JOURNAL OF VIROLOGY, Jan. 1974, p. 186-196

Vol. 13, No. 1
Printed in U.S.A.

Development of Coliphage N4: Ultrastructural Studies

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Received for publication 14 August 1973

The basic properties of bacteriophage N4 development have been investigated in *Escherichia coli* Hfr 3300 under one-step growth and high cell density conditions. N4r^+-infected bacteria are lysis inhibited in mass culture, burst asynchronously starting 180 min postinfection, and release over 3,000 phage per cell. During lysis inhibition the bacteria continuously elongate, increase in girth, and undergo characteristic morphological changes represented by the appearance of dark spots located at the cell poles. In thin sections, during the late stages of replication and assembly, the phage particles are localized exclusively in restricted areas of the cytoplasm near the polar regions. Large paracrystalline arrays of virions are found in over 7% of the cells before lysis. The most common mechanism of lysis consists in the formation of bulges located at random in the cell circumference; these burst and, without extensive disruption of the cell wall, the phage progeny escapes into the medium.

Infection of *Escherichia coli* K-12 cells by bacteriophage N4 (23, 24) is characterized by several unusual features. Early and delayed-early genes of the parental chromosome are transcribed by rifampin-resistant enzymatic activities, and the mRNA originated is translated into proteins that carry out replication of the phage DNA (Schito, Rothman-Denes, Giraldi, and Toni, manuscript in preparation). During these stages most host RNA and protein species are synthesized at an approximately normal rate (19) although bacterial DNA replication is completely shut off (22). Correct phage DNA synthesis and production of N4 structural subunits, on the other hand, require the continued functioning of the host RNA polymerase. Bacterial macromolecular syntheses are depressed but not halted in this phase which proceeds for over 3 h (16).

Although assembly of N4 has been demonstrated in vitro using phage products synthesized by nonpermissive cells infected with amber mutants (22), little is known about the patterns of in vivo morphogenesis of this phage. Therefore, the purpose of this paper is to illustrate some of the basic properties of N4 development as assessed by biological, optical, and electron-optical methods.

MATERIALS AND METHODS

Media. M9 medium supplemented with 0.2% Casamino Acids (Difco), M9S, was prepared by the formula of Studier (26).

Cells and virus. *E. coli* Hfr 3300 was used in all experiments. The origin and characteristics of N4 have been recently summarized (22).

Growth of virus. One-step growth experiments were modeled after those reported by Adams (1). For phage growth curves the cells were grown in M9S, harvested during logarithmic growth (2×10^8 cells per ml), resuspended at the same concentration in fresh, warm medium, and infected with purified phage (20) to give a multiplicity of about 10. All experiments were performed at 37 C on a rotary shaker. Lysis was measured as a decline in the turbidity of the cultures at 650 nm. Free, intracellular, and total virus were assessed after artificial opening of the cells by the chloroform-lysozyme method (11).

Preparation of specimens for electron microscopy. For ultrastructural studies, samples (30 ml) of an Hfr 3300 culture infected as described above were withdrawn at specified times and fixed by a modification (14) of the procedure of Kellenberger, Séchaud, and Ryter (12). Epon 812 was used for embedding. Thin sections, cut with an LKB ultramicrotome and stained with uranyl acetate and lead citrate (18), were examined in an Akashi Tronscope electron microscope.

RESULTS

Growth of bacteriophage N4 in E. coli Hfr 3300. The fate of an N4-infected cell is dominated by the genotype of the invading virus since a different timing of lysis is observed depending on whether the phage carries the *r* or r^+ character (16). In fact, under one-step growth conditions, the latent period with both phages is

34 min, but even after 100 min with N4r^+ there is a continued slow increase in phage titer (Fig. 1). The yield reaches about 900 PFU per cell with N4r^+ and 110 for the rapid lysis mutant.

Development of N4 under conditions of high multiplicity of infection and high cell concentration is shown in Fig. 2. With N4r^+ the optical density of the culture increases for about 180 min, then the turbidity begins to fall, and, by the 6th h of incubation, the optical density returns almost to the preinfection value. On the other hand, the turbidity of an N4r-infected culture rises slightly in the first 30 min of incubation and this rise is followed by a fall with clearing of the cultures about 30 min later. Given these results, infection with N4r^+ in mass culture was chosen for the ultrastructural studies to be reported below. Quantitation of phage production under these conditions is given in Fig. 3. Mature virus can be detected after 30 min of infection, and the phage progeny matures linearly thereafter at a constant rate of 21 phage per cell per min for about 2 h. From Fig. 3 it is apparent that, although a large amount of

