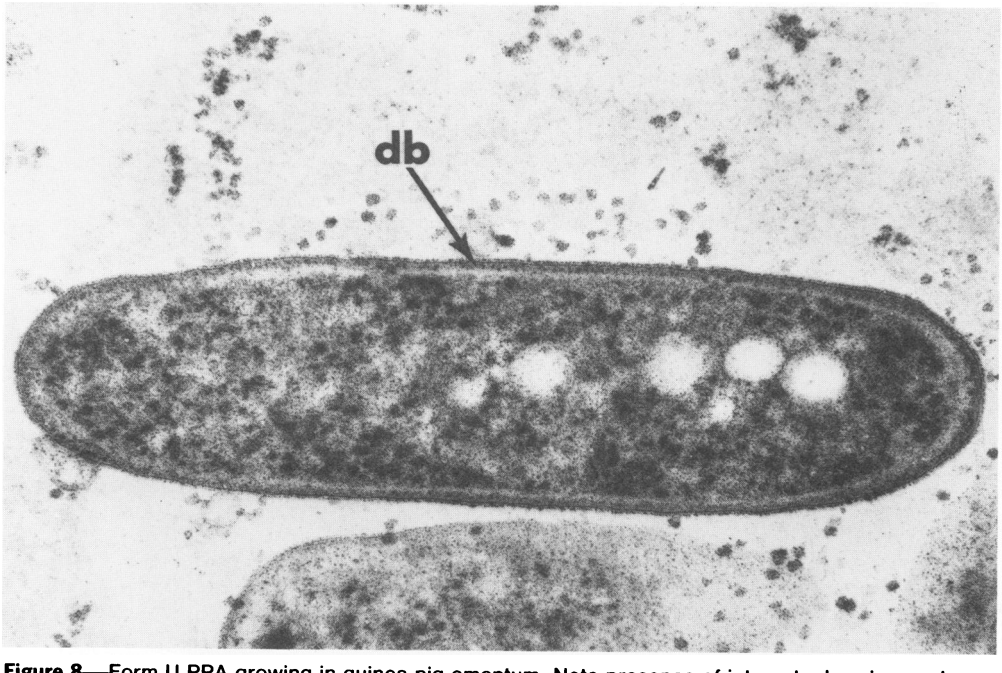


7

Figure 6—Unbanded (form U) PPA growing in Vero cell culture harvested at 4 days following inoculation. Inner (*im*) and outer (*om*) membranes are clearly visualized. No dense band is present in the periplasmic space (*ps*), and cytoplasmic vacuoles are few in number. (×112,900) **Figure 7**—Form U PPA growing in Vero cell culture. Note elaborate surface plications (*arrows*) formed by outfoldings of the outer membrane. (×87,800)

