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Bacteriophage T4D Receptors and the *Escherichia coli* Cell Wall Structure: Role of Spherical Particles and Protein b of the Cell Wall in Bacteriophage Infection

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The nature of the interaction of bacteriophage T4D and the outer cell wall of its host, *Escherichia coli* B, has been investigated. Bacteria with altered or modified cell walls have been obtained by two different growth procedures: (i) growth in high osmolarity medium or (ii) growth in broth in the presence of divalent heavy metal ions. When these altered host cells were washed and subsequently added to regular growth medium, they interacted with added phage particles, but successful infection did not occur. Most of the phage particles released from these treated cells were observed to have full heads and an altered tail structure. The altered phage tails had contracted sheaths and unusual pieces of the bacterial cell wall attached to the distal portion of the exposed phage tail tube. Phage released from bacteria grown in the high osmolarity medium had attached cell wall pieces of two major types, these pieces being either 40 or 21 nm in diameter. The smaller-type cell wall pieces (21 nm) were formed by three spheres each measuring 7 nm in diameter. Phage particles released from cells previously exposed to the divalent metal ions had only one 7-nm cell wall sphere attached to the distal end of the tail tube. It was found that these 7-nm spheres (i) are normal components of the cell wall and are morphologically similar to endotoxin, (ii) are held in place on the cell wall by a component of the cell wall called protein b, and (iii) are most likely the site of penetration of the phage tail tube through which the phage DNA enters the host cell.

The substructure of *Escherichia coli* bacteriophage T4 particles which attaches to the host bacterium consists of a hexagonal baseplate with six projecting short tail fibers and six long tail fibers. The infection of *E. coli* by T4D is initiated by attachment of the long tail fibers to the bacteria (27); subsequently, the main body of the phage moves closer to the cell wall until its short tail fibers bind to the bacterial surface. At this time, T4D gene product 12, which makes up the short fibers, must interact specifically with its receptor site. The binding of gene product 12 to its bacterial receptor seems to immediately precede contraction of the tail sheath. This is followed by tube penetration through the host cell wall and, finally, DNA injection (4, 27). The binding of long tail fibers appears to be a reversible step, and, if short tail fibers are absent, the subsequent steps are not completed (28). In this case the phage can be eluted from the host bacteria. If the short tail fibers are present, the subsequent events lead to an irreversible attachment, and the phage can no longer be eluted. Because at least three structurally different phage components are involved in this infection process, it is to be expected that a number of different host cell wall components are also involved. Notably, isolated *E. coli* B lipopolysaccharide (LPS) has been shown to bind irreversibly to phage tails and to lead to DNA release (31). This seems to indicate that *E. coli* B LPS contains receptors for all the phage elements involved in this process. Specific LPS hexose residues have been identified as necessary for T4 attachment (6, 20), but the organization of the cell wall LPS which is in contact with the different phage structures is still unclear. Some aspects of the morphology of the receptor sites for the T phages were described previously by Bayer (1, 2). He found that bacteriophages T1 to T7 adsorb preferentially in areas where the cell wall and the inner cellular protoplasmic membrane remain adhered when the cell is plasmolyzed, but further details on the morphology of the receptor sites for these phages have not been reported.

The cell wall of gram-negative bacteria, which covers the inner cytoplasmic cell membrane, is

built of two major elements, the peptidoglycan complex layer and an outer membrane (7). The peptidoglycan layer consists mainly of polysaccharides covalently linked by oligo-amino acid peptide cross-bridges (30). In *E. coli* B this complex layer also includes a variety of other proteins, including two tightly bound proteins, one of which is lipoprotein described by Braun (3), and the other is the "matrix" protein described by Rosenbusch (22). This matrix protein has been named protein b by Lugtenberg et al. (13), protein 1 by Henning et al. (8), and protein 1 by Schnaitman (25). The relative amounts of the various proteins in *E. coli* B cell walls are quite different from those found in *E. coli* K-12. *E. coli* B contains largely protein b (the term proposed by Lugtenberg et al. and used in this paper) and a small amount of another protein, called d, and lacks completely a protein closely related to b, called protein c. On the other hand, *E. coli* K-12 strains contain relatively much less protein b (6 to 20%), more protein d (35 to 50%), and a significant amount of protein c (14 to 48%). In *E. coli* B, protein b and Braun's lipoprotein appear to connect the peptidoglycan with the outer membrane (12). The outer membrane of the gram-negative bacterial cell walls contains additional proteins, phospholipids, and LPS (19), as well as the receptor sites for bacteriophages (11).

In this paper we report some progress in characterizing the morphology of part of the host cell wall receptor for T4D and in understanding its relationship to the major matrix protein, protein b. This part of the cell receptor which reacts with the tail tube was isolated as a structure separated from the cell wall. This has been accomplished by using bacteria whose cell walls have been altered by growth either in a medium of high osmolarity or in the presence of certain heavy metal ions. Electron microscopic examination of phage particles which have reacted with these cells and then become detached showed that the phage tails carried with them specific and unusual fragments of the cell wall which are part of the phage receptor.

