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Isolation of Bacteriophages T2 and T4 Attached to the Outer Membrane of *Escherichia coli*

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Phage T2 or T4 was adsorbed to *Escherichia coli*, and the outer (L) membrane was then isolated with the phage still attached in their usual postinjection appearance. T2 was readily inactivated by isolated cell walls but very poorly by purified L membrane. T4 was inactivated by neither.

Specificity of bacteriophage adsorption resides in the cell wall (18). The cell wall of gram-negative bacteria is composed of two major components: the peptidoglycan layer and the outer or "L" membrane (7). L membrane is composed of lipopolysaccharide (LPS), protein, and phospholipid (11) and contains the receptor sites for gram-negative bacteriophages. Phages T3 (2, 10, 20), T4 (2, 8, 20, 21), T7 (2, 10, 20), C21 (13), and ϵ^{15} (9) attach to the LPS component. Phages T2 (2, 10) and T6 (2, 10) attach to a protein or lipoprotein component. These conclusions are based on the isolation of chemical moieties that inactivate phage and on the analysis of phage-resistant bacterial mutants.

Simon and Anderson (16) showed that phage first attach to the cell surface and then inject their needle about 12 nm into the cell wall. The present communication describes a novel experiment which extends their work one step further. Phage T2 or T4 was first adsorbed to *Escherichia coli*, and then the L membrane was purified with the phage still attached.

A suspension of *E. coli* K-12 (AW330) (4), 10^9 cells/ml, was prepared in 0.035 M potassium phosphate (*p*H 7.1), 0.08 M NaCl, and 10% (w/w) sucrose at 37 C. An equal volume (3 ml) of either T2 or T4 (Doermann's strain, no tryptophan requirement) in the same medium was added to give 100 particles per cell. In 4 min, 50 to 60% of the phage had adsorbed. Spheroplasts were then formed by a 10-min incubation at 30 C in 0.1 M tris(hydroxymethyl)aminomethane (Tris), *p*H 7.8, 60 μg of lysozyme per ml, and 2 mM ethylenediaminetetraacetic acid (EDTA). The spheroplasts were adjusted to 30 mM $MgCl_2$, stirred for 2 min, and then lysed by dissolving their exposed plasma membranes in 0.5% Triton X-100 (4). The $MgCl_2$ prevents solubilization of L membrane by the detergent (3). Deoxyribonuclease I (10 μg/ml) was then added to reduce the viscosity.

The following procedure is a modification of a method for purifying L membrane (4). In a Spinco SW-25 tube, 2 ml of 20% (w/w) sucrose in 0.1 M Tris (*p*H 7.8) and 10 mM $MgCl_2$ (Tris-Mg^{2+} buffer) was layered over 3 ml of 60% sucrose in Tris-Mg^{2+} buffer. The lysate was diluted to 25 ml with Tris-Mg^{2+} buffer containing 0.1% Triton X-100 and layered over the sucrose. After centrifuging at 8,000 rev/min (6,000 × *g*) for 1 hr, the material at the interface between the sucrose layers was combined with the 60% sucrose layer and dialyzed against Tris-Mg^{2+} buffer. This procedure may be repeated to reduce further the number of unattached phage in the preparation. The material was then subjected to isopycnic sedimentation in CsCl, Tris-Mg^{2+} buffer by centrifuging in a Spinco SW-39 rotor at 30,000 rev/min for 35 hr. The isolated phage-L membrane complexes found at a density of 1.33 to 1.34 g/ml were dialyzed for 12 hr against Tris-Mg^{2+} buffer.

When the material was negatively stained with phosphotungstic acid and viewed in an electron microscope, it consisted of empty vesicles, 60 to 200 nm in diameter, with about 80% of the vesicles having one or more phage attached (Fig. 1). Results obtained with either T2 or T4 were indistinguishable. These vesicles were identified as L membrane by comparing the physical and chemical properties of *E. coli* outer membrane and purified LPS with purified L-membrane vesicles as previously described (3, 4).