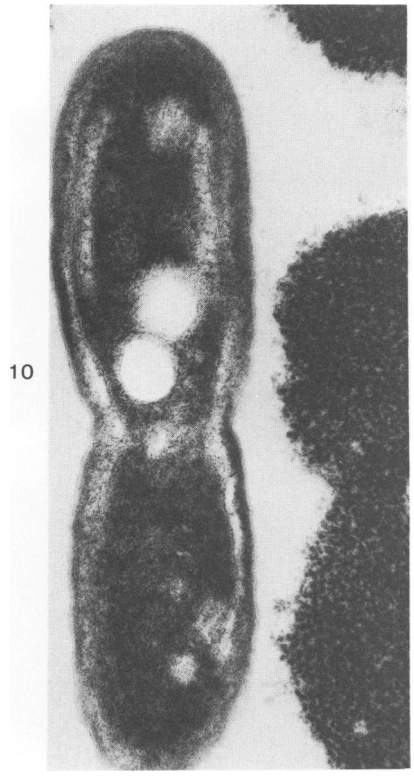


8

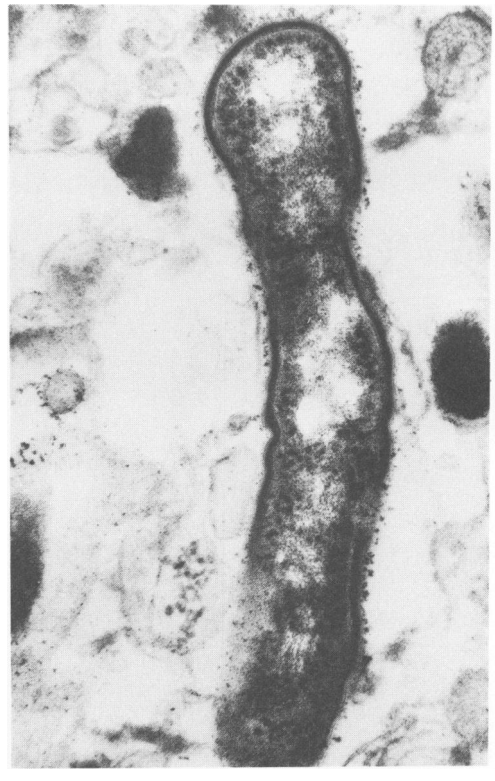


9

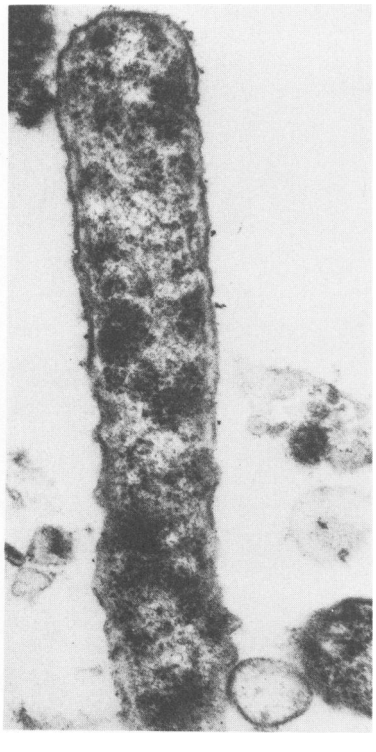
Figure 8—Form U PPA growing in guinea pig omentum. Note presence of intracytoplasmic membrane configurations (*arrows*). (×107,800) **Figure 9**—Transitional form of PPA growing in guinea pig omentum. Dense band (*db*) is evident, but its decreased thickness and reduced opacity suggest that formation is incomplete. (×87,800)