MATERIALS AND METHODS

Bacterial and phage strains and growth conditions. *E. coli* B strains and *E. coli* K-12 bacteria strain CR63 were used as hosts for T4D phage (27, 31). Phage were assayed by standard procedures. Normally the bacteria were grown in tryptone broth media containing per liter 8 g of tryptone (Difco) and 5 g of NaCl (85 mM) as the standard medium. In some experiments, to achieve high osmolarity, the NaCl (or KCl) concentration was raised to 300 mM, and in others the NaCl was omitted and sucrose was added to a final concentration of 600 mM. After growth in the high-osmolarity medium, the cells were washed and resuspended in standard broth containing 85 mM NaCl.

E. coli B was also grown in the presence of added divalent metal ions. The cells were incubated, usually overnight, at 35°C in broth media containing the various divalent metals until the turbidity of the culture, i.e., the mass of the cells, was 50% of the turbidity of a control culture. The concentration of the different metal ions in the growth medium was: for Zn^{2+}, 0.5 mM; for Cd^{2+}, 0.4 mM; for Co^{2+}, 0.1 mM; for Ni^{2+}, 0.75 mM; and for Cu^{2+}, 1.8 mM. After growth in medium containing one of these heavy metal ions, the cells were centrifuged at low speed and washed twice with fresh media, free of added heavy metal ions, and then resuspended in fresh media containing 85 mM NaCl to give a final cell concentration of 5×10^8 to 8×10^8 cells per ml. They were then infected with T4D at a multiplicity of 50 to 100 T4D per cell and incubated for 15 min at 35°C. The infected cells were centrifuged at low speed and then resuspended in a small volume of phosphate buffer 0.1 M, pH 7.0, and samples were removed for electron microscopy.

Chemicals and other methods. All chemicals were of analytical grade. Samples were prepared for electron microscopy by negatively staining with 0.3 or 1% phosphotungstic acid, pH 7.0, on carbon-coated collodion grids. They were examined and photographed with an RCA EMU 4 electron microscope. Gel electrophoresis was carried out on 10% sodium dodecyl sulfate-polyacrylamide gels at a current of 3 mA per gel in glass tubes (7 by 120 mm) in 0.025/0.2 M tris(hydroxymethyl)aminomethane-glycine buffer, pH 8.8, with 0.1% sodium dodecyl sulfate. Each gel was loaded with 100 μl of extract. Protein bands were stained with Coomassie brilliant blue.

RESULTS

Effect of previous bacterial growth in high-osmolarity media on the infection of *E. coli* B with T4D. The phage-induced lysis of *E. coli* B cells previously grown in high concentrations of sucrose, NaCl, or KCl was delayed with respect to cells grown in low-salt media (Fig. 1). Under these circumstances it is known that the relative concentration of the specific matrix protein, protein b, in the cell wall is greatly decreased (29). One possible explanation for the resistance to lysis is to assume that the phage cannot be adsorbed until the growth of the cells in the standard medium results in normalization of protein b content of the cell wall (1.5 generations, according to Van Alphen and Lugtenberg [29]) and only then the bacteria are infected. This would account for the longer time before lysis occurred. Alternately, it can be assumed that the phage interacted with the host cells with the altered cell walls but that this interaction did not lead to a productive infection. Figure 2A shows phage attachment to cells previously grown in medium of standard osmolarity at multiplicity of infection of 30 phage per bacterium. Figure 2B and C shows the effect on

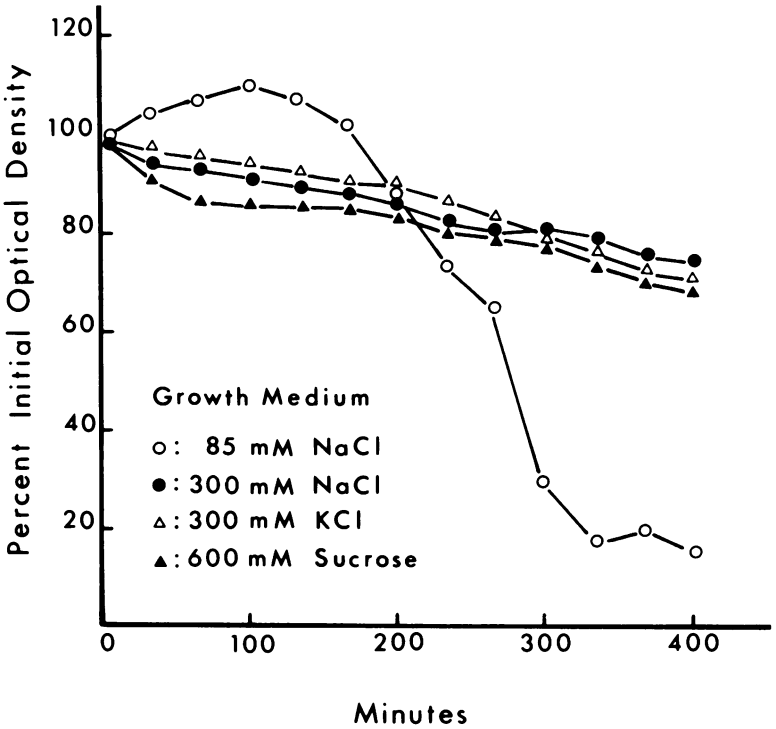


FIG. 1. *Sensitivity of E. coli B grown in high-osmolarity media to T4D infection. The cells were grown overnight in broth media containing 85 mM NaCl (○), 300 mM NaCl (●), 300 mM KCl (△), or 600 mM sucrose (▲). After washing twice with fresh media (85 mM NaCl), the cells were resuspended in fresh media, 6 × 10^8 to 8 × 10^8 per ml, infected with T4D (multiplicity of infection, 1/1), and the light transmission of each sample was read in a Klett photoelectric colorimeter.*