The appearance of phage seen in isolated phage-L membrane complexes was very similar to (15) the description of Simon and Anderson of T2 and T4 attached to *E. coli* cells. The phage (Fig. 1A and 1B) were perpendicular to the vesicle and displayed contracted tail sheaths and

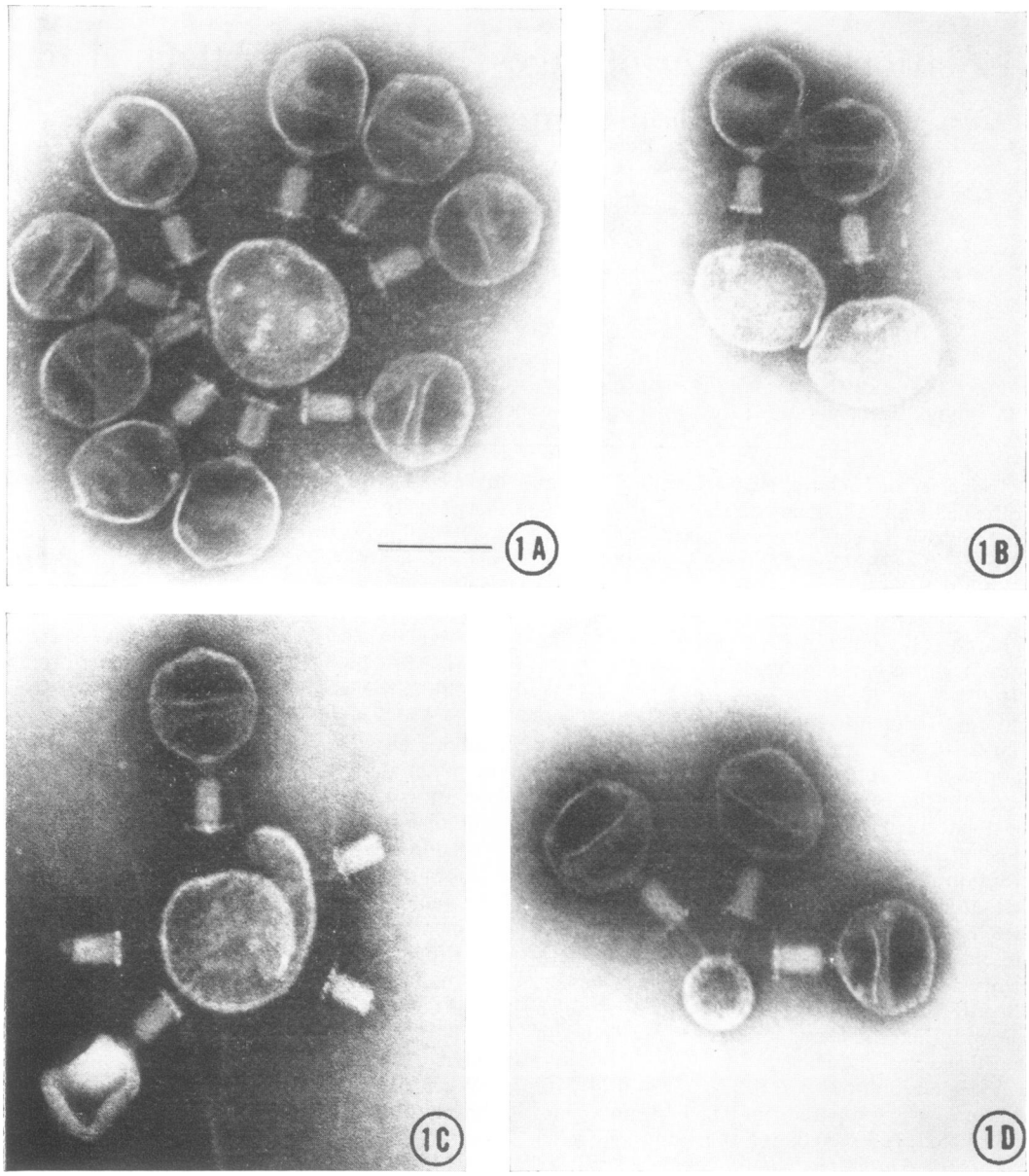

FIG. 1. *(A) Nine T2 phages attached to a single L-membrane vesicle, 124 nm in diameter. (B) Single T4 phage bound to a single L-membrane vesicle. Phage needles are visible and have a fragment of material on their tips. (C) T2 phage tails still attached to L membrane after their heads have broken off. (D) Three T2 phage attached to a small L-membrane vesicle, 65 nm in diameter. The needles have all injected within a 25-nm distance of each other. Bar represents 100 nm. Phosphotunstic acid. × 154,000.*

empty heads. This was also true before the use of CsCl. Base plates generally appeared 30 to 35 nm from the vesicle, and phage needles were often seen penetrating the vesicle with a fragment of material at the tip of the needle (Fig. 1B). The distances between base plates and vesicles, as well as the penetration of the needle, can vary due to superposition effects (16). The points of injection of phage needles were sometimes clustered within a short distance (ca. 25 nm) of each other (Fig. 1D). Although tail pins could not be located, it seems very likely that their permanent

attachment to the L membrane would be necessary to maintain the postinjection orientation and distance of the phage from the L membrane throughout the isolation procedure (17). Figure 1C shows that during the isolation, forces occurred which were sufficient to break off some phage heads and yet the tails remained attached in the same fashion as undamaged particles.

