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A Visualization Method of Filamentous Phage Infection and Phage-Derived Proteins in *Escherichia coli* Using Biotinylated Phages

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Direct visualization of filamentous phage infection in *Escherichia coli* (*E. coli*) was attempted using biotinylated phages (BIO-phages). The biotinylation of the phages did not influence their infectivity into *E. coli*. *E. coli* infected with BIO-phages could be detected by using fluorescein-conjugated avidin with confocal laser scanning microscopy, and BIO-phages and BIO-phage-derived proteins in *E. coli* could be directly observed by using the avidin–biotin–peroxidase complex method with electron microscopy. This is the first report of direct visualization of phage infection and phage-derived proteins in the host cell using a biotin–avidin interaction. This simple and powerful method is applicable to the study of infection by various viruses. © 2001 Academic Press

Key Words: **filamentous phages; infection; biotin; avidin; microscopy.**

Visualization of the viral infection process is useful for studying the molecular and cellular mechanisms of viral infection, and could provide information valuable for the development of antiviral drugs. Previous studies have reported various methods for the visualization of viral infections, including fluorescein labeling of viral particles using chemical modifications (1–4), intercalation of fluorescent lipids into envelopes (5, 6), and genetic fusion of green fluorescent protein and viral proteins (7, 8). However, the resolution of fluorescence microscopy is not as high as that of electron microscopy. Electron microscopy remains the major method for visualizing directly the interaction between a virus and its host cell. However, this method is laborious if many cells must be examined, and it is difficult to detect the virus in infected cells. Therefore, highly sensitive and simple methods to detect phages by using electron microscopy are required. The biotin–avidin interaction can be used to improve the visualization of phages because avidin is known to bind to biotin with extremely high affinity ($K_d \sim 10^{-15}$).

The filamentous phage is a virus that infects *Escherichia coli* (*E. coli*) carrying F-pili, and it has been used not only as a tool of genetic engineering but also as a model for studying various virus actions, for example, infection. Here we report the direct visualization of filamentous phage infection and phage-derived proteins in *E. coli*. Filamentous phages were labeled with D-biotin–*N*-hydroxysuccinimide ester, and the biotinylated phage (BIO-phage) infections were examined by using avidin–biotin binding.

MATERIALS AND METHODS

Materials. D-Biotin-*N*-hydroxysuccinimide ester and Sephadex G25 column was purchased from Roche Molecular Biochemicals (Tokyo, Japan). Horseradish peroxidase (HRP)-conjugated anti-M13 antibody, enhanced chemiluminescence Western blotting detection reagents, nitrocellulose membranes, and polyvinyldifluroide (PVDF) membranes were purchased from Amersham Pharmacia Biotech (Tokyo, Japan). OsO_4 and paraformaldehyde were purchased from Merck (Darmstadt, Germany), 3,3′-diaminobenzidine (DAB) from Dojin (Kumamoto, Japan), the Vectastain ABC kit, fluorescein Avidin D, and VECTASHIELD mounting medium from Vector Laboratories (Burlingame, CA), and Epon-812, dodecenyl succinic anhydride, methyl nadic anhydride, and 2,4,6-tridimethylaminomethyl phenol from TAAB (Reading, UK). All other reagents were of biochemical research grade.

Preparation of biotinylated M13KO7 phages. M13KO7 phages (8×10^8 cfu) were suspended in phosphate-buffered solution (PBS) and then mixed with 50 μg of D-biotin-*N*-hydroxysuccinimide ester (BIO) for 2 h to prepare biotinylated phages (BIO-phages). The reactions were stopped with glycine (final concentration, 50 mM), and remaining BIO was separated using a Sephadex G25 column.

Western blotting of BIO-phages. BIO-phages were subjected to 15–25% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Proteins were then transferred electrophoretically to

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a nitrocellulose membrane. After blocking with PBS containing 2% skim milk (MPBS), the membrane was incubated with an avidin–biotin–peroxidase complex for 30 min at room temperature. After washing with PBS containing 0.1% Tween 20, the blots were developed using a chemiluminescent substrate, and analyzed by a Luminescent Image Analyzer LAS-1000 (Fuji Photo Film, Tokyo, Japan).

BIO-phage detection on a PVDF membrane. Detection of BIO-phages was performed using quantitative dot blotting as described previously (9). Various amounts of BIO-phages were blotted on PVDF membranes according to the manufacturer's recommendations. The membranes were washed once in PBS, blocked with MPBS, and incubated with HRP-conjugated anti-M13 antibody, which had been diluted 20,000 times, or with avidin–biotin–peroxidase complex for 30 min at room temperature, and the detection was performed as described above.