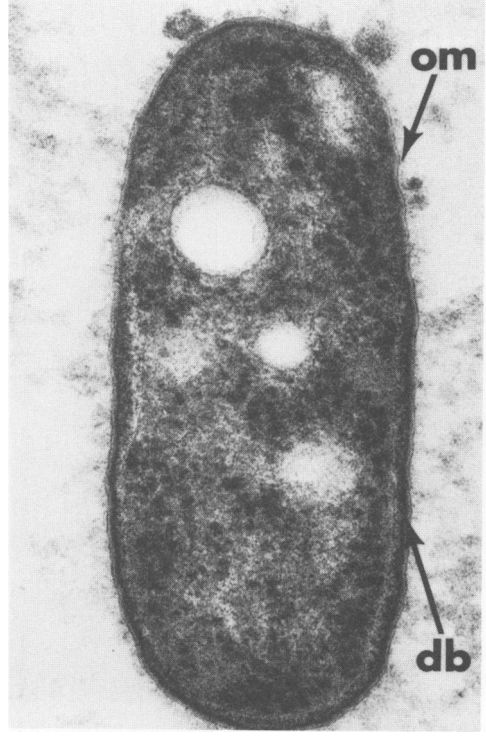
10



11

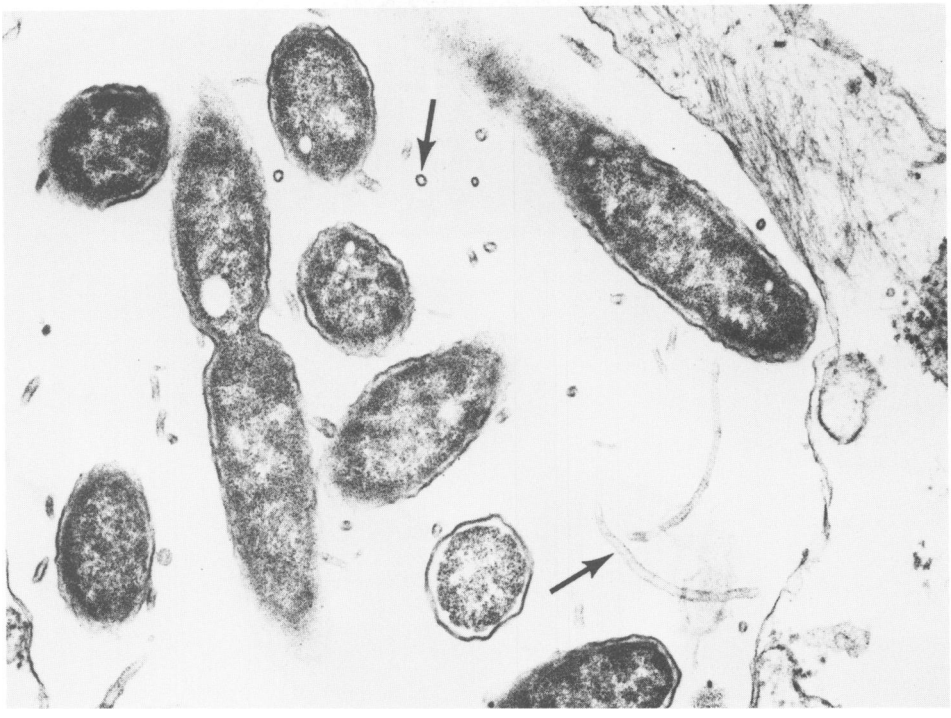


12

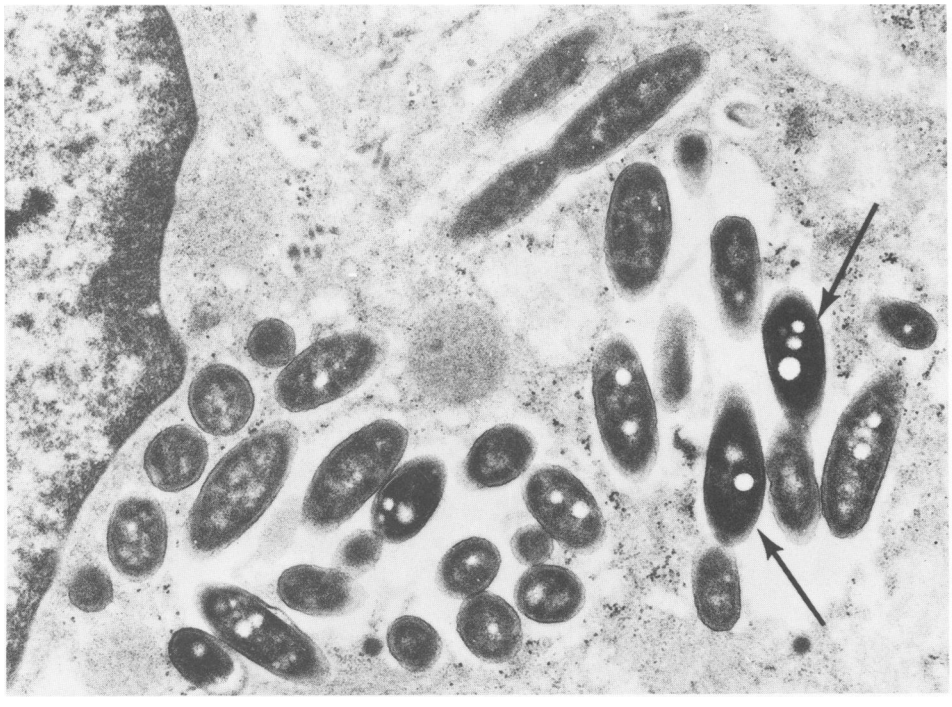


13

Figure 10—Possibly dividing organism of form B PPA from egg yolk pellet. (×75,200) **Figure 11**—Form B organism from lung autopsy specimen of a patient with purulent pneumonia due to PPA. (×65,200) **Figure 12**—Form U PPA from same specimen as Figure 11. (×60,200) **Figure 13**—Outer membrane (*om*) and dense band (*db*) are clearly visualized in PPA grown in yolk sac membrane. Eggs were inoculated with homogenate of autopsy lung specimen depicted in Figures 11 and 12. (×91,500)