phage attachment when the host cells were previously grown in high concentrations of NaCl or sucrose. Most of the phage particles still have full heads, and very few (if any) phage particles are seen attached to the cell walls of bacteria previously grown in the medium of high osmolarity. However, the majority of phage particles near the cell walls have altered tails with contracted tail sheaths. These results suggest that although the phage particles did not remain attached to host bacteria when the cells were grown previously in high-osmolarity medium, a primary interaction of the phage with the bacterial receptor sites had occurred, resulting in a physical alteration of the phage tail. Figure 3 shows that the content of protein b per cell wall from *E. coli* B cells grown in 300 mM NaCl in these experiments was diminished three- to fourfold as compared with the protein b content of cells grown under standard conditions. The same was true for cells grown in 300 mM KCl or 600 mM sucrose (data not shown). Because most of the other cell wall components appear to be unchanged (29), it seems likely that the decrease in protein b content was directly related to the resistance of these cells to successful T4D infection.

Interaction of *E. coli* K-12 with T4D after prior bacterial growth at high osmolarity. In contrast to *E. coli* B, *E. coli* K-12 grown in high-osmolarity medium showed somewhat different changes in cell wall composition and function. Whereas *E. coli* B lost both protein b and concurrently certain cell wall transport functions, *E. coli* K-12, while losing protein b, replaced it in the cell wall with a quite similar protein called c and retained these same transport functions (14, 15, 29).

Similar experiments to that shown in Fig. 1 did not demonstrate any difference in the lysis time between cells of *E. coli* K-12 grown in low or high osmolarity and infected with T4D (data not shown). Figure 4 shows microphotographs of *E. coli* K-12 cells grown in high osmolarity and then infected with T4D. Figure 4A illustrates phage attachment to cells grown in normal medium. Figure 4B and C shows T4 attachment to *E. coli* K-12 cells previously grown in high concentration of NaCl or sucrose. The attachment in this case is mostly shown over large