Assessment of the infectivity of BIO-phages. Various concentrations of BIO-phages or M13KO7 phages were applied to determinations of colony forming units and quantification assays. To determine the colony forming unit, 495 μL of *E. coli* JM109 solution that had been cultured overnight was added to 5 μL of various concentrations of BIO-phages or M13KO7 phages and incubated for 1 h at 37°C. After incubation, 50 μL of serially diluted cells were plated on LB plates containing 50 μg/mL kanamycin, incubated overnight at 37°C, and then the number of bacteria colonies were counted. The phages applied to the colony forming units were quantified by using quantitative dot blotting with HRP-conjugated anti-M13 antibody as described above.

Preparation of E. coli JM109 infected with BIO-phages. One hundred microliters of *E. coli* that had been cultured overnight were mixed with BIO-phages ($0.1–1.2 \times 10^8$ cfu) and incubated for 1 h at 37°C. After incubation, the cells were washed with PBS, fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 10 min, and washed again with PBS. For control experiments, we prepared *E. coli* infected with M13KO7 phages with the same procedure.

Confocal microscopic observation of E. coli infected with BIO-phages. *E. coli* infected with BIO-phages were reacted with avidin–fluorescein conjugate (25 μg/mL) for 30 min. After washing, the cells were pelleted, resuspended with VECTASHIELD mounting medium, and observed with confocal laser scanning microscopy (Leica TCS NT, Herderberg, Germany).

Electron microscopic observation of E. coli infected with BIO-phage. *E. coli* infected with BIO-phages were incubated with PBS containing 2% skim milk for 1 h at room temperature and then incubated with avidin–biotin–peroxidase complex for 30 min at room temperature. After washing with PBS, peroxidase reactions were performed with 0.02% DAB and 0.03% H_2O_2. The cells were washed, treated with 2% OsO_4, dehydrated in a graded series of ethanol, and embedded in Epon epoxy resin. Ultrathin sections were cut with a Reichert Ultracut E (Leica Microsystems, Wetzlar, Germany) and were observed with a Hitachi H500 electron microscope (Hitachi, Tokyo, Japan).

RESULTS

Assessment of the biotinylation of BIO-phages. BIO-phages were subjected to Western blotting, and biotinylated proteins derived from the BIO-phages were detected (Fig. 1). In addition to bands of about 10 kDa, a band of about 50 kDa was the main band detected. Possible proteins near 10 kDa and about 50 kDa in size were capsid proteins, including pVII, pVIII, and pIX and pIII, respectively, according to their molecular weight. Therefore, coat proteins of the phages could be biotinylated. Phage particles consisted mainly of about

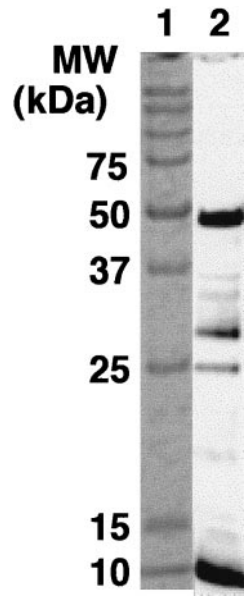

FIG. 1. Assessment of the biotinylation of phage using Western blotting. BIO-phages were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and then transferred to nitrocellulose membrane. After blocking, the membrane was incubated with avidin–biotin–peroxidase complex and visualized using a chemiluminescent substrate. Lane 1, molecular weight marker; lane 2, BIO-phages.

2700 copies of the major capsid protein pVIII. Possible extracellular labeling sites of pVIII by BIO are the amines of the N-terminus, Lys-8, Asn-12, and Gln-15. If pVIII were fully labeled with BIO, more than 10,000 molecules of BIO would be conjugated with one phage. As shown in Fig. 1, however, biotinylation of pIII, a minor coat protein, is almost identical to that of the other coat proteins, indicating that the biotinylation of pIII could be more efficiently performed in comparison with the other coat proteins. Accessibility of the pIII protein to the solvent would increase the efficiency of biotinylation.

