A morphologic study of filamentous phage infection of *Escherichia coli* using biotinylated phages

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Abstract **Using biotinylated phage (BIO-phages), we observed the infection of filamentous phages into *Escherichia coli* JM109 morphologically. BIO-phages and BIO-phage-derived proteins, mainly pVIII, were detected in *E. coli* by using the avidin–biotin–peroxidase complex method with electron microscopy. Infected cells revealed positive staining on the outer and inner membranes and in the periplasmic space. Some cells showed specific or predominant staining of the outer membrane, whereas others showed predominant staining of the inner membrane or equivalent staining of the outer and inner membranes. The periplasmic spaces in some infected cells were expanded and filled with reaction products. Some cells showed wavy lines of positive staining in the periplasmic space. BIO-phages were detected as thick filaments or clusters covered with reaction products. The ends of the infecting phages were located on the surface of cells, in the periplasmic space, or on the inner membrane. These findings suggest that phage major coat proteins are integrated into the outer membrane and that phages cause periplasmic expansion during infection.**

Key words: Phage; Infection; Biotin; Periplasm; Membrane; Electron microscopy

1. Introduction

Filamentous bacteriophages are viruses that infect strains of *Escherichia coli* that carry F-pili. The infection process of viruses is best understood for the filamentous phages, particularly the Ff phages (e.g. M13, fd, and f1). Infection of *E. coli* by the Ff phages is at least a two-step process [1]. 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 coat proteins and DNA of the phages into the bacterium. During the infection, where and how the phage coat proteins integrate into *E. coli* has not been investigated in detail. In addition, an effective morphologic approach to investigating phage infection has not been available until recently.

Previously we reported the preparation of biotinylated phages (BIO-phages) and their usefulness for various studies [2]. In the present paper, we describe a morphologic study in which we used BIO-phages, the avidin–biotin–peroxidase complex method, and electron microscopy to investigate the process of phage infection. The detection of BIO-phage-derived proteins is specific for the parental phage, because phage proteins newly synthesized by *E. coli* are not biotinylated. In light of our various findings, we discuss the integration process of the phage coat proteins into *E. coli*, the significance of intracellular morphologic changes, and two possible phases during infection by phages.

2. Materials and methods

2.1. Materials

D-Biotin-*N*-hydroxysuccinimide ester (BIO) and Sephadex G25 columns were purchased from Roche Molecular Biochemicals (Tokyo, Japan). OsO_4 and paraformaldehyde were purchased from Merck (Darmstadt, Germany), 3,3′-diaminobenzidine (DAB) from Dojin (Kumamoto, Japan), the Vectastain ABC kit and fluorescein Avidin D from Vector Laboratories (Burlingame, CA, USA), and Epon-812, dodecenyl succinic anhydride, methyl nadic anhydride, and 2,4,6-triphenol from TAAB (Reading, UK). All other reagents were of biochemical research grade.

2.2. Preparation of BIO-phages

Preparation of BIO-phages was performed as described previously [2]. In brief, M13KO7 phages (8×10^9 cfu) were biotinylated by adding 50 μg BIO to the phage solution; the reactions were stopped by adding 100 mM glycine. Excess BIO was removed by using a Sephadex G25 column pre-equalized with phosphate-buffered saline (PBS).

2.3. Detection of BIO-phage proteins in gels

Detection of the biotinylated proteins of BIO-phages was performed by using a gel method as described previously [3]. BIO-phages (4.4×10^8 cfu) were subjected to 15.0% polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate. After electrophoresis, the gels were washed three times with fresh PBS for 5 min and incubated with the avidin–fluorescein conjugate (25 μg/ml) for 1 h. The gels were rinsed using two changes of PBS containing 0.1% Tween-20, and then washed once for 15 min and twice for 5 min with fresh buffer changes. The gels were analyzed by using a FM BIO II Multi-View with FM BIO Analysis version 8.0 (Takara, Tokyo, Japan).

2.4. Preparation and electron-microscopic observation of E. coli JM109 infected with BIO-phages

Preparation and electron-microscopic observation of *E. coli* JM109 infected with BIO-phages was performed as described previously [2]. In brief, 100 μl of an overnight culture of *E. coli* JM109 was mixed with BIO-phages ($0.1–1.2\times10^8$ cfu; multiplicity of infection [MOI], 5–25) and incubated for 1 h at 37°C. After incubation, the cells were washed, fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 10 min, and washed again with PBS. For controls, we performed the same experiments but with the F^- bacterium *E. coli* HB101.

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E. coli infected with BIO-phages were blocked with 2% skim milk and then incubated with avidin–biotin–peroxidase complex for 30 min at room temperature. After washing the cells 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 (thickness, 80 nm) were cut with a Reichert Ultracut E (Leica Microsystems, Wetzlar, Germany) and were observed with a Hitachi H500 electron microscope (Hitachi, Tokyo, Japan). For control experiments, uninfected *E. coli* JM109 cells were prepared by using the same procedure, including the detection with the avidin–biotin–peroxidase complex.

