ROLE OF CELL SURFACE MOBILITY ON BACTERIOPHAGE INFECTION: TRANSLOCATION OF SALMONELLA PHAGES TO MEMBRANE ADHESIONS

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SUMMARY:

Phage ϵ^{15} adsorbed at a low temperature (or by short-time incubation) to the outer surface of Salmonella anatum gathers on further incubation at a high temperature to a certain region where the inner and outer membranes may join. This was demonstrated by separating the inner and outer membranes of the cells in sucrose gradient after addition of ^{35}S-labeled ϵ^{15} to the cells. Radioactivity adsorbed at 4° was first recovered mainly with the dense outer membrane but disappeared by further incubation at 35° within 5 min. Instead, the radioactivity was recovered with the membrane fraction which had intermediate density. Such phage traslocation was not observed when phage ϵ^{15} was added to a pep mutant of S.anatum to which the phage can adsorb but fail to infect. A host range mutant phage which can infect the pep mutant migrated to the intermediate dense region.

The smooth specific Salmonella phages such as ϵ^{15}, ϵ^{34}, g341 and P22 adsorb to O antigen, the peripheral portion of lipopolysaccharide, which is an important constituent of the outer surface of Gram negative organisms. The base plate of these phages consists of a single species of enzyme protein which cleaves or deacetylates the host O antigen polysaccharide (1-6) and is responsible for phage adsorption (3,7). After phage adsorption, the release of phage DNA may be triggered and the nucleic acid may penetrate into the bacterial cytoplasm. Events involved in these early stages of infection, especially those which occur between attachment of phage to its receptor and the completion of DNA transfer into the cytoplasm, however, are not yet clearly understood.

It was demonstrated by Bayer (8) that various phage particles including the smooth specific Salmonella phages preferentially located over the adhesion sites between the outer and inner membranes of Gram negative bacteria. If the adhesion sites are the place where phage penetrates its nucleic acid, two kind of possibilities can be considered for the phages how to reach to the adhesion sites. One possibility is that over the adhesion sites, the phage receptors reside preferentially and the phages directly recognize these limited numbers of binding sites. The other is that the receptors are distributed randomly all over the outer membrane and the phages adsorbed to the receptor at the sites other than the adhesions migrate subsequently to the adhesion sites during incubation.

In this communication we show the migration of phage ϵ^{15} from the attachment to adhesion sites. The step seems to be essential for the accomplishment of phage infection.

MATERIALS AND METHODS:

Most of the methods and materials used in this experiment were the same as those described previously (9).

Bacteria and phages: Salmonella anatum A1, S. anatum SK59 (a pep mutant) (9) and phages ϵ^{15}vir, ϵ^{15}hp32 (a host range mutant phage which infects the pep bacterium) (9) and c341 were used.

^{35}S-labeled phages: ^{35}S-labeled phages ϵ^{15}vir, ϵ^{15}hp32 and c341 were prepared and purified according to the method described previously (9).

Adsorption of radioactive phage to bacteria: Bacteria grown in L broth (5×10^8 cells/ml) and the radiactive phage (approximately 100,000 cpm/250 ml) (see figure legends) were mixed at 0° or 35° and incubated at 0° or 35°. After the incubation, the mixtures were quickly chilled and phage-adsorbed cells were recovered by centrifugation.

Membrane separation: This was done according to a modified method (10) of the Schnaitman procedure (11). The position of the inner and outer membrane were usually defined by protein profile (12), distribution of cytochrome b_1 (13), and contents of rhamnose (14). The density of the resultant peaks is comparable to that observed by Smit et al. (10). The densest peak is outer membrane since it containes the majority of rhamnose. The middle band is unseparated inner and outer membrane as indicated by an intermediate amount of rhamnose and cytochrome b_1. The lightest bands are inner membrane as indicated by the lack of rhamnose and high absorbance of cytochrome b_1.

RESULTS:

Salmonella anatum cells and ^{35}S-labeled phage ϵ^{15}vir were mixed and incubated at 4° for 30 min. The cells were collected by centrifugation and disrupted in a French pressure cell. The membranes collected by centrifugation were applied onto a discontinuous sucrose gradient. As shown in Fig. 1a, almost 60% of radioactivity of the phage were recovered in the region of the dense outer membrane and the rest of radioactivity was associated with the membrane of intermediate density. Similar results were obtained when the cells and the radioactive phage were mixed and incubated at 35° for 15 sec (Fig. 1b). When the cells and the phage were incubated further at 35°, the radioactivity in the outer membrane region decreased while that in the intermediate dense region increased (Fig. 1c). After 5 min of incubation, no radioactivity was found with outer membrane (Fig. 1d). The results indicate that the phage initially attaches to its receptor lipopolysaccharide which distributes randomly over the outer membrane. The results also suggest that the phage adsorbed to the receptor site other than the region where the inner and outer membranes are fused, subsequently migrates and gathers to the membrane adhesions.

