Ultrastructure of Bacterial Cells Infected with Bacteriophage PM2, a Lipid-containing Bacterial Virus

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The cytological pattern of infection of a host pseudomonad with PM2, a lipid-containing bacterial virus, was investigated by electron microscopy. Normal and infected cells frequently contain a myelin figure, which is found in the nucleoid region or at the periphery of the cell. The most striking finding in this investigation was that completed virions are found in the cell adjacent to or in association with the cytoplasmic membrane. This localization is precise; virions are not found elsewhere in infected cells. The completed virions occasionally appear to be attached to the cytoplasmic membrane. The virus contains a darkly staining core surrounded by a tripartite envelope of a thickness of approximately 70 Å, which is identical to the thickness of the cytoplasmic membrane. Lysing cells appear to undergo extensive damage of the cytoplasmic membrane prior to rupture of the L layer of the cell wall.

Two marine bacteriophages active against *Pseudomonas* species were shown to be markedly inactivated by organic solvents (9). The sensitivity of bacterial viruses to organic solvents is not a property heretofore recognized as significant among bacteriophages; in fact, resistance to chloroform is commonly utilized to facilitate the study of the adsorption of bacteriophages to their host (10). It must be remembered that obligately parasitic procaryotic cells such as *Bdellovibrio* (11), which form plaques on lawns of appropriate host bacteria, are inactivated by organic solvents. Thus, the inhibition of plaque-forming ability by chloroform must not necessarily be construed as the inactivation of a bacterial virus by an organic solvent. However, it is true that a number of animal viruses are inactivated by organic solvents (2), and with certain of these viruses it has been clearly shown that some of those viruses which contain phospholipids are readily disrupted by lipid solvents (3).

Espejo and Canelo (*submitted for publication*) recently described the chemical and physical properties of a unique deoxyribonucleic acid-containing bacteriophage, PM2, which infects a marine pseudomonad. These workers showed that

PM2, which contains up to 10% of its dry weight as lipid, is inactivated by ethyl ether and is disrupted by dilute solutions of the detergent Sarkosyl. Electron microscopy of PM2 reveals that this virus possesses a polyhedral structure with a diameter of 600 Å. PM2 stained with uranyl acetate frequently reveals an apparently double-layered envelope surrounding an inner core.

The presence of appreciable levels of lipid in PM2, plus the "apparent" envelope seen by electron microscopy, suggested that PM2 might be a bacteriophage counterpart of animal viruses such as influenza virus or Newcastle disease virus. The structure of such viruses has been studied extensively in thin sections by electron microscopy. It was suggested by Hoyle (4) that such viruses derive their outer coat from the cytoplasmic membrane of the host cell. The electron microscopic studies of Morgan et al. (7) offered convincing support to Hoyle's suggestion.

This report describes the ultrastructure of host pseudomonad cells during the replication of PM2, the structure of the virus within infected cells, and the pattern of cell lysis leading to viral release. We have observed a pattern of viral distribution within infected cells which is quite different from that recently described by Valentine and Chapman (12) for the bacteriophage NCMB 385 which infects another marine bacterium, *Cytophaga marinoflava*.

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MATERIALS AND METHODS

Cultivation of bacteria and bacteriophage. The host bacterium, which has been tentatively identified as a member of the *P. stutzeri* subgroup, was routinely cultivated with forced aeration at 29°C in a medium containing 8 g of dehydrated nutrient broth per liter of AMS solution. AMS solution contains 0.7 g of KCl, 1.5 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 12.0 g of $\text{MgSO}_4 \cdot 12\text{H}_2\text{O}$, and 26.0 g of NaCl per liter of distilled water. The bacterial cells were cultured to a population density of 3×10^8 cells per ml, at which point they were infected with lysates of PM2 at a multiplicity of infection of 5 to 10. In such infected cultures, lysis begins at about 60 min after infection and is complete by 90 min after infection.

Electron microscopy. Samples of infected cells were removed at 30, 45, 60, 70, 80, and 90 min after infection, fixed for 1 hr in 10% Formalin, followed by the standard Kellenberger and Ryter (6) osmium fixation procedure, stained for 2 hr in uranyl acetate, dehydrated through a graded acetone series, and embedded in Vestopal W. Sections were cut with diamond knives on an LKB Ultratome, and stained with 1% uranyl acetate in 60% methanol for 10 min and with lead citrate by the method of Venable and Coggeshall (13). Stained sections were examined in a Hitachi Hu 11 electron microscope or in a Philips EM 300 electron microscope.