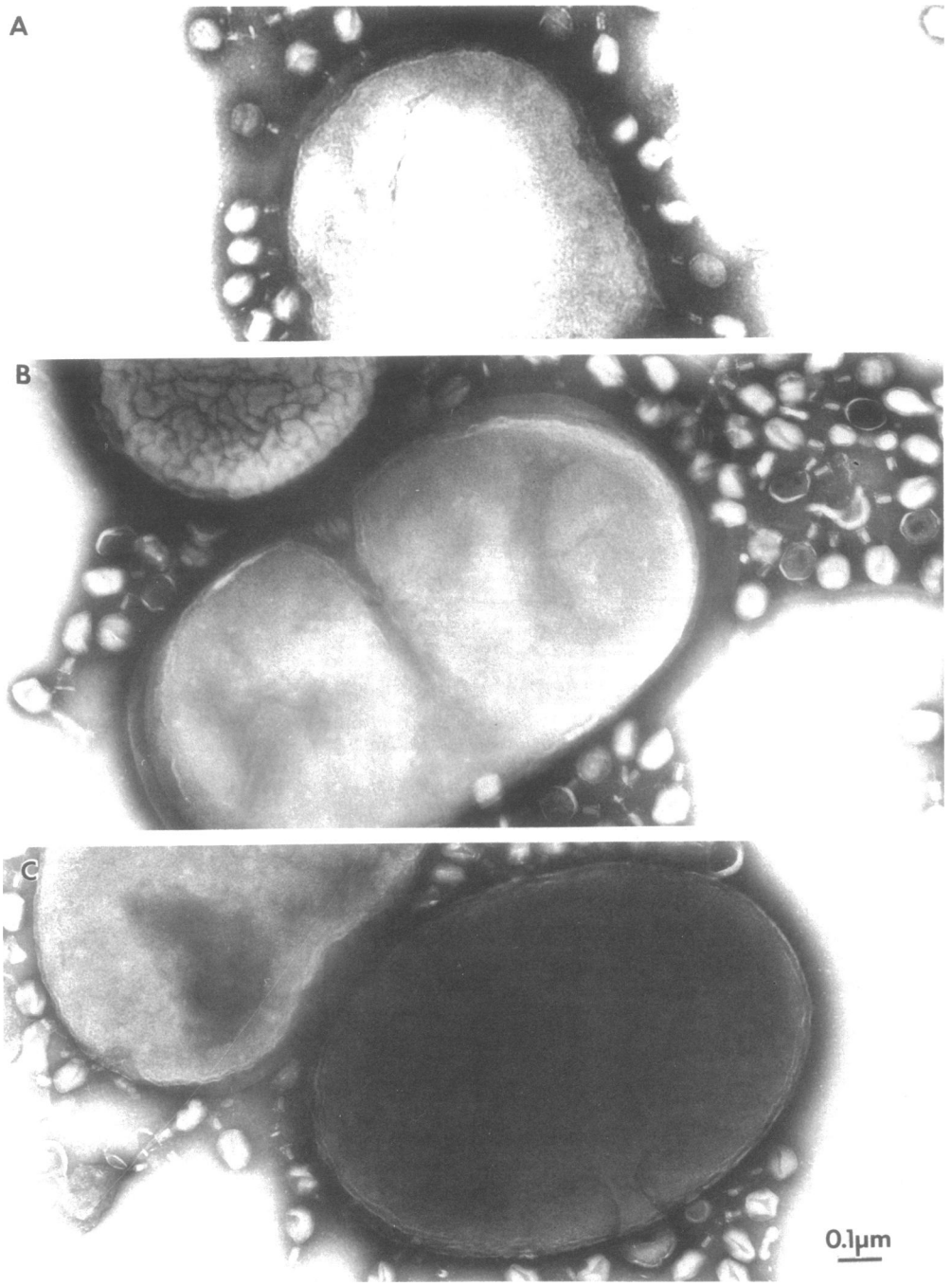


FIG. 2. *Negatively stained E. coli B grown in broth media containing 85 mM NaCl (A), 300 mM NaCl (B), or 600 mM sucrose (C) and infected with 30 T4D/cell in fresh medium (85 mM NaCl).*

sections of cell wall (bacterial ghost), because the infection was made at high multiplicity and almost all the bacteria were lysed from without.

There was a marked contrast in the reaction of T4 with *E. coli* B as compared with *E. coli* K-12. *E. coli* K-12 showed no alterations in the efficiency of phage attachment when grown under these different conditions. This suggests that

in *E. coli* K-12 cells grown at high osmolarity protein c, which was incorporated into the cell wall in place of protein b, also functioned to replace protein b in the structure of the phage receptor site. As *E. coli* B does not have protein c after growth at high osmolarity when protein b is diminished, part of the phage receptors appears to be defective.

Phage morphology and the phage tail relationship to the phage receptor after prior growth at high osmolarity. The results shown in Fig. 2 indicate that, upon infection of *E. coli* B grown at high osmolarity, phage and receptor interact, but normal infection does not occur. A detailed analysis of these infected cells (Fig. 5A) shows that phage particles near the cell wall have contracted tail sheaths and various-sized pieces of the bacterial wall are attached to the distal portion of the exposed tail tube. These host cell wall pieces can be classified into two distinct groups (Fig. 5B and C): (i) those pieces which are 40 ± 4 nm in diameter and 10 ± 2 nm wide and (ii) a cell wall structure of 18 to 24 nm in diameter which is apparently formed by three spheres, 7 ± 1 nm in diameter each. It is important to note that three spheres of 7 nm each in a chain were also observed near the site of tail tube penetration in phages interacting with the cell wall of *E. coli* K-12 or of *E. coli* B grown under normal conditions (Fig. 6A and B). These structures are only visible where the phages are bound to cell wall ghosts but are not visible over intact cells because of the high density of the cell material. The diameter of the larger cell wall pieces (group I) is similar to the diameter of the phage tail baseplate, suggesting that the places where cell wall disruption occurred are in the same area where the short fibers of the phage baseplate attach to the cell wall. On the other hand, the group II cell wall pieces show a marked similarity to the chain-like spherical structures forming the endotoxin isolated from *E. coli* and *Pseudomonas aeruginosa* cell walls (17, 21).