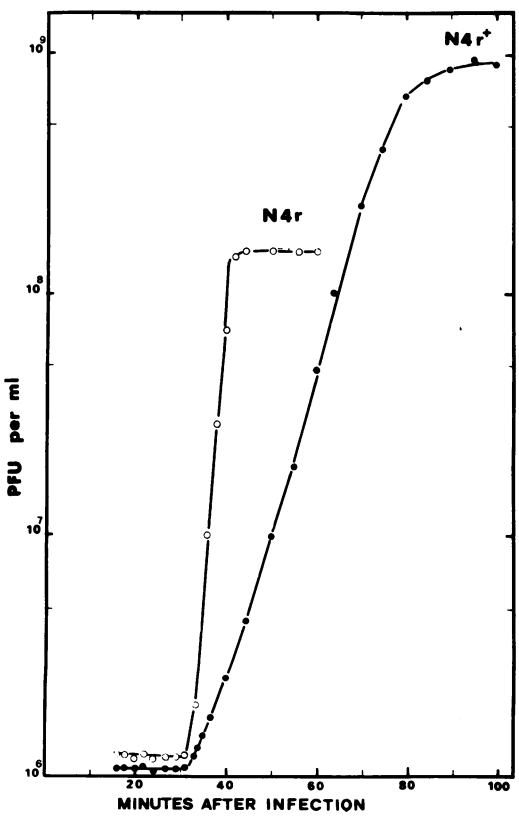


FIG. 1. *Development of bacteriophage N4r$^+$ (●) and N4r (○) in E. coli Hfr 3300 under one-step growth conditions in M9S at 37 C.*

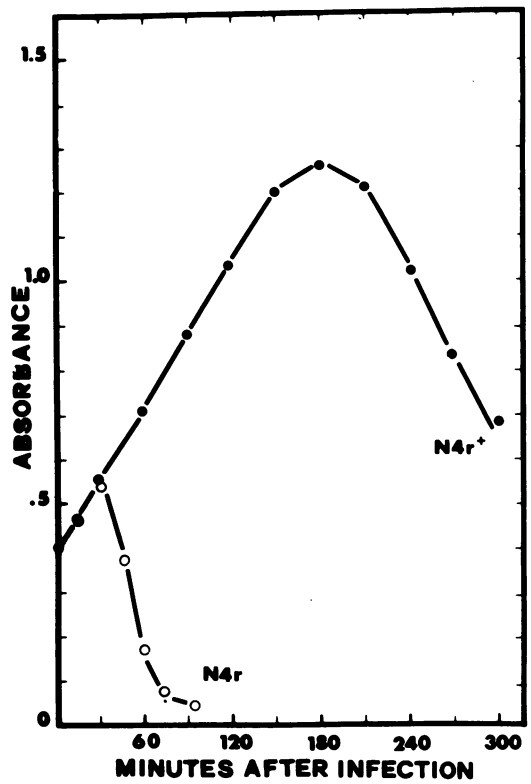


FIG. 2. *Cell lysis in E. coli infected with N4r$^+$ or N4r. Cultures of E. coli Hfr 3300 were grown to a density of 2 × 10^8/ml in M9S, whereupon each culture was infected with N4r$^+$ (●) or N4r (○) at a multiplicity of 10. At intervals, the turbidity of the cultures was measured at 650 nm.*

virions accumulates within the cells, the titer of the extracellular phage remains one order of magnitude lower than that of total phage for at least 150 min. The mean yield of N4 per cell, in mass culture, is 3,300.

Gross morphology of N4-infected cells. As mentioned above, the total mass on an infected culture increases severalfold during 3 h. This is due to the fact that although N4-infected cells do not divide and are efficiently killed (22), they elongate and increase in mean cell volume at a rate of 2.3 × 10^9 nm^3 per cell per h.

The gross morphological changes which occur after phage infection are illustrated in Fig. 4. Early after addition of the phage the rod-shaped cells (Fig. 4a) lengthen and thicken. After about 1 h of incubation dark spots become visible at cell poles (Fig. 4b). The bacteria grow progressively larger and, after about 3 h (Fig. 4c), the hypertrophic complexes are irregular in shape and might bulge in places. They show a central zone of low density and, by contrast, sharply defined regions located at one or, more frequently, both poles.

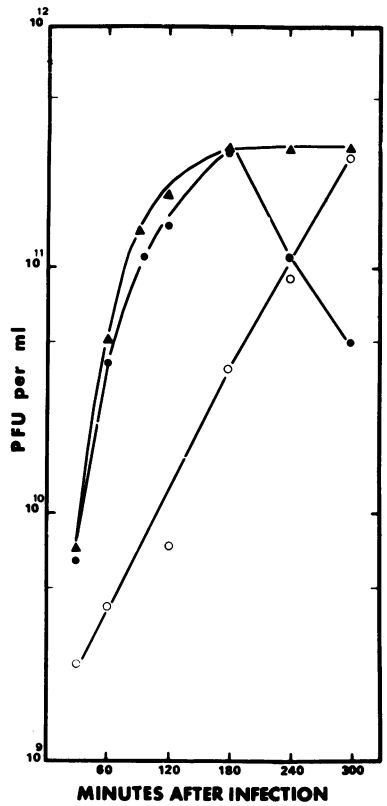


FIG. 3. *Time course of N4r⁺ bacteriophage replication in E. coli Hfr 3300 under conditions of high cell density. Cells grown in M9S to a density of 10⁸/ml were infected with N4r⁺ at a multiplicity of about 10. At prescribed intervals intracellular (●), free (○), or total phage (▲) was measured.*

Ultrastructure of infected bacteria. Few distinct ultrastructural changes were noted in the infected bacteria up to the time of virus maturation. By 30 min, a consistent proportion of the cells contain virus-like particles. The cell shown in Fig. 5 is distended, with a seemingly intact cytoplasmic membrane and cell wall. The nuclear area is enlarged and the cytoplasm is packed with fine granular particles. Developing phage heads are visible as round, irregular, electron-dense bodies located within the polar regions. Cells observed at 120 (Fig. 6) and 180 (Fig. 7) min were similar, but the average number of particles per bacterium increased from 20 to 200 and over 300, respectively.