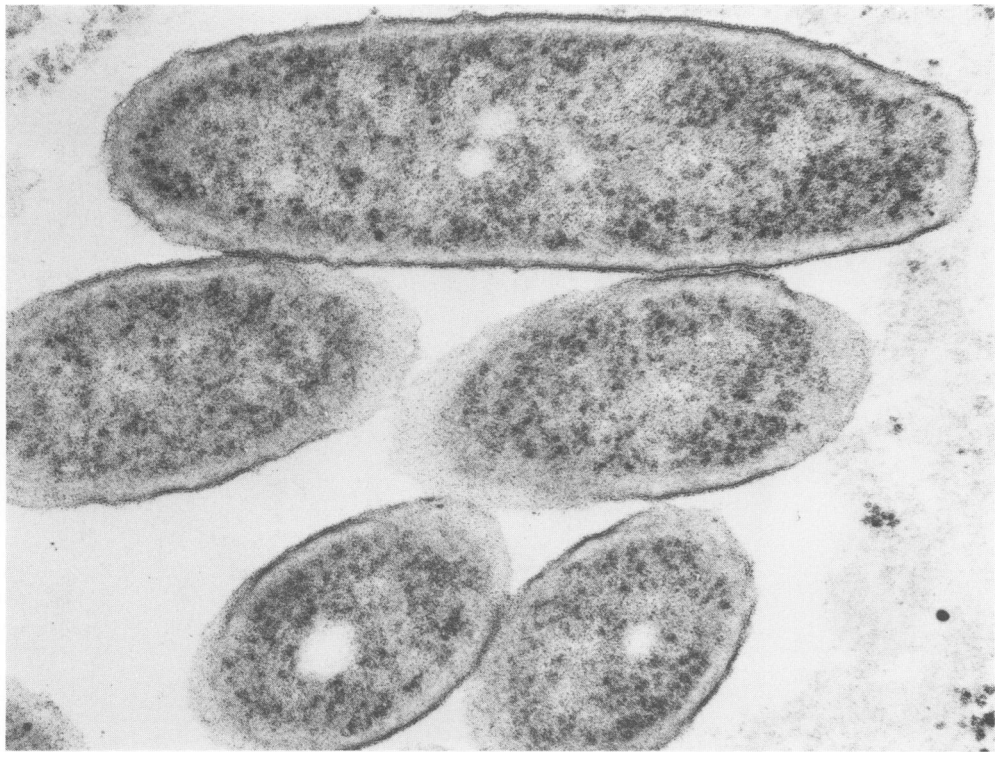
14



15

Figure 14—Form U PPA grown in yolk sac membrane. Same specimen as Figure 13. Circular and elongated profiles (*arrows*) probably represent cuts through cell surface plications like those seen in Figure 7. (×45,100) **Figure 15**—Infected cell from guinea pig omentum. At this magnification form B PPA (*arrows*) are identified by their greater electron opacity as compared with form U bacilli. Note the dividing form U organism in upper portion of micrograph. (×17,900)