RESULTS AND DISCUSSION

The host bacterium has the structural appearance of a typical gram-negative rod (Fig. 1). We have been able to preserve only the outer membrane component of the cell wall [the L layer of the cell wall of *Escherichia coli* described by De Petris (1)]. Although the host pseudomonads appear as typical gram-negative rods, it has become increasingly apparent that these cells frequently contain an internal body which has the appearance of a myelin figure. This structure is seen in Fig. 2 and is quite similar to myelin figures observed by Kaye and Chapman (5) in colistin-treated *E. coli*, by Valentine and Chapman (12) in phage-infected *C. marinoflava*, and by Ryter and Jacob in plasmolyzed cells of *E. coli* (8). The myelin figures are most frequently found in the nucleoid region of the cell; however, they are occasionally observed at the periphery of the cell, as is clear in Fig. 3. Figure 4 is a particularly striking demonstration of the myelin figure; one can clearly detect that the periodicity between each dark component of the myelin figure is quite regular and measures approximately 25 Å.

The multiplicities of PM2 used to infect the host bacterium in these experiments has not permitted us to observe in detail the initial stages of infection, i.e., the attachment of the phage to the bacterial cell. However, after 30 min one can see that the infected cells have suffered no structural alteration. Figure 5 clearly shows that, after 30

min, there has been no change in the appearance of the nucleoid region. Again, one can see myelin figures in the cells; Fig. 6 demonstrates one myelin figure at the periphery of a dividing cell. The L layer of the cell wall can occasionally be seen to bleb away from the cell proper. Figure 7, which is a micrograph of cells 45 min after infection, shows one such bleb within which one can observe a myelin figure encased. It is of interest to note that the myelin figure seen in Fig. 7 is actually between the L layer and the cytoplasmic membrane. Such a location means either that the myelin figure has been extruded from the cytoplasm, or that it never truly exists within the cytoplasm but that it normally is found in the periplasmic space. However, if the myelin figure were normally in the periplasmic space, one cannot easily understand why it can be found so frequently in the nucleoid region unless the periplasmic space actually is not limited to the periphery of the cell but projects into the nucleoid region. We have seen numerous examples of the myelin figure in the nucleoid region and in protruding blebs of the L layer. We hope to be able to explain the structural relationship of the myelin figure within the cell by the more detailed ultrastructural examination of the normal host bacterium which is currently under way in our laboratory. The function of this structure is unknown; however, Ryter and Jacob (8) suggested that those myelin figures found in *E. coli* may be storage deposits for lipids localized near the membrane.

The bulk of the cells seen at 45 min after infection appear quite normal (Fig. 7). However, an occasional cell has the appearance which we have found to be characteristic for cells in the later stages of infection with PM2. One such cell is seen in Fig. 8. This cell is highly distended, with an intact L layer and intact cytoplasmic membrane. The nucleoid is somewhat enlarged and the cytoplasm is packed with ribosomes. Within the cytoplasm one can detect small but distinct particles, of a diameter of 500 Å, which lie close to cytoplasmic membrane. These particles generally reveal a densely staining central region which is surrounded by a less densely staining outer region. We believe that these particles are examples of partially assembled or completed PM2 virions. It is clear that these particles are not randomly distributed throughout the cytoplasm but are always found at the periphery of the cell close to or adjacent to the cell membrane.

The characteristic localization of the phage particles in cells is quite clear 60 min after the onset of the infection (Fig. 9). A number of viral particles can be seen in two of the three cells depicted. A tripartite envelope surrounds a

FIG. 1. Thin section of normal, exponential-phase host pseudomonad. Only the L layer (L) of the cell wall has been preserved. The bar in all electron micrographs represents 0.5μ . $\times 60,000$.

FIG. 2. Normal cell of host bacterium containing myelin figure (MF) in nucleoid (N) region. $\times 60,000$.

FIG. 3. Normal cell of host pseudomonad containing myelin figure (MF) at the periphery of the cell. $\times 45,000$.

FIG. 4. Normal cell of host pseudomonad showing a well-resolved myelin figure in the nucleoid region. The lamellae contained in this myelin figure are approximately 25 A apart. This micrograph was taken with a Philips EM 300 electron microscope. $\times 105,000$.

FIG. 5. Host cells 30 min after onset of infection. Myelin figures (MF) can be seen in the nucleoid (N) region of one cell. $\times 60,000$.

FIG. 6. Host cell 30 min after infection showing myelin figure (MF) at periphery of cell. $\times 60,000$.

FIG. 7. Host cells 45 min after infection. A myelin figure (MF) is visible within a protruding bleb of the L layer (L) of the cell wall. $\times 30,000$.

FIG. 8. Host cell 45 min after infection. The cell appears swollen and deformed. PM2 particles (P) can be seen at the periphery of the cell close to the cytoplasmic membrane. $\times 59,000$.

FIG. 9. Host cells 60 min after infection. A triple-layered envelope can be seen surrounding the PM2 phage particle labeled A. The phage particle labeled B appears to be in juxtaposition to the cytoplasmic membrane (CM). $\times 57,000$.

densely staining core in a number of the phage particles, particularly the particle designated A. The viruses are close to the cytoplasmic membrane, and one in particular, labeled B, is virtually in apposition to the membrane. Cells at this stage of infection are frequently extremely distended, with a massive nucleoid region (Fig. 10). One of the viral particles, labeled C, appears to be attached to the cell membrane. This is made clear (inset, Fig. 10) when the phage particle and membrane are further enlarged. Our most careful measurements cannot distinguish any difference between the thickness of the tripartite structure which surrounds the phage core and the thickness of the cytoplasmic membrane. These measurements prove to be approximately 70 Å.