3. Results

To investigate biotinylated proteins of phage, we used the detection in gel methods for detecting the labeled products [3]. The detection of biotinylated protein by using avidin–fluorescein conjugates showed that the labeled proteins were mainly major coat proteins, such as pVIII (Fig. 1). This result suggests that the positive signals that appeared with avidin–biotin–peroxidase originated from biotinylated pVIII. Partially

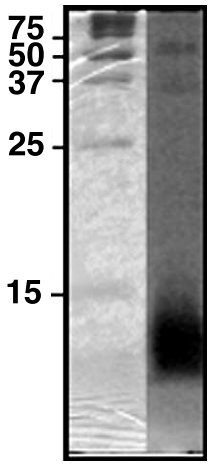

Fig. 1. Detection of BIO-phage proteins in gels. BIO-phages were subjected to denaturing polyacrylamide gel electrophoresis. After electrophoresis, the gels were stained with the avidin–fluorescein conjugate and analyzed by using a fluorescence image analyzer.

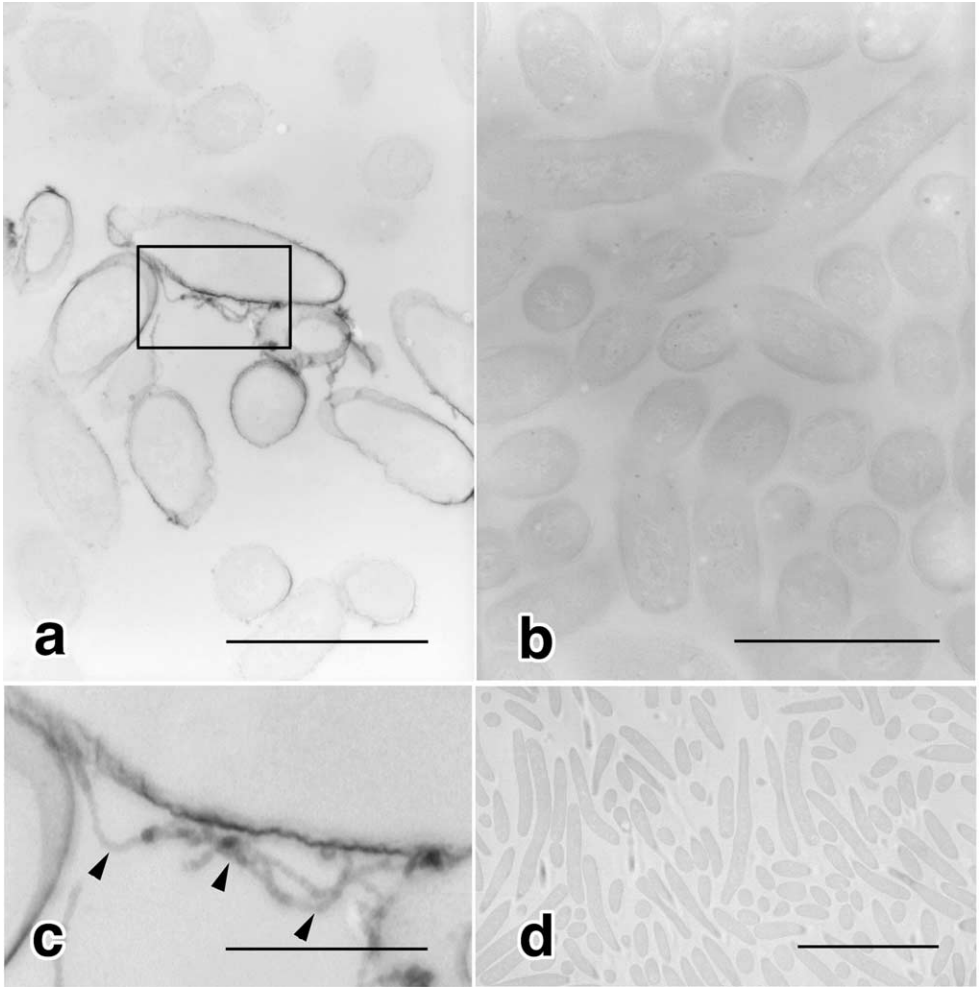

Fig. 2. Electron-microscopic observation of *E. coli* infected with BIO-phages. *E. coli* JM109 was cultured overnight and mixed with BIO-phages. After 1 h, the cells were fixed, reacted with avidin–biotin–peroxidase complex and DAB, and observed with an electron microscope. BIO-phages and BIO-phage-derived proteins appeared clearly as black deposits of peroxidase reaction products (a,c). 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. c: Close-up of the boxed region in a. BIO-phages were detected as thick filaments or clusters covered with reaction products (arrowheads). In control experiments, uninfected *E. coli* JM109 cells (b) and F^- bacterium *E. coli* HB101 mixed with BIO-phages (d) were observed with the same procedure. Original magnifications: a–c, ×10 000; d: ×2400. Bars: a,b, 3 μm; c, 1 μm; d, 5 μm.