These phage-L membrane complexes are reminiscent of T5 "receptor particles" (6) which are evidently also L-membrane vesicles (5). These receptor particles can attach to the tip of the phage tail, triggering the release of deoxyribonucleic acid (6).

Purified L membrane was therefore tested for its ability to inactivate T2 and T4. Figure 2 shows that, although intact cells inactivated 82% of the T2 and T4 in 12 min, purified L membrane (4) at a concentration equivalent to 16 times that amount of intact cells inactivated only 18% of the T2 and none of the T4. Lower concentrations did not inactivate T2. Isolated cell walls at three different concentrations all inactivated 89 ± 2% of the T2 but none of the T4 (Fig. 2). Addition of 100 μg of tryptophan per ml had no effect on T4 inactivation. To determine whether the methods used to purify L membrane (4) affected its ability to inactivate T2, isolated cell walls were treated under the same conditions except that lysozyme was not used to preserve cell wall integrity. These treated cell walls (equivalent to 5.3 mg of cells/ml) inactivated 84% of the T2 in 12 min. Therefore, unless lysozyme affects the receptor sites, purified L membrane should still be able to inactivate T2. One would also expect that since purified LPS can inactivate T4 (2, 8, 18, 20, 21) so should isolated cell walls or L membrane.

Beumer et al. (2) measured the inactivating ability of a variety of cell extracts from both *Shigella* and *E. coli* on a number of phages. Their results show that the potency of an inactivator depends on both the source and method of preparation and, in addition, can differ unexpectedly among phages. For example, their cell wall preparation (19) inactivated T2 1,000 times more effectively than T4, and their LPS was 200 times more effective than cell walls for inactivating T4. The above results suggest to me that phage absorption is sensitive to configurational changes that cause a distortion of the pattern of tail fiber attachment points or a physical masking of the sites by other cell wall components, or both. Thus, the sharp curvature of L-membrane vesicles compared to the relatively flat surface of an intact cell may distort the T2 attachment sites, whereas receptors in the LPS such as for T4 may also become masked by lipoprotein when the cell wall changes its shape. Masking of T3, T4, and T7 sites by lipoprotein was demonstrated on T5 receptor particles (18). In contrast, LPS prepared by the Westphal method exists in the configuration of long thin ribbons (15) that evidently leave the receptor sites exposed and accessible.

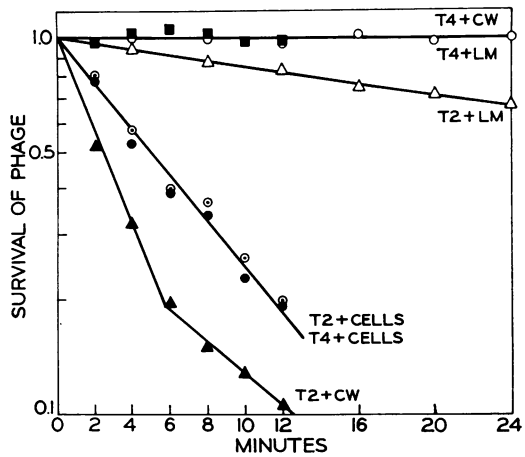

FIG. 2. *Phage adsorption rates were measured at 37 C in 0.06 M NaCl and 0.07 M potassium phosphate at pH 7.0 by using the chloroform dilution technique (1). Conditions described for T2 were also used for T4. Closed circles: T2 adsorption to 10^9 E. coli AW330/ml (0.5 mg of cells/ml) at a multiplicity of infection of 0.001. Dotted open circles: T4 adsorption to E. coli AW330. Open triangles: T2 adsorption to 0.8 mg of purified L membrane (LM) per ml which was first dialyzed for 12 hr against the adsorption medium at 5 C. This amount of L membrane is equivalent to about 8 mg of cells/ml. Open circles: T4 adsorption to purified L membrane. Closed triangles: T2 adsorption to 0.38 mg of cell walls (CW) per ml isolated in 0.1 M Tris (pH 7.8) and 0.2 M KCl with a French press and the procedure of Osborn et al. (12). The walls were dialyzed 12 hr against adsorption medium at 5 C before using them. Cell wall concentrations of 0.17 and 1.0 mg/ml gave results within ± 2% of the values shown. These cell wall concentrations are equivalent to 2.5, 1.1, and 6.7 mg of cells/ml, respectively (14). Solid squares: T4 adsorption to isolated cell walls.*

Isolation of phage attached to L membrane offers a new system in which the details of phage attachment can be explored. This technique can also be used to identify L membrane in bacterial lysates.