Maturing N4 virions are not distributed randomly throughout the hypertrophic cell but are generally found at the periphery of the bacterium, at the poles. Morphogenesis of phage particles was never observed to occur, in close proximity to the low-density nuclear material.

A striking feature noted during the development of N4 is the coalescence of the mature viral progeny into paracrystalline arrays (21). These structures are rare prior to 120 min, but they are found in about 7 to 10% of the thin sections 180 min postinfection (Fig. 8 to 10). The paracrystals, consisting exclusively of hexagonal nets, are located in one pole of the bacteria. As many as 500 to 600 particles can be counted in well-preserved crystals. The diameter of the virions in these arrays is about 66 nm, and the individual particles appear to be composed of a central dense core surrounded by a less dense zone possibly corresponding to the capsid.

Analysis of the most frequent alterations noted in the late stage of N4 development points to the following tentative picture of the mechanism of cell lysis. Weakening of the cell wall structures by a phage-directed endolysin (22) induces localized bulges in a segment of the cell contour (Fig. 11 and 12). In these regions, the cell wall collapses and large holes are created where the outer cell membrane curls back (Fig. 13 and 14). For a short-lived phase the cytoplasmic membrane maintains the cell content within the main body of the bacterium, and then even this membrane bursts and the progeny virus streams out through the aperture (Fig. 15). Negative staining of lysing cells gave further evidence for this mechanism of cell lysis. Fig. 16 shows a giant cell releasing its enormous phage content into the medium. In Fig. 17 the rod shape of the bacterium is preserved and the process of extrusion of the intracellular material is limited to a few sites in the cell circumference. Most virus particles have the appearance of normal, DNA-filled virus with attached base plates.

DISCUSSION

E. coli Hfr 3300 cells infected with N4*r*⁺ or N4*r*, although showing the same latent period in one-step growth conditions, differ in the time required to lyse and in the burst size. In fact, even in the absence of superinfection (8), the yield of N4*r*⁺ exceeds 900 virions per cell as compared with about 110 for N4*r*. In mass culture, N4*r*⁺-infected bacteria manifest a long delay in lysis, lasting over 180 min, and release a mean phage burst of 3,300 PFU per cell. The behavior of N4 lysis-inhibited complexes therefore differs in a number of details from that shown by T-even phage-infected cells (3, 6, 8, 15), and the mechanisms that sustain this phenomenon may deserve further study.

Although it has been reported that the host bacteria sometimes enlarge after phage infection (2, 5, 10, 13), in most cases the modest