Assessment of the sensitivity of BIO-phages. The sensitivity of BIO-phages was assessed by quantitative dot blotting (9) using HRP-conjugated anti-M13 antibody or avidin–biotin–peroxidase complex. BIO-phage detection with avidin–biotin–peroxidase complex was more sensitive than that using anti-M13 antibody (Fig. 2). The ratio of avidin–biotin–peroxidase complex to anti-M13 antibody was higher when high titration levels were used, and the maximum ratio was about 25 to 1, indicating the high sensitivity of the avidin–biotin interaction.

Infectivity of BIO-phages. To examine the influence of biotinylation on phage infectivity, we examined the relation between the number of colony forming units and the quantity of phages. The relation was not significantly different for BIO-phages and M13KO7

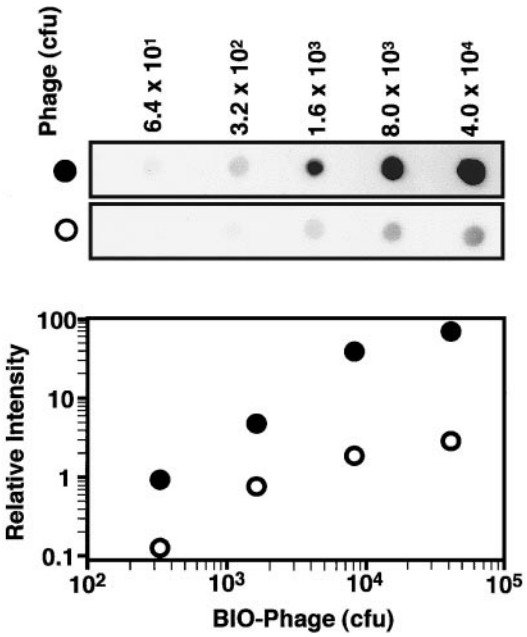

FIG. 2. The sensitivity of BIO-phages. Fivefold dilutions of BIO-phages or M13KO7 phages were blotted on PVDF membranes, incubated with HRP-conjugated anti-M13 antibody (open circle) or avidin–biotin–peroxidase complex (solid circle), and visualized using a chemiluminescent substrate. Relative intensity was expressed as a ratio with the intensity of 3.2×10^2 cfu of BIO-phages defined as 1.

phages (Fig. 3); therefore, biotinylation did not influence the infectivity with *E. coli* JM109 carrying the F-pili.

Confocal microscopic observation of E. coli infected with BIO-phages. *E. coli* JM109 infected with BIO-phages were reacted with avidin–fluorescein conjugate

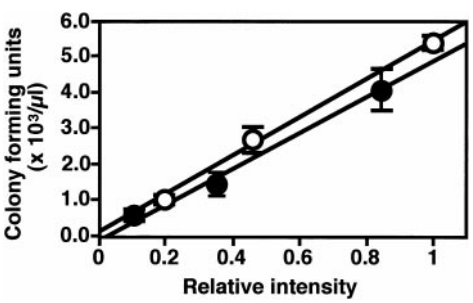

FIG. 3. The infectivity of BIO-phages. To determine the colony forming unit, *E. coli* JM109 was cultured overnight and mixed with various concentrations of BIO-phages (solid circle) or M13KO7 phages (open circle). After incubation, the solutions were plated and incubated overnight, and then the number of bacteria colonies were counted. Values are means ± standard deviation from three separate experiments. The quantification of the phages applied to the colony-forming units was performed by using quantitative dot blotting with HRP-conjugated anti-M13 antibody. BIO-phages were blotted on PVDF membranes, then incubated with HRP-conjugated anti-M13 antibody, and visualized using a chemiluminescent substrate. Relative intensity was expressed as a ratio with the intensity of 5.4×10^3 cfu of the M13KO7 phage being defined as 1.