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LITERATURE CITED

1. Adams, M. 1959. Bacteriophages, p. 467–468. Interscience Publishers, Inc., New York.

2. Beumer, J., M. P. Beumer-Jochmans, J. Dirkx, and D. Dekegel. 1966. Localization des récepteurs de phages chez des souches de *Shigella* et d' *Escherichia*. Ann. Inst. Pasteur **110**:727–736.
3. DePamphilis, M. L. 1971. Dissociation and reassembly of *Escherichia coli* outer membrane and of lipopolysaccharide, and their reassembly onto flagellar basal bodies. J. Bacteriol. **105**:1184–1199.
4. DePamphilis, M. L., and J. Adler. 1971. Attachment of flagellar basal bodies to the cell envelope: specific attachment to the outer lipopolysaccharide membrane and cytoplasmic membrane. J. Bacteriol. **105**:396–407.
5. Frank, H., and D. Dekegel. 1967. Electron microscopical studies on the localization of the different components of the cell walls of gram negative bacteria. Folia Microbiol. **12**: 227–233.
6. Frank, H., M. Zarnitz, and W. Weidel. 1963. Über die Rezeptorsubstanz für den Phagen T5, VII. M.tt: Elektronenoptische Darstellung und Längenbestimmung der aus T5/R5 Komplexen freigesetzten DNS. Z. Naturforsch. **18b**:281–284.
7. Glauert, A. M., and M. J. Thornley. 1969. The topography of the bacterial cell wall. Annu. Rev. Microbiol. **23**:159–198.
8. Jessaitis, M. A., and W. F. Goebel. 1955. Lysis of T4 phage by the specific lipopolysaccharide of phase II *Shigella sonnei*. J. Exp. Med. **102**:733–752.
9. Losick, R., and P. Robbins. 1969. The receptor site for a bacterial virus. Sci. Amer. **221**:121–124.
10. Michael, J. G. 1968. The surface antigens and phage receptors in *Escherichia coli* B. Proc. Soc. Exp. Biol. Med. **128**:434–438.
11. Osborn, M. 1969. Structure and biosynthesis of the bacterial cell wall. Annu. Rev. Biochem. **38**:501–538.
12. Osborn, M., S. M. Rosen, L. Rothfield, and B. L. Horecker. 1962. Biosynthesis of bacterial lipopolysaccharide. I. Enzymatic incorporation of galactose in a mutant strain of *Salmonella*. Proc. Nat. Acad. Sci. U.S.A. **48**:1831–1838.
13. Rapin, A. M. C., H. M. Kalckar, and L. Alberico. 1968. The metabolic basis for masking of receptor sites on *E. coli* K12 for C21, a lipopolysaccharide core-specific phage. Arch. Biochem. Biophys. **128**:95–105.
14. Salton, M. R. J. 1964. The bacterial cell wall, p. 246. Elsevier Publishing Co., New York.
15. Shands, J. W., J. A. Graham, and K. Nath. 1967. The morphologic structure of isolated bacterial lipopolysaccharide. J. Mol. Biol. **25**:15–20.
16. Simon, L., and T. Anderson. 1967. The infection of *Escherichia coli* by T2 and T4 bacteriophages as seen in the electron microscope. I. Attachment and penetration. Virology **32**: 279–297.
17. Simon, L. D., J. G. Swan, and J. E. Flatgaard. 1970. Functional defects in T4 bacteriophages lacking the gene 11 and gene 12 products. Virology **41**:77–90.
18. Weidel, W. 1958. Bacterial viruses (with particular reference to adsorption/penetration). Annu. Rev. Microbiol. **12**:27–48.
19. Weidel, W., H. Frank, and H. H. Martin. 1960. The rigid layer of the cell wall of *Escherichia coli* B. J. Gen. Microbiol. **22**:158–166.
20. Weidel, W., G. Koch, and F. Lohss. 1954. Über die Zellmembran von *Escherichia coli*. B. II. Der Rezeptorkomplex für die Bakteriophagen T3, T4 and T7 vergleichende chemisch-analytische untersuchungen. Z. Naturforsch **9b**:398–406.
21. Wilson, J. H., R. B. Luftig, and W. B. Wood. 1970. Interaction of bacteriophage T4 tail fiber components with a lipopolysaccharide fraction from *Escherichia coli*. J. Mol. Biol. **51**:423–434.