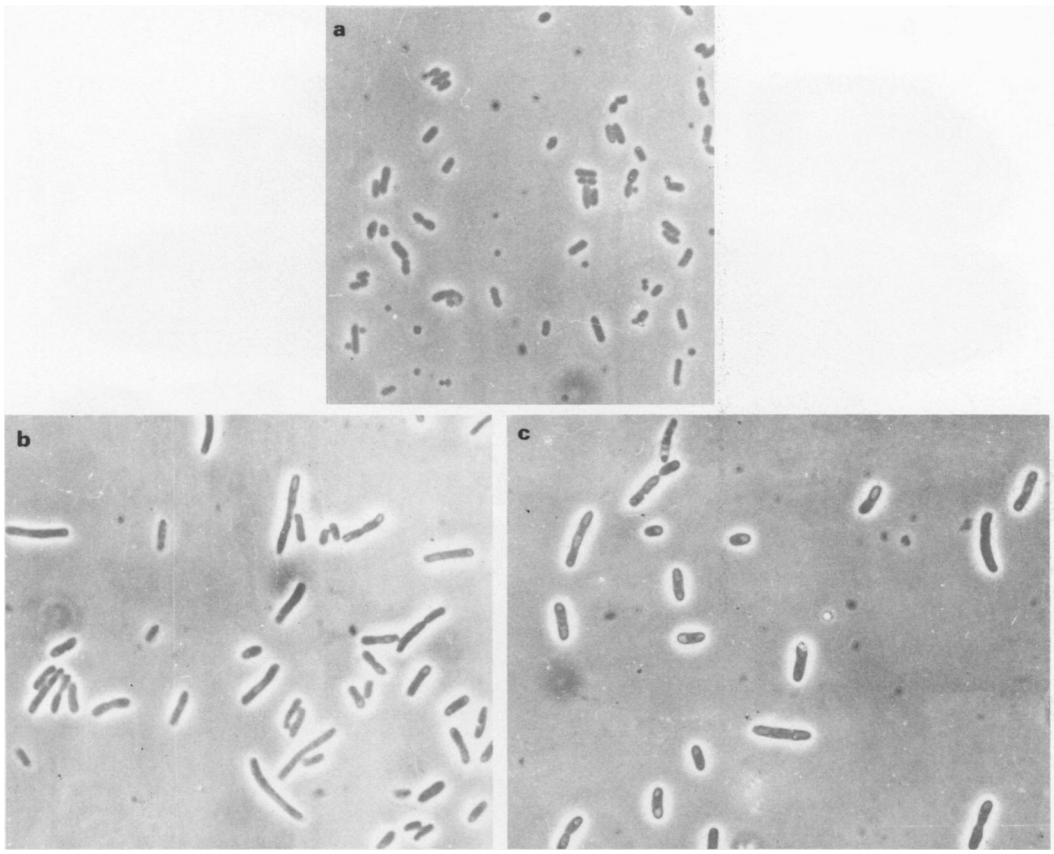
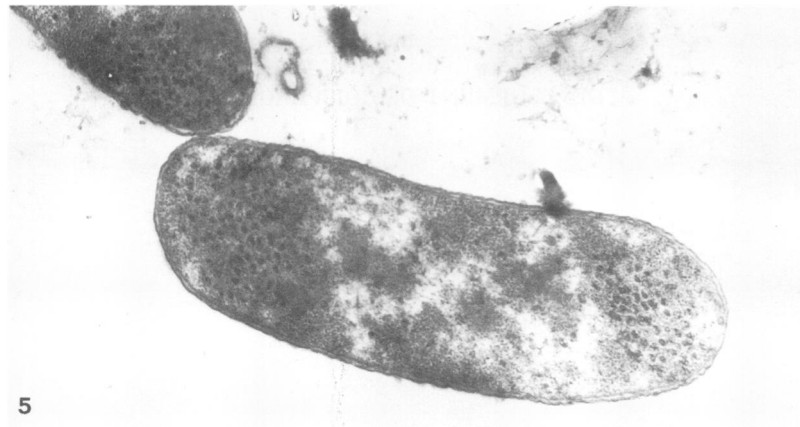
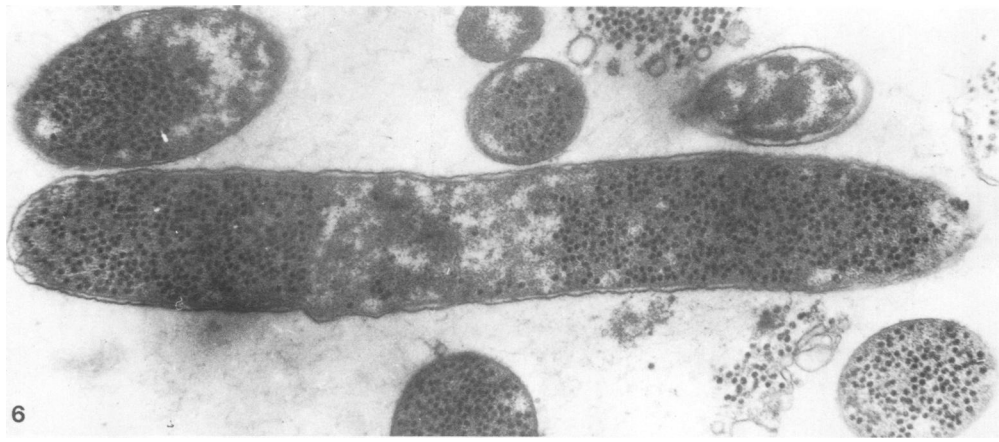


FIG. 4. *Sequence of gross morphological changes during replication of bacteriophage N4 in E. coli Hfr 3300. (a) Uninfected cells. (b) At 60 min postinfection the bacteria have increased in length and girth and a few individuals show differentiated polar regions. (c) At 180 min after addition of the phage. Typical bipolar spots are visible in large, hypertrophic cells.* ×1,600.