We have previously reported bacterial mutants designated as pep to which phage ϵ^{15} can adsorb but fail to lyse (9). One such mutant cells (SK59) were mixed with the

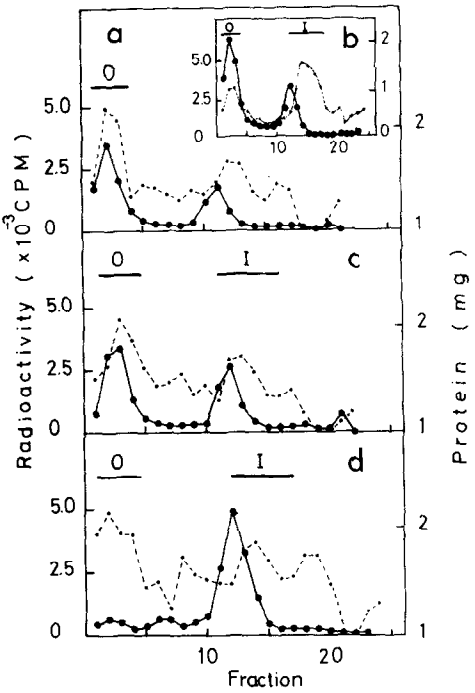

Fig. 1. Translocation of ^{35}S-labeled phage ϵ^{15}vir from the primary adsorption sites (the outer membrane) to the membrane with intermediate density.
A1 cells (5.5×10^8/ml) in 150 ml of chilled L-broth were mixed with ^{35}S-labeled ϵ^{15}vir (89,000 cpm:MOI=0.15) in a ice bath. After 30 min for adsorption, the mixture was divided into three 50 ml portions (a,c and d) and two portions were further incubated at 35° for 1 (c) or 5 min(d). In another experiment, the L-broth culture of A1 cells (5.5×10^8cells/ml) in 50 ml was mixed with ^{35}S-labeled ϵ^{15}vir (88,000 cpm:MOI=0.15) and incubated for 15 sec at 35° (b). After incubation, the mixtures were poured into 200 ml (a,c and d) or 150 ml (b) of precooled L-broth containing carrier cells(1.1×10^{11} or 8×10^{10} cells, respectively). The cells washed twice with ice-cold N-2-hydroxyethylpiperazine-N'-2'-ethanesulfonic acid (HEPES) buffer, pH 7.4 (10 mM) were suspended in 25 ml of the same buffer, and after addition of 0.5 mg each of deoxyribonuclease and ribonuclease, disrupted in a French pressure cell at 1,200 kg/cm^2. Membranes were collected by centrifugation in a Spinco type 60Ti rotor for 3 hrs at 40,000 rpm, suspended into 1 ml of the HEPES buffer and applied to a discontinuous sucrose density gradient (1.5 ml of 2.02 M, 5.5 ml of 1.44 M and 4 ml of 0.77 M sucrose in the buffer). The gradients were centrifuged in a Spinco SW 41 Ti rotor for 15 hrs at 20,500 rpm. The fractions were collected by puncturing the bottom of the tubes. Aliquots of the fractions were spotted on 25 mm filter discs (Whatman GF83) and radioactivity was measured (—●—). The protein profile was also shown (- - • - -). O and I indicate the positions of the outer and inner membrane, respectively.

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radioactive phage ϵ^{15}vir and incubated at 35° for 5 min. The membranes were separated as described above and the results were shown in Fig. 2. Almost all radioactivity adsorbed to the pep bacteria were recovered with the outer membrane. The results suggest that this pep mutant lacks an anchoring mechanism of phage particles at the membrane adhesions. Lack of migration of the phage-receptor complex in the mutant is less likely (see below).

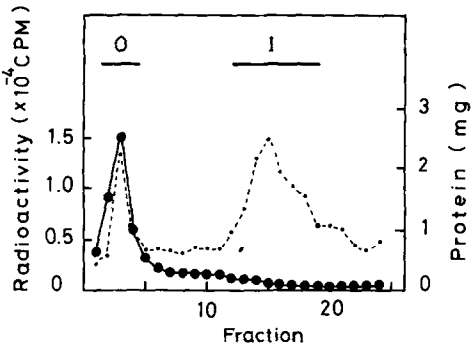

Fig. 2. Association of ^{35}S-labeled ϵ^{15}vir with the outer membrane of pep mutant cells. SK 59 cells (5.5×10^8/ml) in 250 ml of L-broth were mixed with ^{35}S-labeled ϵ^{15}vir (107,000 cpm, MOI=0.04) and incubated for 5 min at 35°. For disruption of the cells and separation of membranes, the similar conditions as described in the legend of Fig. 1 were employed. Radioactivity (—●—) and protein profile (- - ● - -) were shown. O and I indicate the positions of the outer and inner membrane, respectively.