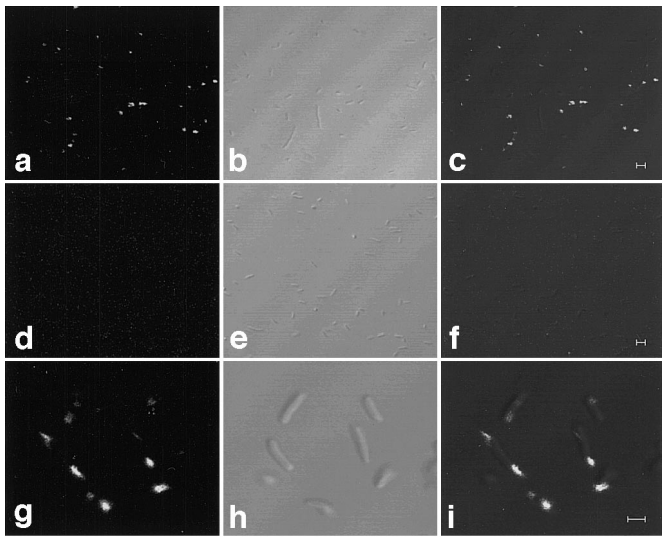

FIG. 4. Microscopic observation of *E. coli* infected with BIO-phages. *E. coli* JM109 were cultured overnight and then mixed with BIO-phages (a–c, g, h) or M13KO7 (d, e). After incubation, the cells were fixed, reacted with avidin–fluorescence conjugate, and observed with a confocal laser scanning microscope. The images represent fluorescence (a, d, g), phase-contrast microscopy (b, e, h), and superimposed images of a and b, d and e, and g and h (c, f, i), respectively. Bars: a–f, 4 μm; g–h, 2 μm.

and observed with confocal laser scanning microscopy. The infected *E. coli* showed fluorescence (Figs. 4a–4c, 4g, and 4h). On the other hand, *E. coli* JM109 infected with M13KO7 showed no fluorescence (Figs. 4d and 4e), indicating that the fluorescence was generated by BIO-phages. The infected cells showed various intensities of fluorescence, and intracellular differences in fluorescence intensity were observed in the infected cells (Figs. 4g and 4h).

Electron microscopic observation of E. coli infected with BIO-phages. *E. coli* JM109 infected with BIO-phages were examined using electron microscopy. BIO-phages and BIO-phage-derived proteins appeared clearly as black deposits of peroxidase reaction products (Figs. 5a–5c). BIO-phages were detected as thick filaments or clusters covered with reaction products (Fig. 5b). The phage particle was observed as a thin line in the filament (Fig. 5c). In the cells, the reaction products of BIO-phage-derived proteins were observed in the outer membrane, periplasm fraction, and inner membrane, but not in the cytoplasm. The density of products near the phage-infected sites was higher than near noninfected sites.

DISCUSSION

Visualization of the viral infection process is useful for studying molecular and cellular mechanisms of viral infection. In previous studies, viral infections were visualized by using fluorescein labeling of viral parti-