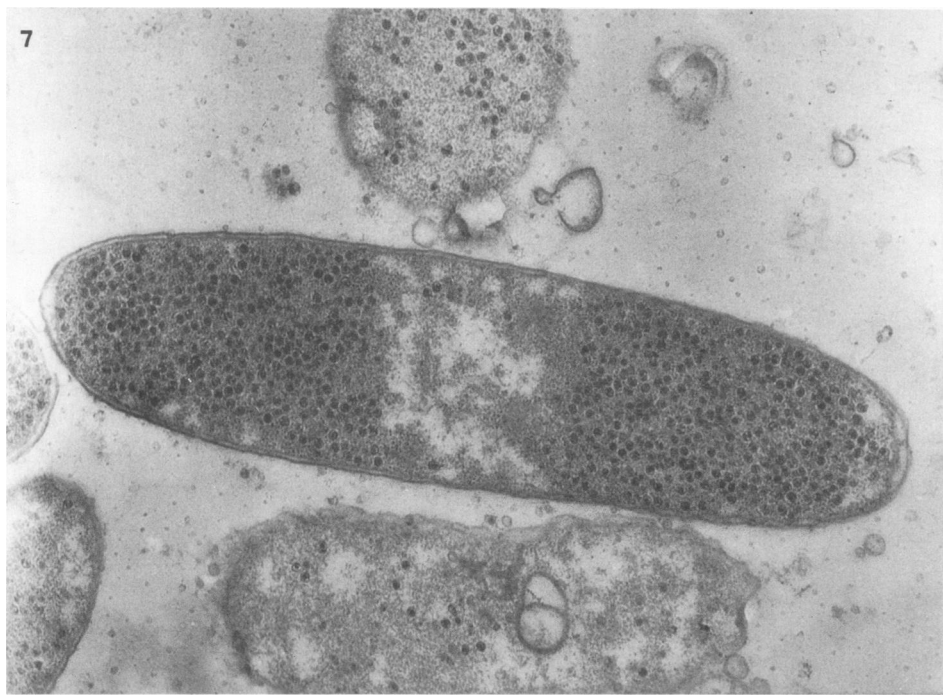
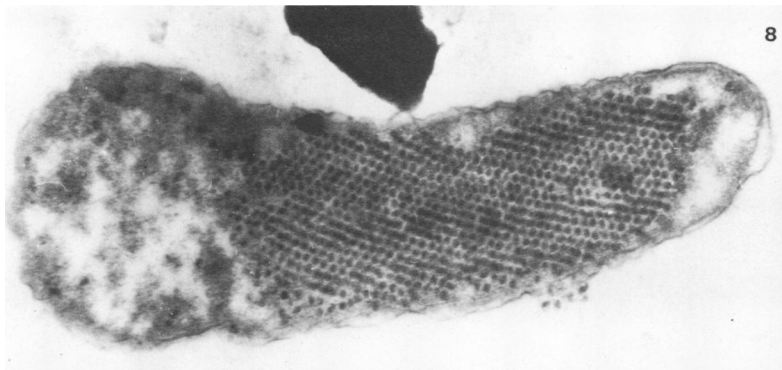
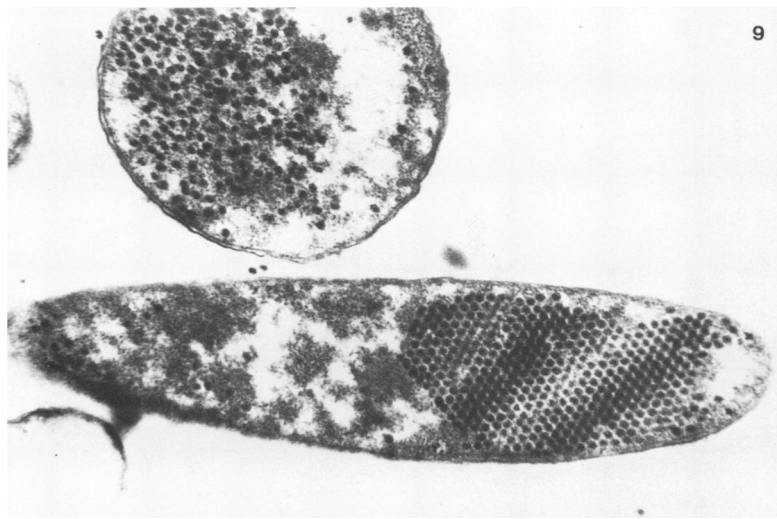


FIG. 5. *Distended cell after 30 min. Phages at various stages of development are seen. The nuclear area is enlarged.* ×*45,000.*

FIG. 6. *E. coli Hfr 3300 cells 120 min after infection. The cell is hypertrophic, and areas of phage morphogenesis are evident in localized zones of the cytoplasm. Note absence of virus particles from the nuclear area of the bacteria.* ×*18,000.*

FIG. 7. *Over 600 phage particles in various stages of development are observed in this cell at 180 min after infection. Note typical segregation of the areas of viral morphogenesis in the poles of the bacteria. Note also possible identification of the dark spots noted by phase contrast microscopy with regions of phage assembly.* ×*27,000.*