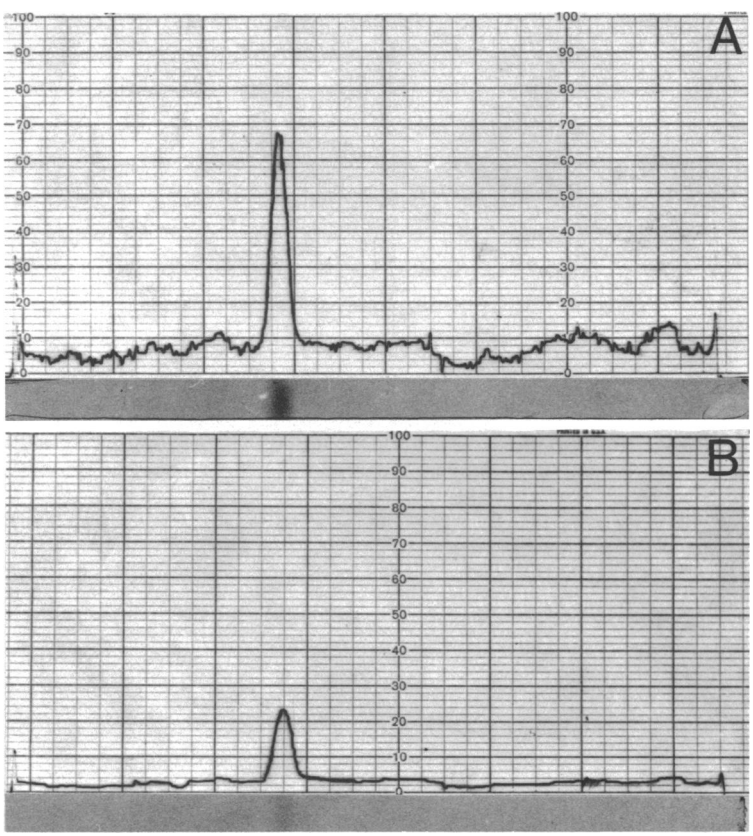
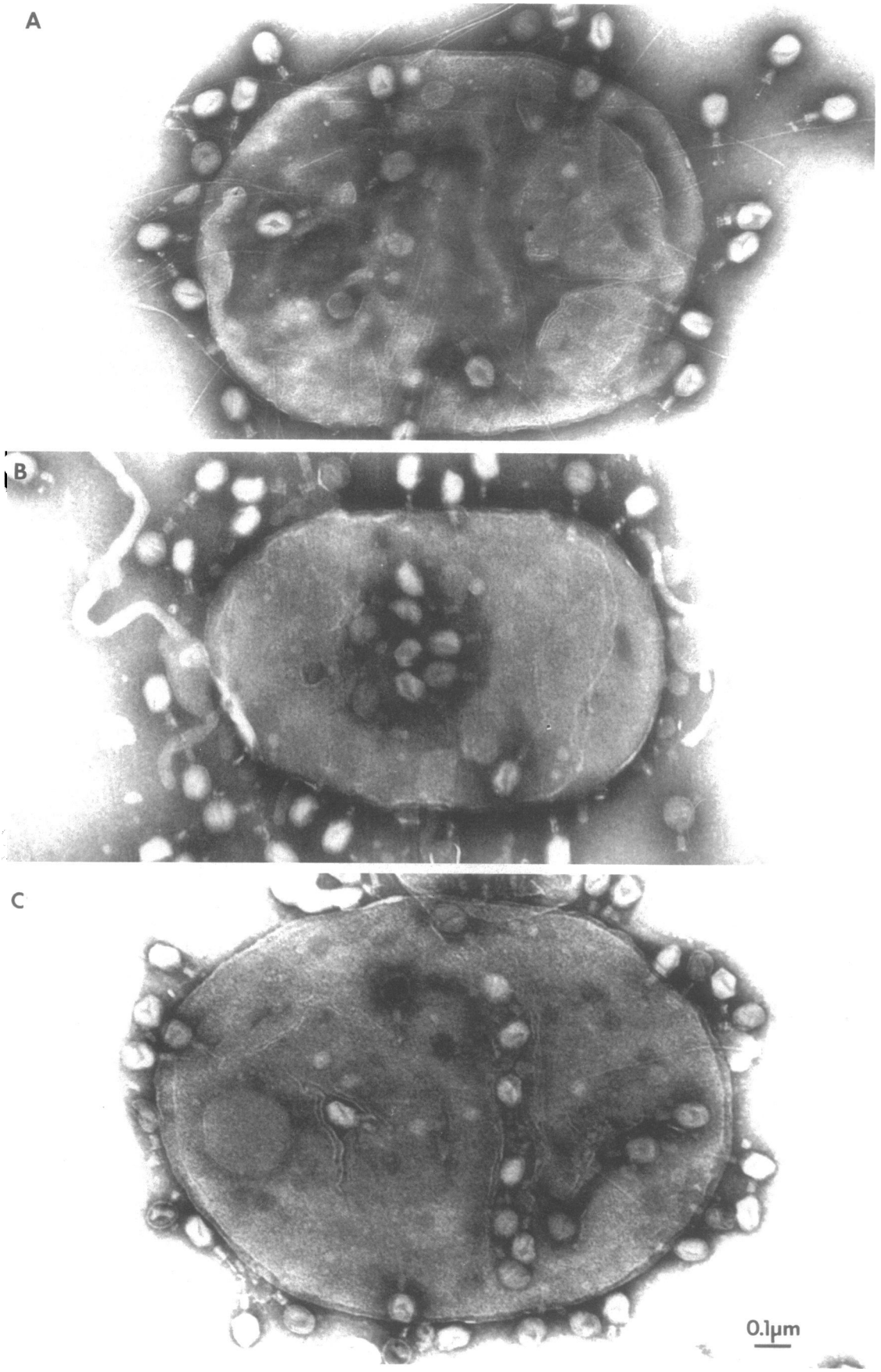


FIG. 3. *Densitometric tracing and electrophoresis gels of peptidoglycan-associated outer membrane proteins of E. coli B grown in broth media containing 85 mM NaCl (A) or 300 mM NaCl (B). The proteins were extracted from the cells by successive heatings at 55 and 100°C in a buffer containing 2% sodium dodecyl sulfate as described by Rosenbusch (22). The gels were stained with Coomassie brillant blue.*


A
B
C
0.1µm

These results with *E. coli* B or K-12 strongly suggest that the cell wall matrix component, protein b (or c), is structurally involved with the T4D receptor site and serves primarily to stabilize it, because in its absence the contraction of the phage sheath leads to the detachment of the phage receptor area of the cell wall.