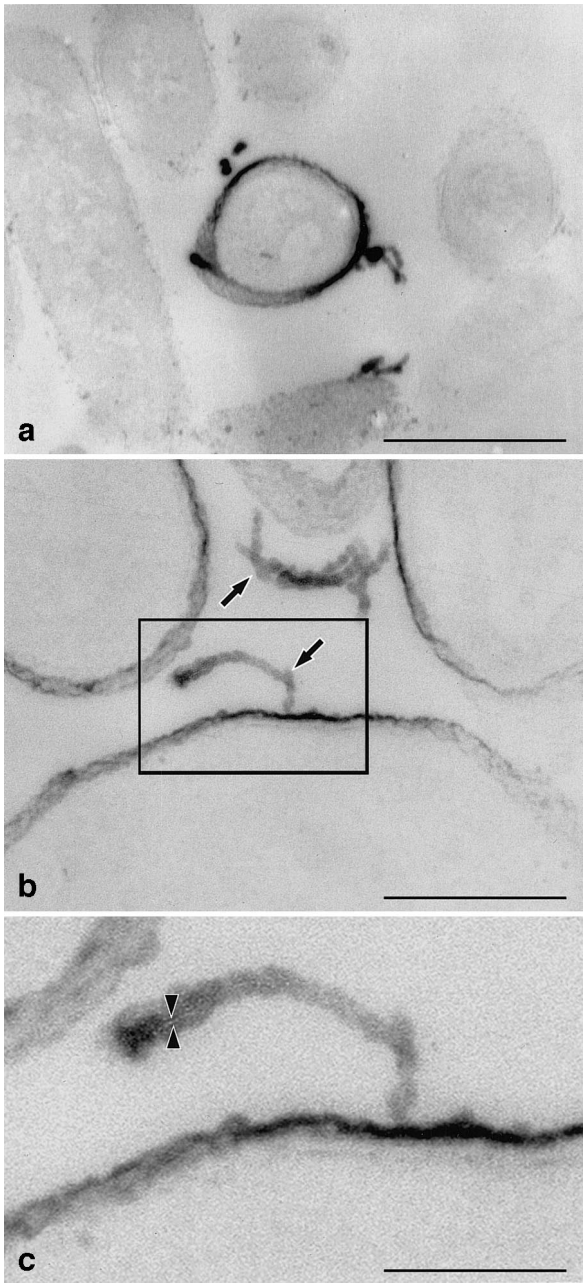

FIG. 5. Electron microscopic observation of *E. coli* infected with BIO-phages. *E. coli* JM109 was cultured overnight and mixed with BIO-phages. After incubation, the cells were fixed, reacted with avidin–biotin–peroxidase complex and DAB, and observed with an electron microscope. BIO-phages and BIO-phage-derived proteins appeared clearly as black deposits of peroxidase reaction products (a–c). BIO-phages were detected as thick filaments or clusters covered with reaction products (arrows) (b), and the phage particle was observed as a thin line in the filament (arrowheads). (c) Closeup of the boxed region in b. In the cells, the reaction products of the BIO-phage-derived proteins were observed in the outer membrane, periplasm, and inner membrane, but not in the cytoplasm. Original magnifications: a, ×12,000; b, c, ×24,000. Bars: a, 1 μm; b, 0.5 μm; c, 0.125 μm.

cles with chemical modification (1–4), intercalation of fluorescent lipids into the particle envelopes (5, 6), and the fusion of green fluorescent protein and viral proteins (7, 8). Labeling of the virus using fluorescence and its observation using fluorescence microscopy made it possible to observe the movement of the virus within the cells. To understand the interaction of the virus and the host cells in detail, including the fusion of the cellular and viral membranes, and to detect the locations of virus-derived proteins, more highly sensitive high resolution methods were required.

The filamentous phage is a virus that infects *Escherichia coli* (*E. coli*) carrying F-pili. It has been proposed that the infection is at least a two-step process (10). The first step is the interaction of the pIII end of the phage with the tip of the F-pilus of *E. coli,* and the second step is the integration of the capsid proteins and DNA of the phages into *E. coli.* No research, however, has been reported describing direct observations of phages infected into *E. coli,* or, especially, of the locations of coat proteins during infection.

The ability of avidin and biotin to bind to each other has been used as a molecular tool in biotechnological, diagnostic, and therapeutic applications (11–13). Here, we demonstrated the sensitivity of BIO-phage detection using avidin–biotin binding. In comparison to detection with HRP-conjugated anti-M13 antibody, detection using avidin–biotin–peroxidase complex was obvious highly sensitive with dot blotting (Fig. 2). In addition, the infectivity into *E. coli* carrying the F-pili (Fig. 3) was not decreased by biotinylation, and *E. coli* infected with BIO-phages could be detected using the avidin–fluorescein conjugate. Many cells exhibited various intensities of fluorescence (Fig. 4). Detection using avidin–biotin binding also has many potential advantages. The binding ability of biotin to avidin is very high ($K_d \sim 10^{-15}$ M) (14). Because each protein has many possible biotinylation sites, there are more binding sites for avidin than for antibodies. Therefore, the detection using avidin–biotin binding provides high sensitivity.

The electron microscopic study demonstrated that BIO-phages were present as thick filaments or clusters (Fig. 5). BIO-phage-derived proteins could also be detected in the cells. The reaction products were observed in the outer membrane, periplasm and inner membrane, but not in the cytoplasm. These results clearly support the previous proposal (10) that the integration of DNA into the host cell can be achieved via the assembly of phage coat proteins in the outer and inner membranes of the host cell. Further developments, including site-specific biotinylation and the modification of the detection method would be required to determine the location of each of the component phage proteins in the cells.

In conclusion, this is the first report of visualization of phage infection using biotinylated phages. Biotinylated coat proteins were localized in the outer membrane, periplasmic fraction, and inner membrane could be detected using the high sensitivity of the biotin–

avidin interaction. BIO-phages are useful for the detection of phage both separate from and inside infected cells. Furthermore, BIO-phage-derived proteins could also be detected in the cells. In addition, filamentous phage have been used for phage display (15), which has been successfully applied to the display of antibody fragments (16) and peptides (17–19). Recently, phage display libraries were applied to the selection of internalizing antibodies (20) or peptides (21) for delivery into mammalian cells. BIO-phages could be used to detect the phage in the cells and to assess the internalization pathway in mammalian cells. Our BIO-phages would also provide some advantages for the study of filamentous phage infection into *E. coli* and the internalization of filamentous phage into mammalian cells. Finally, this biotinylation and detection method may be applicable to various viruses, and would thus benefit studies of viral infections.

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