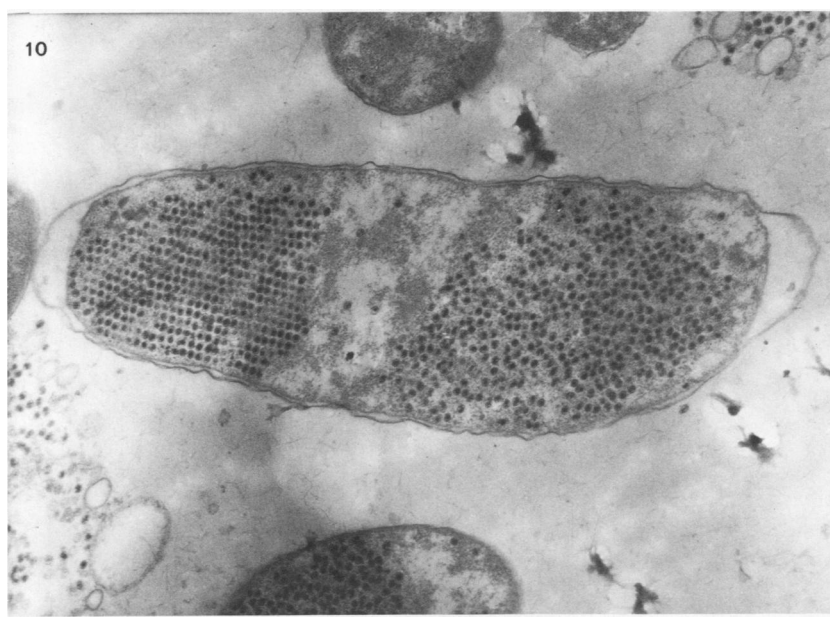
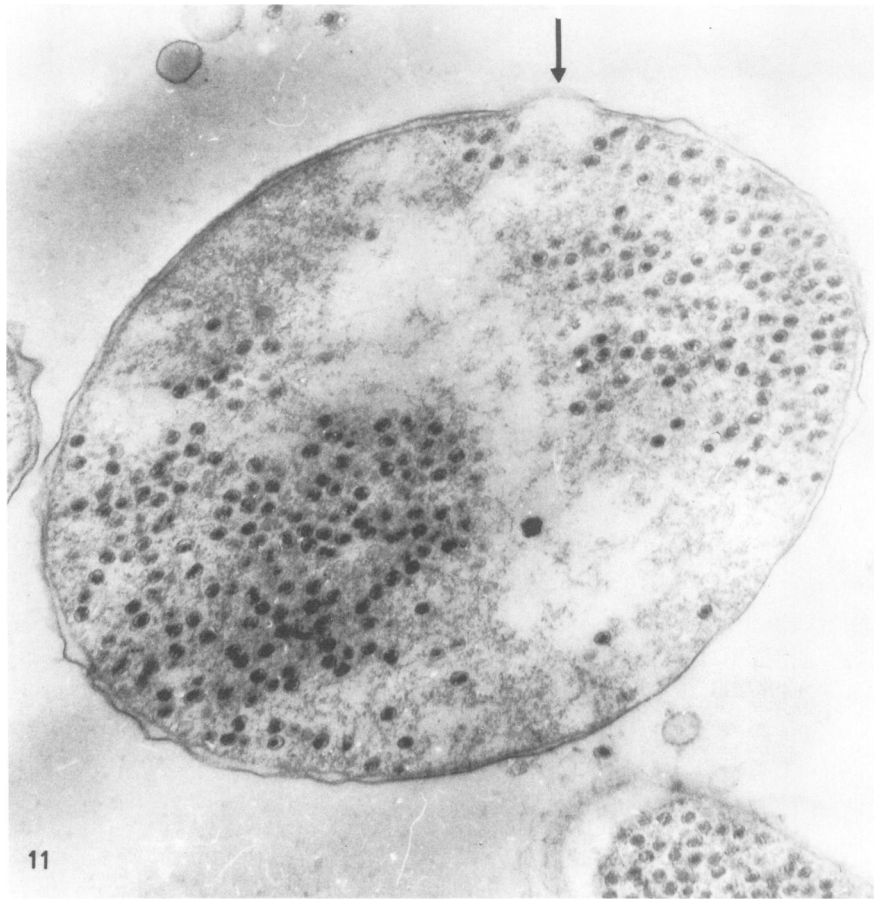


FIG. 8, 9, and 10. *E. coli Hfr 3300 cells with intracellular phage particles condensed into paracrystalline formations. Note preferential location of the crystals in the polar region of the bacteria. The crystal lattice is composed of hexagonal nets. Depending on the dimensions of the aggregate, up to 600 individual particles can be counted in the surface plane of the crystal. Viral capsids are often visible as thin contours around an electron-dense central core. Enlargements: ×34,000 (Fig. 8); ×36,000 (Fig. 9); and ×27,000 (Fig. 10).*