16



17

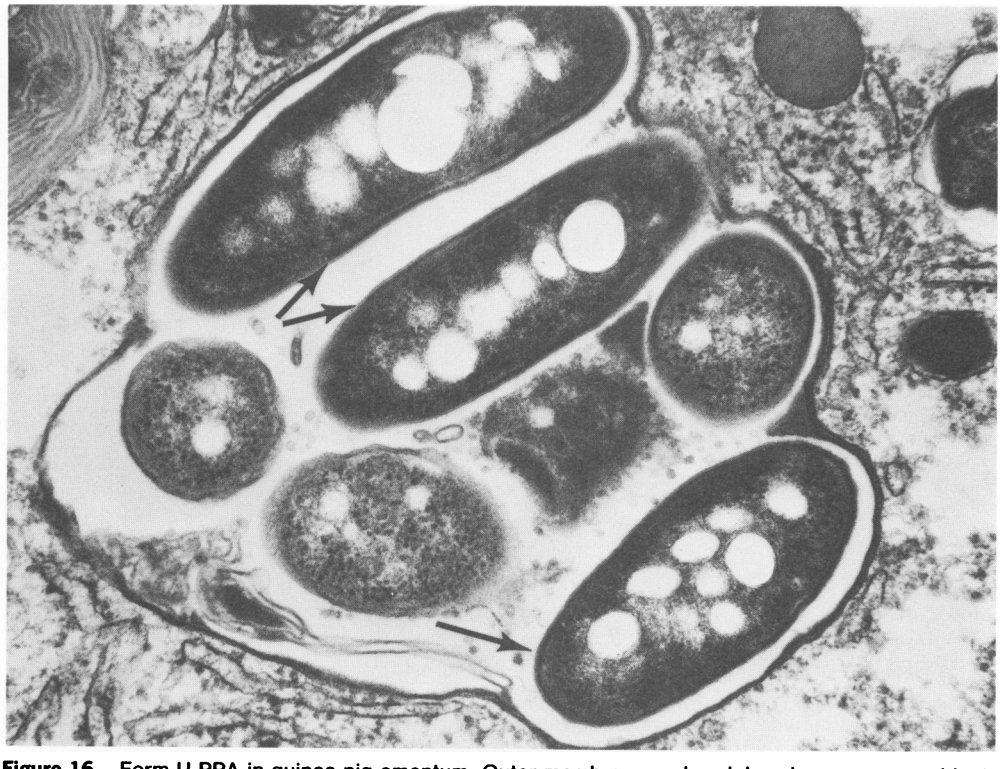
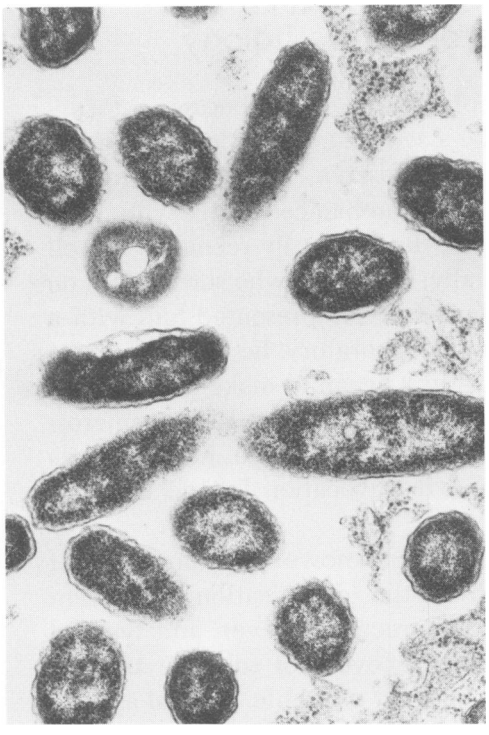
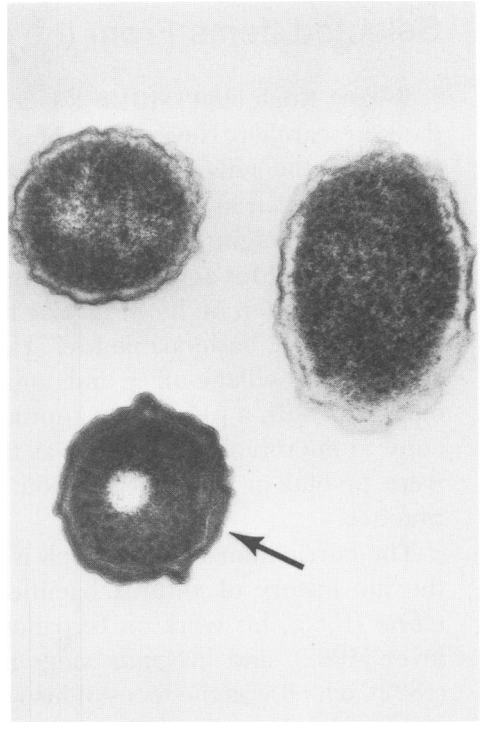