Interaction of T4D particles with host bacterial cells having modified cell walls after growth in the presence of various divalent metal ions. It is known that the presence of heavy metal ions in the growth medium causes a number of changes in the cell wall structure (18). Analysis of these cell walls (to be described elsewhere) has indicated that the major change is a decrease in the amount of the Braun lipoprotein. When *E. coli* B cells previously grown in high concentrations of divalent cations such as Zn^{2+}, Cd^{2+}, Co^{2+}, or Cu^{2+} were infected with T4D at low multiplicity of infection, lysis from without was observed, and the cell wall was broken down to many smaller fragments. The supernatant fluid contained spherical particles similar to the known cell wall endotoxin (17) and also contained a large number of phage associated with these particles (Fig. 7A). Most significantly, altered phage particles were observed showing just one of these spheres located at the distal tip of the tail-tube (Fig. 7B and C). The micrographs do not clearly show whether the tube penetrated through the center of the sphere or if the sphere was bound to the side of the tail tube. However, because the spheres in all the pictures appeared to be centered with respect to the tube, it seems likely that the tail tube goes through the center of the sphere.

DISCUSSION

It is likely that the coordination of all sequential steps during bacteriophage attachment and infection demands the existence of a well-defined structure on the bacterial cell wall, fitting the symmetry of the phage baseplate. Our results lend support to this inference because under conditions in which the receptor is destabilized by a decrease in the amount of protein b in the cell wall, in many cases the piece of cell wall detached during the contraction of the phage sheath is the same size as the baseplate. It should be emphasized here again that protein b is a matrix protein which links the outer structure of the cell wall to the peptidoglycan layer.

It is important to note that the host receptor seems to have a central portion where the tail tube penetration occurs (Fig. 5 and 6). After reaction with protein b-deficient cells, the phage tail tube appears to be attached to a piece of the cell wall consisting of a linear array of three spheres 7 nm in diameter with a morphology identical to those described for the free endotoxin isolated from *E. coli* cultures (17) or to the lipid protein-LPS complexes released from cell walls of *P. aeruginosa* by ethylenediaminetetraacetic acid (21). This central part of the receptor appears to be torn off the cell wall during phage tail sheath contraction when the protein b content of the cell has been lowered (Fig. 5C). The decrease in protein b appears to create at least two weak points in the receptor, one at the probable site of attachment of the short tail fibers and the other 10 nm away from the tube penetration site. Consequently, the phage particles near the bacteria show either a piece of the bacterial wall approximately 40 nm in size (Fig. 5B) or chains 21 nm long formed by three spheres 7 nm each in diameter (Fig. 5C). Although these spheres are clearly not protein b, it is interesting to note that 7.5 nm is the diameter measured for aggregates of protein b linked to the peptidoglycan layer (22). The similarity in size of these protein b aggregates to the spheres released with phage particles from protein b-deficient cells suggests a possible overlapping of these protein b aggregates with the lipid-protein-LPS complex constituting the endotoxin in some gram-negative bacteria (9, 10, 17, 21, 23).

The Cu^{2+}-resistant mutants of *E. coli* B described by Lutkenhaus (15) show a marked decrease in the protein b content of the cell wall. These mutants show altered permeability to various hydrophylic chemicals. In *E. coli* K-12, a decrease in both protein b and c is necessary to alter permeability in the outer membrane to the same hydrophilic chemicals (15). These observations support those reported here on loss of stability of the phage receptor in cells grown at high osmolarity for *E. coli* B but not for receptors in *E. coli* K-12. These facts strongly suggest that both b and c proteins could perform the same role for some functions. This is not surprising given the similarity in structure for these two proteins (24).

When *E. coli* B was grown in high concentrations of four different divalent heavy metal ions and infected with T4D at high multiplicity, phage particles showed only one 7-nm sphere on the distal portion of the tail tube (Fig. 7). The symmetrical position of the sphere on the tail tube suggests that the tube penetrates through

FIG. 4. *Negatively stained E. coli K-12 grown in broth media containing 85 mM NaCl (A), 300 mM NaCl (B), or 600 mM sucrose (C) and infected with 30 T4D/cell in fresh medium (85 mM NaCl).*