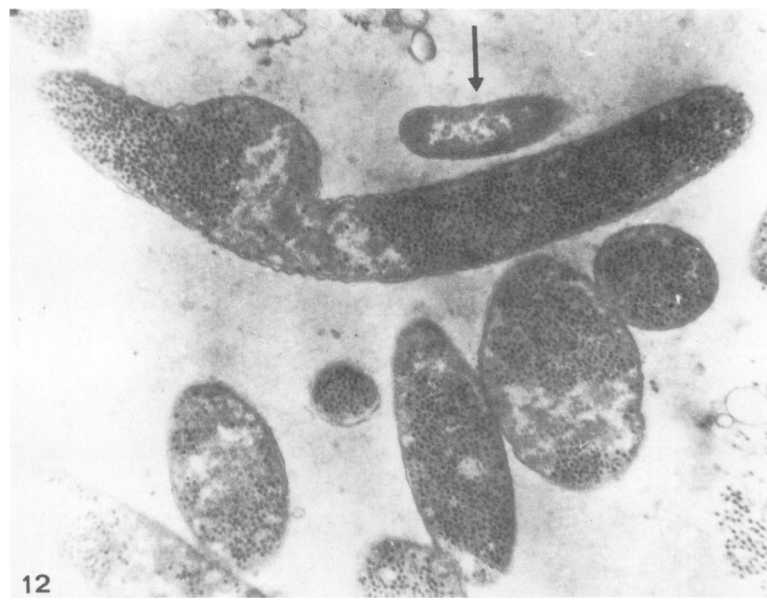
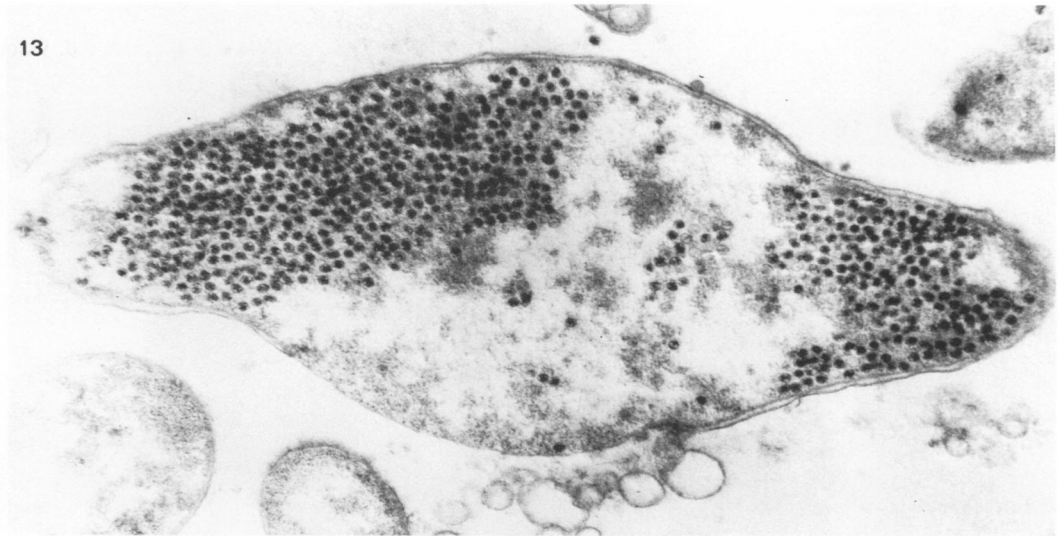
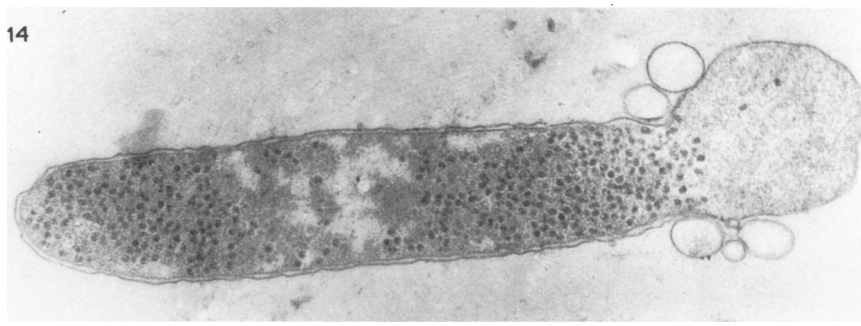


FIG. 11. *Host cell 240 min after infection. The cell appears swollen and the cytoplasmic membrane exhibits a small hole in its circumference (arrow).* ×*31,000.*

FIG. 12. *Deformed cell on the verge of lysing 240 min postinfection. Arrow points to a thin section of an N4-resistant bacterium displaying normal morphology.* ×*16,000.*





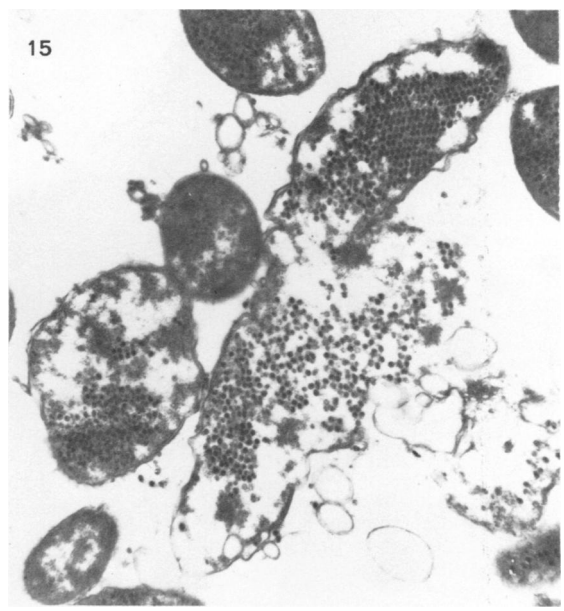
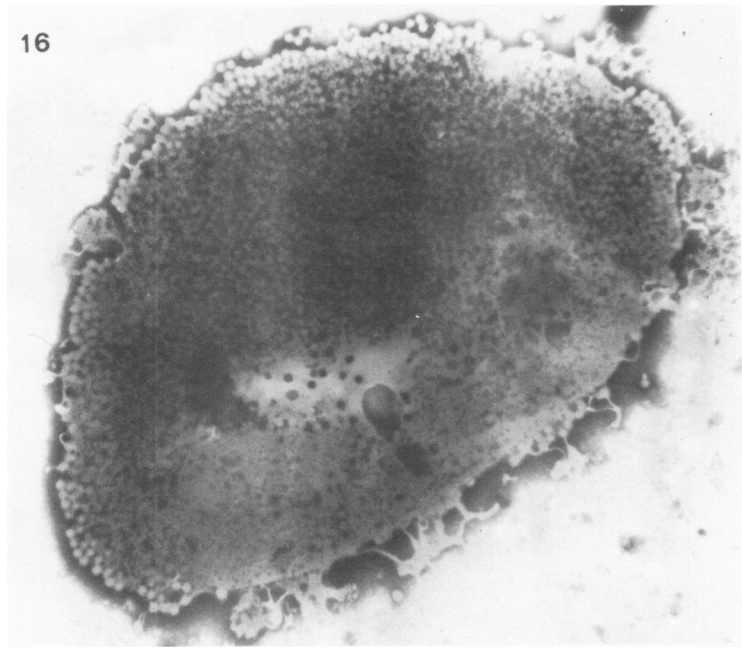