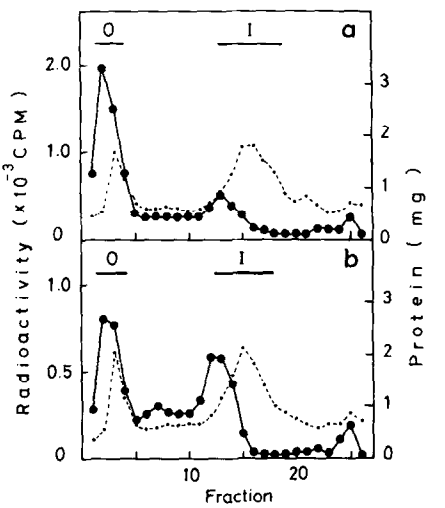

Fig. 3. Migration of a host range mutant phage, ϵ^{15}hp32, on the cell surface of pep mutant cells.
SK 59 cells (5.5×10^8/ml) in 50 ml of L-broth were infected with ^{35}S-labeled ϵ^{15}hp32 (28,000 cpm:MOI=0.005) and incubated for 15 sec (a) or 5 min (b) at 35°. The conditions for disrupting the cells and separating membranes were similar as those described in the legend of Fig. 1. Radioactivity (—●—) and protein profile (- - ● - -) were shown. O and I indicate the positions of the outer and inner membrane, respectively.

As previously reported, the inhibition of phage infection in pep mutants can be overcome by a host range mutation of phage ϵ^{15} (9). Using such mutant phage, ϵ^{15}hp32 and the pep bacteria, the similar experiments were performed. As shown in Fig. 3a and b, a significant portion of radioactivity of the host range mutant phage which was originally associated with the outer membrane of the pep bacteria, migrated into the membrane fraction with the intermediate density. These results strongly suggest that the phage migration from the outer membrane to the intermediate dense mambrane is essential for the accomplishment of the infection.

DISCUSSION:

The cytoplasmic membrane of Gram-negative bacteria is surrounded by a rigid layer of the peptideglycan and the outer membrane. The latter consists of lipopolysaccharides, proteins and phospholipids (15). Between the cytoplasmic (inner) and outer membranes, there is a limited number of adhesion sites which are visible after plasmolysis of the cells (8). These adhesions are considered to be the sites where newly synthesized lipopolysaccharide molecules are translocated to the outer membrane (16) and through which the phage nucleic acid penetrates into cytoplasm. In this paper, we demonstrated that the phage ϵ^{15} initially adsorbed to the receptor in the dense outer membrane migrated to the membrane with the intermediate density. The membrane of this region is considered to be (or at least include) the adhesion sites between the inner and outer membrane. The rate of migration was fast and the migration was accomplished within 5 min at 35°.

The translocation of the phage was not observed in a bacterial mutant which blocks phage ϵ^{15}infection at the early stage but this difficulty was overcome by a host range mutant phage. This phage mutant can infect the pep mutants of S. anatum more efficiently than the wild type bacteria (9). These results strongly suggest that the traslocation of phages to the adhesions is essential for accomplishing phage infection. In the pep mutant, the wild type phage seems not to be trapped at the adhesion sites, although the mechanism of fixation of the phages at the adhesions is still unknown.

The mechanism of traslocation of the phage (or the phage-receptor complex) is also unclear at the present. The mobility of the receptor molecules (in this case, the lipopolysaccharide) on the cell surface may play an essential role for the lateral movement of the complexes. Although the lateral diffusion of the newly synthesized lipopolysaccharides has already demonstrated by Mühlradt et al. (17) and Bayer (18) under the electron microscope, the direction of the diffusion of the newly formed lipopolysaccharides and that of migration of phage-receptor complexes are reverse. In addition, neither cleavage of

O antigen polysaccharide shown by phage ϵ^{15} nor even the enzymatic action of the base plate of the smooth specific phages may sufficiently explain the lateral movement of the phages, since phage g341 (c341), which does not cleave O antigen polysaccharide (5) or phage Felix O, the receptor of which is known to be the R-core portion of the lipopolysaccharide (19) and whose morphology is quite different from the smooth specific phages, also gathered to the intermediate dense region after incubation (unpublished observation). The enzymatic action of the base plate of the smooth specific phages (1-6) may be required especially for the perpendicular movement of the phages, which allow correct orientation of the phages above the cell surface to eject their DNA.

Whatever the mechanism of the traslocation is, most of the radioactive phage in the intermediate dense region seemed to have ejected its DNA, since few plaques were formed after treatment of the membrane fraction with EDTA-Triton X100. This treatment can release a significant number of phage particles associated with the outer membrane (unpublished observation). Further experiments are needed to clarify whether the penetration of the phage nucleic acid into the cytoplasm takes place through the region.

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