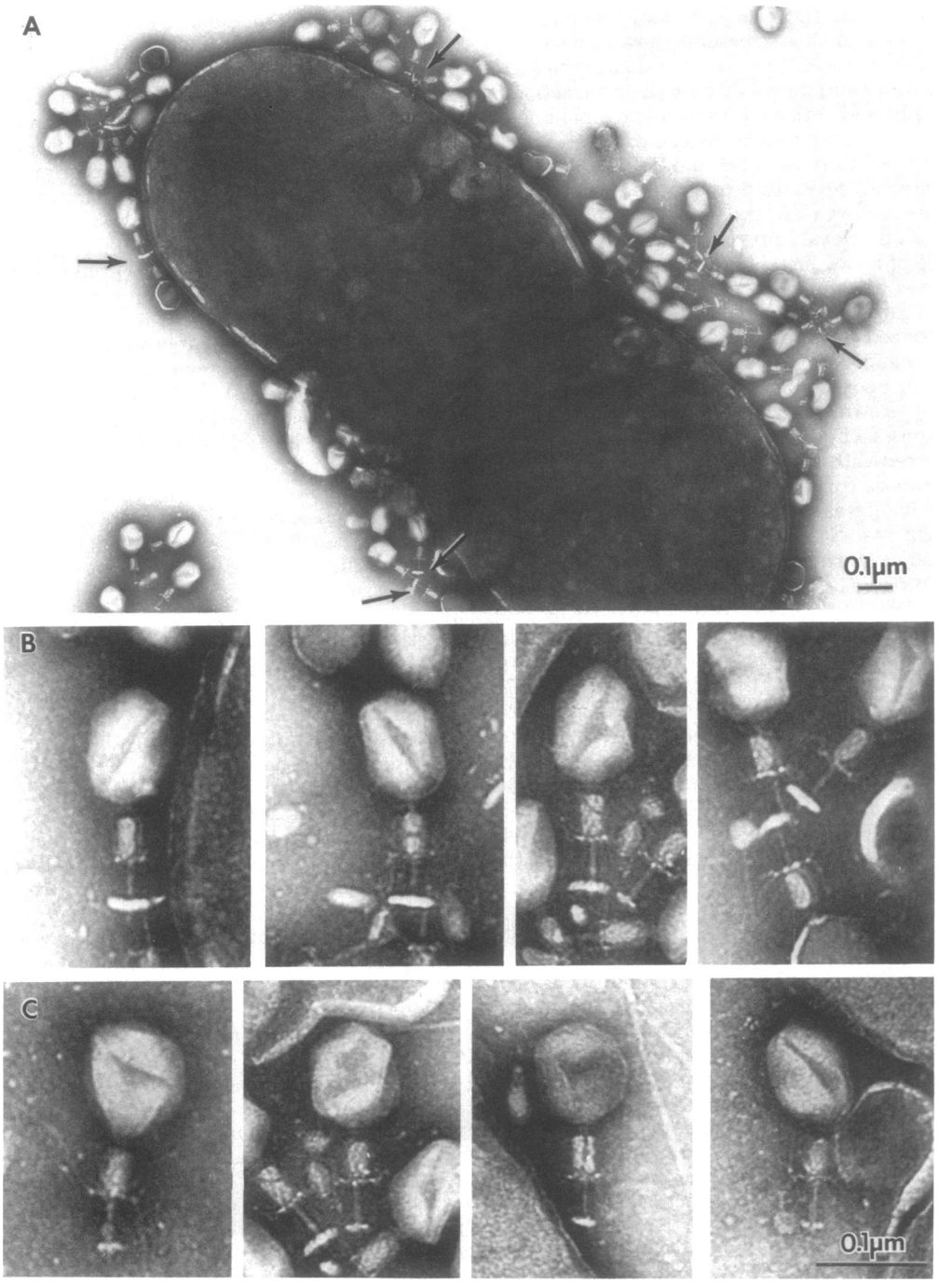


FIG. 5. *Negatively stained E. coli B grown in broth media containing 300 mM NaCl and infected with 30 T4D/cell in fresh media (85 mM NaCl). (A) Unattached phages with contracted sheath and various-size pieces of bacterial wall attached to the tail tube; (B) 40-nm cell wall pieces attached to the tail tube; (C) 21-nm cell wall pieces attached to the tail tube.*