FIG. 13. *Lysing cell 240 min postinfection showing the plasma membrane bulging at the midpoint and retracted cell wall layers.* ×*30,000.*

FIG. 14. *Lysing E. coli cell showing cytoplasmic membrane bulging through ruptured cell wall layers. Note retraction of cell wall.* ×*32,000.*

FIG. 15. *Cell in last stage of lysis, showing phage particles and cytoplasmic material streaming out through a large hole in the cell envelope.* ×*22,000.*



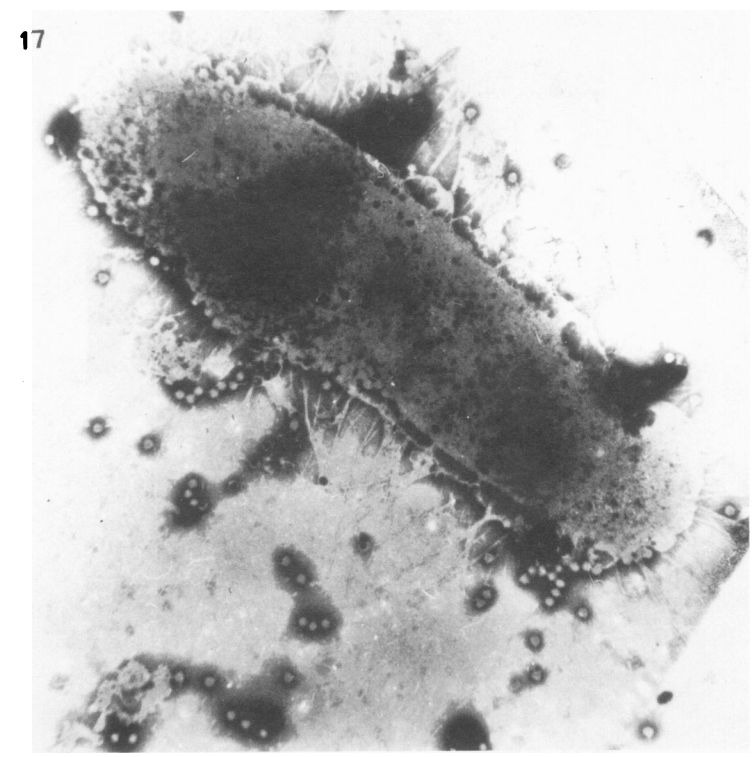


FIG. 16. *Negative staining of this giant cell shows partial lysis of the cell envelopes and dissolution of phage intracellular aggregates occurring at the cell periphery possibly as a result of streaming.* ×30,000.

FIG. 17. *Negatively-stained E. coli cells at 240 min postinfection. Mature phage escapes through small holes of the cytoplasmic sac. The cell wall still confers a rod shape to the lysing bacterium.* ×30,000.

increase in cell volume can be ascribed to osmotic swelling (17), residual growth of the bacterial envelopes, or an association of both processes (10). Since invasion by N4 does not completely shut off host macromolecular synthesis (19, 22), continuing growth of the cell combined with blockage of cell division can account for the remarkable hypertrophy of the complexes.

The ultrastructural changes which occur during N4 reproduction are in general agreement with those summarized by Bradley (4). Significant deviations include the preferential clustering of maturing particles in the polar regions, and this feature can be tentatively explained in a number of ways. Receptors for the phage could be confined to the poles, and replication could then be so compartmentalized as to hinder diffusion of virus precursors. Segregation of N4 development might contribute to an explanation of the incomplete arrest of host macromolecular synthesis already discussed (22). Alternatively, receptor sites could be distributed over the entire surface of the cell and the phage products could secondarily move from the sites of synthesis toward the poles where local microenvironmental conditions might favor the morphogenetic process.

Crystalline arrays of N4 virions are observed in about 10% of the cells 180 min postinfection, a frequency somewhat higher than that previously reported (21). At this time there is an average of over 3,000 physical particles per cell. Considering the phage volume (1.8×10^5 nm^3) and that of the host (about 9×10^9 nm^3, due to the continued cytoplasmic growth), the phage progeny fills about 7% of the cell volume. This figure is comparable to that described for the viroplasm of the RNA coliphages during whose replication crystalline aggregates were also noted (7, 9, 25).

Intracellular virus crystallization represents a rare event in the morphogenesis of bacteriophages. This is so possibly because conditions such as a high yield of phage, lysis inhibition, and maturation in restricted areas of the host that increase the local concentration of particles and thus favor crystal formation sledom occur in association.

The physiological significance, if any, of phage crystallization in vivo remains a matter of speculation.

ACKNOWLEDGMENTS

I thank C. A. Romanzi (Institute of Microbiology, University of Genoa) in whose laboratory part of this work has been performed, for hospitality and generous support. I also wish to acknowledge the excellent technical assistance of I. Conti who patiently maintained the electron microscope in working conditions throughout this investigation.

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