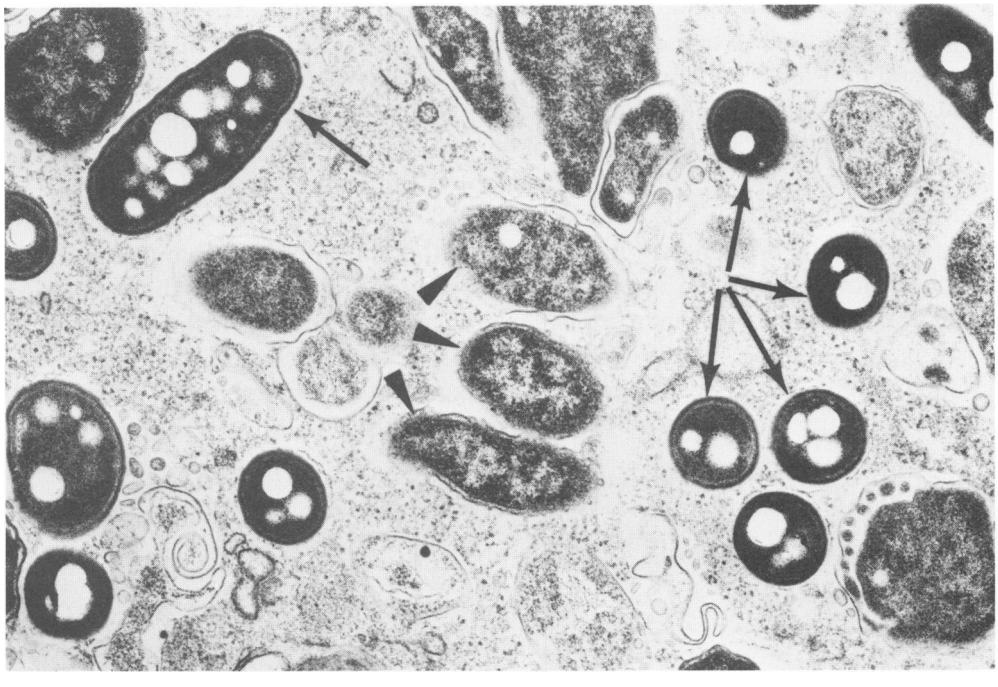
Figure 16—Form U PPA in guinea pig omentum. Outer membrane and periplasmic space are evident, but the inner membrane is not visualized. Ribosomes are numerous, and a few cytoplasmic vacuoles are present. (×77,700) **Figure 17**—Macrophage within guinea pig omentum containing both form B and form U PPA. Form B cells stain more densely and contain numerous cytoplasmic vacuoles. Note the well-defined, membrane-delimited phagosome that contains the bacilli. (×50,200)



18



19



20

Figure 18—Form U PPA harvested after 4 days in Vero cell culture. (×28,700) **Figure 19**—PPA grown 4 days on artificial medium. Form B (*arrow*) and form U organisms are present. (×70,200) **Figure 20**—Form B and form U PPA harvested after 9 days in Vero cell culture. Form B cells (*arrows*) are more opaque and vacuolated than are form U cells (*arrowheads*). (×35,100)