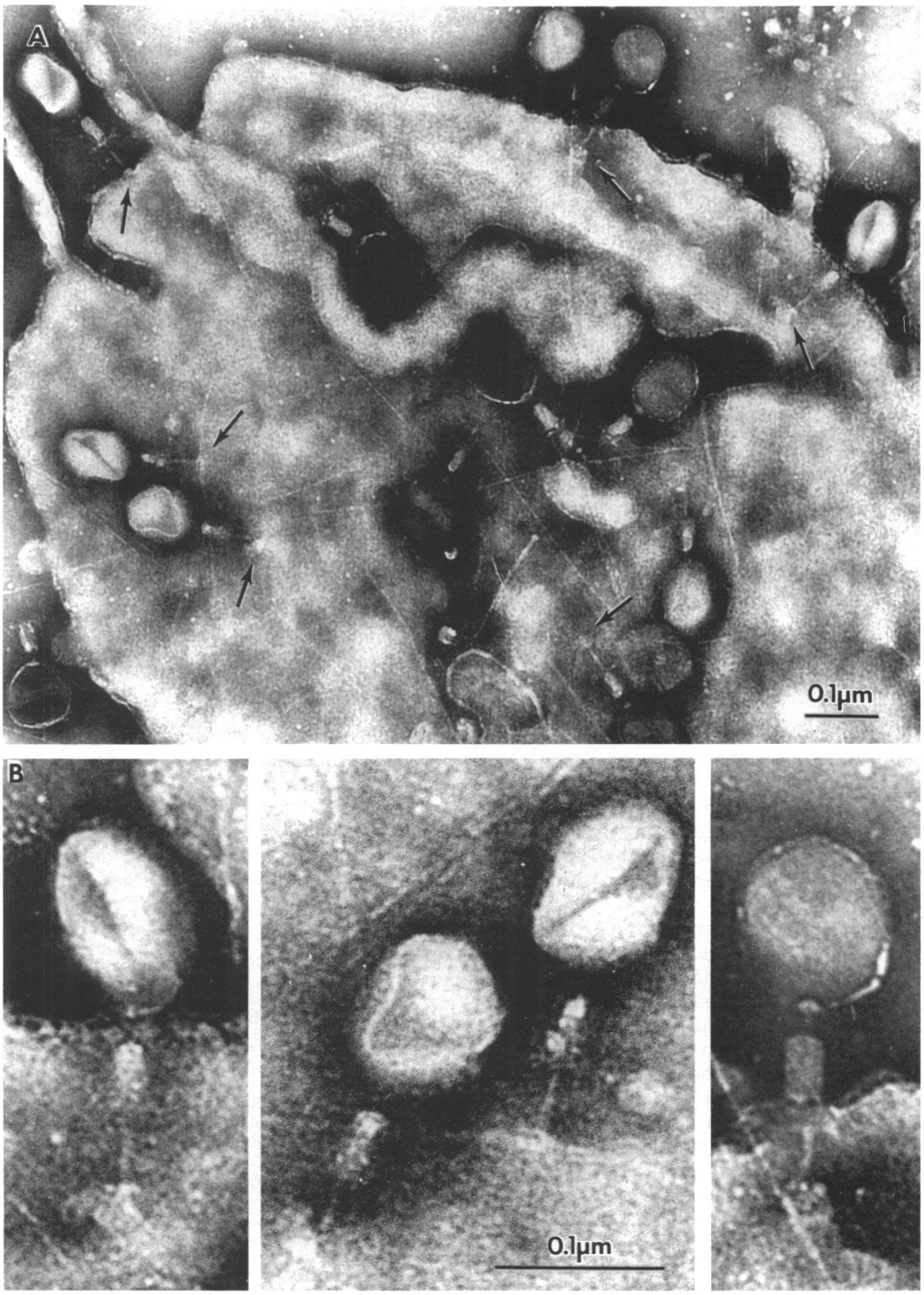


FIG. 6. *(A) Negatively stained E. coli K-12 cell wall (ghost), showing a large number of phage attached. The arrow indicates a typical formation of the three 7-nm each spherical particles seen distributed over the cell wall in the site of tail tube penetration. (B) The same particles, but at higher magnification.*

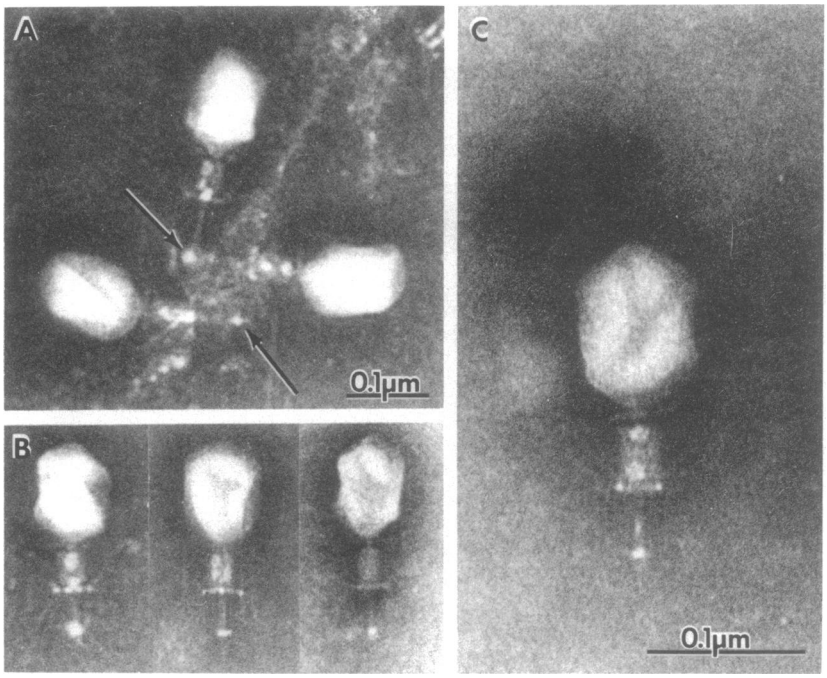


FIG. 7. *Negatively stained phages, showing 7-nm spheres bound to the distal portion of the phage tail tube. The phages were detached during a high-multiplicity infection from cell wall of E. coli B previously grown in high concentration of* Zn^{2+}.

it. If it were just a lateral association, we would be able to see occasionally such spheres attached tangentially to the tubes. It seems then that during the infection the phage is positioned directly over a cell wall pore in the center of a spherical cell wall particle, so that its tail tube can pass through the sphere and through the cell wall.

The infection process might be then somewhat similar to bacterial conjugation. The importance of both LPS and one of the major outer cell wall proteins (protein II of Henning) in the recipient cell wall during conjugation has been shown (26). It has been proposed by Manning and Reeves (16) that the alteration of LPS affects recipient function by "altering the local environment of protein 3A (II of Henning) or other receptor in the outer membrane." The importance of the association between LPS and outer membrane proteins has also been established for phages Tu Ia, Tu Ib, and Tu II* receptors in *E. coli* (5).

In case of T4D infection, the sites of penetration are probably the 7-nm spheres, presumably LPS, free of any associated additional internal proteins so that the phage tail tube itself can act as an outer membrane protein and thus form a continuous pore or channel from the phage head through the cell wall spherical particle and into the cell. These spherical particles without any internal proteins would probably be found in the newly synthesized cell wall. This is in agreement with Bayer's observation that the sites of attachment for the T phages are located where the cell has been newly synthesized (2); during "maturation" of the cell wall, the spheres may be filled with different outer membrane proteins which then block the opening of the site of penetration of the T4 tail tube.

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