The fact that the virions are not distributed randomly throughout the infected cell is underscored in Fig. 11. At one pole of this cell, one can see a striking array of six phage particles which virtually abut against one another and against the underlying cytoplasmic membrane. Continued observation of virions adjacent to or in contact with the cytoplasmic membrane forces us to conclude that this localization is not fortuitous nor trivial. Such a precise association can be explained by one of the two following alternatives: (i) the phage envelope is acquired from the cell membrane of the host cell, i.e., the virus is coated with portions of the cytoplasmic membrane, or (ii) the phage envelope is synthesized *de novo* at the membrane by the enzymatic apparatus which synthesizes cytoplasmic membrane. It is apparent that the crude electron microscopic technique utilized in this investigation will not permit us to resolve this matter. Thus, one of us (R. T. E.) is currently investigating the source of the phosphorus in the phospholipid of PM2. Preliminary studies suggest that a considerable amount of this phosphorus is derived from pools of phosphate extant in the cell prior to infection. One such pool could of course be the phospholipid in the cytoplasmic membrane. In any case, it is likely that chemical or immunological means will permit the resolution of the question of phage localization which has been made apparent by electron microscopy.

Some lysing cells are evident in our preparations of cells sampled 60 min after infection, which supports the turbidimetric studies (Espejo and Canelo, *submitted for publication*) made earlier. Figure 12 shows one infected cell in which the L layer is greatly distended, although apparently still intact, whereas the cytoplasmic membrane is ruptured. Some of the cytoplasmic contents have been released into the space delimited by the L layer of the cell wall. Other lysing cells also appear

to suffer an initial degradation of the cytoplasmic membrane prior to a rupture of the L layer. In Fig. 13, the cytoplasmic membrane has been disrupted locally at point D. One bacteriophage particle can be seen at the site of disruption of the cytoplasmic membrane. The extensive lysis which has occurred by 80 min is evident in Fig. 14. In addition to the numerous vesicles, one can see the remains of a cell (CF) in which virions are still in close association to the membrane. Such an observation suggests that the virions are not released immediately upon lysis and that their association with the cytoplasmic membrane may be a real union rather than a casual association. Further evidence for this interpretation is presented in Fig. 15, in which one can see a large contorted cell fragment with a number of phage particles still in association with the cell membrane.

The precise localization of PM2 with the cell membrane of the host pseudomonad underscores another similarity between PM2 and animal viruses such as influenza virus and Newcastle disease virus. The presence of high levels of lipid, the sensitivity to organic solvents and detergents, the demonstration of an envelope in thin sections, and the site of viral completion offer telling evidence in support of the view that PM2 may indeed be a bacteriophage counterpart of certain animal viruses. However, the absence of nucleoprotein from PM2 (Espejo and Canelo, *submitted for publication*) emphasizes that PM2 cannot be considered an absolute counterpart of these animal viruses.

It is conceivable that the unusual properties of PM2 are the result of the rather unique selection pressures exerted in its particular ecological niche, the marine environment. However, it is also possible that lipid-containing bacteriophages are more widespread in nature but that they have not been observed; the tacit assumption that bacteriophages are resistant to organic solvents may have made their discovery unlikely, since organic solvents are frequently used to facilitate the isolation of bacteriophages. We well recognize that the isolation of a previously unexpected bacterium, *Bdellovibrio* (11), would not have been accomplished if organic solvents had been used in the isolation of the plaque-forming entity.

The isolation and characterization of a lipid-containing bacteriophage is interesting in itself; however, further study of the relationship of the lipid envelope of PM2 to the cytoplasmic membrane of the host cell, as well as the study of the synthesis of the lipid envelope, may contribute to the understanding of the synthesis of such important macromolecular species as the phospholipoproteins.

FIG. 10. Swollen host *pseudomonad* 60 min after infection, showing massive nucleoid region (N). Phage particle labeled C appears to be attached to the cytoplasmic membrane (CM). This is emphasized in the inset. $\times 57,000$; inset, $\times 165,000$.

FIG. 11. *Host pseudomonad* 60 min after infection with PM2, showing a row of six virions adjacent to the cytoplasmic membrane. $\times 150,000$.

FIG. 12. Infected cell with highly distended L layer. The cytoplasmic membrane exhibits evidence of extensive disruption. $\times 60,000$.

FIG. 13. Lysing cell 60 min after infection. The cytoplasmic membrane appears degraded at site D; the L layer (L) appears intact. $\times 65,000$.

FIG. 14. Debris observed in lysing culture 80 min after infection. One large cell fragment (CF) contains PM2 virions (P) in association with the cytoplasmic membrane (CM). $\times 60,000$.

FIG. 15. Large cell fragment observed 90 min after infection, showing numerous PM2 particles within the fragment. $\times 152,000$.

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