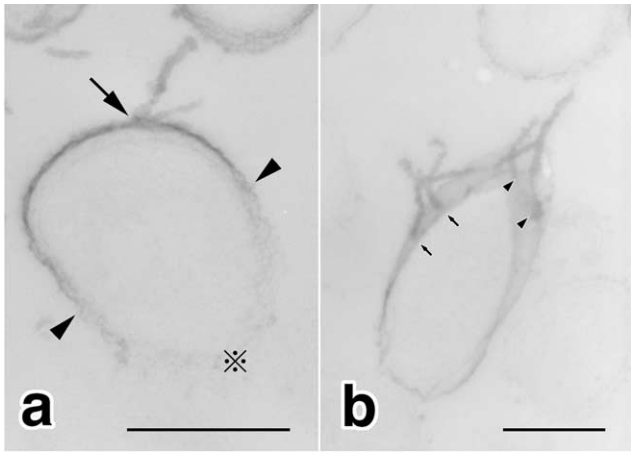

Fig. 3. The location of the ends of infecting phages. In some *E. coli* cells, the ends of the infecting phages were located on the surface of cells (a). In a, the side of the cell that was in contact with BIO-phages showed outer membrane-dominant staining (arrow), the opposite side showed diffuse staining (*), and between those regions, wave-like staining (arrowheads) occurred. b: In some cells, the ends of the infecting phages were located in the periplasmic space (small arrowheads) or near the inner membrane (small arrows) in the other cells. Original magnifications: a, ×24 000; b, ×30 000. Bars: 0.5 μm.

degraded proteins might also be labeled by the peroxidase-mediated reaction.

E. coli JM109 cells were cultured overnight and infected with BIO-phages at a high MOI (5–25). After incubation, the cells were fixed, reacted with the avidin–biotin–peroxidase conjugate and DAB, and observed with an electron microscope under the experimental conditions described previously. BIO-phages and BIO-phage-derived proteins appeared clearly as black deposits of peroxidase reaction products. BIO-phages were detected as thick filaments or clusters covered with reaction products (Fig. 2a,c). The ends of the infecting phages occurred in various places in the section. They were located on the surface of cells (Fig. 3a), in the periplasmic space, or near the inner membrane (Fig. 3b) on the sections. Equivalent experiments performed using the F^- bacterium *E. coli* HB101 yielded no positive signal (Fig. 2d). This finding indicates that the positive staining in JM109 does not originate from non-specific adsorption to bacterial surfaces.

The periplasmic spaces in some positively stained cells were expanded and filled with positive reaction products (Figs. 2a, 3a,b and 5). In control experiments, we observed that uninfected *E. coli* JM109 lacked this expansion of the periplasm space (Figs. 2b and 4b,d). To rule out the possibility of a conformational effect, we observed both long- (Fig. 4a,b) and short-axis (Fig. 4c,d) sections. The periplasmic expansions in BIO-phage-infected cells were confirmed in the sections from the infected cells (Figs. 4a,c) in comparison with those from controls (Figs. 4b,d).

Infected cells revealed various patterns of positive staining on the outer and inner membranes and in the periplasmic spaces. Some cells were stained only at the outer membrane (Fig. 5a); we designated this staining pattern as 'outer membrane-specific'. All other cells were stained on the outer and inner membranes and periplasmic space (Fig. 5b–d). We designated this type of staining as 'diffuse'. Diffuse staining was present as several patterns: 'outer membrane-dominant' (Fig. 5b); 'inner membrane-dominant' (Fig. 5c); and 'non-specific' staining, which showed equivalent staining of the outer and inner membranes (Fig. 5d). These findings also observed the sample that fixated after 5 min of the mixture of *E. coli* JM109 cells and BIO-phages (Fig. 6). These various types of staining often occurred simultaneously in the same cell.

Many infected cells showed diffuse-type staining in their periplasmic spaces (Figs. 5b–d), and in some cells, the staining was wave-like in appearance (Figs. 5e,f). This wave-like staining occurred with other staining patterns. For example, in the cell shown in Fig. 3a, the side in contact with BIO-phage was categorized as having outer membrane-specific staining, the opposite side had diffuse staining, and the intervening area showed the wavy pattern of staining.

4. Discussion

Infection of *E. coli* by the Ff phages is proposed to proceed via a two-step (at least) process [1]. 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 coat proteins and DNA of the phages into *E. coli* [4]. Infection by Ff phages requires pIII, F-pili, and the products of the bacterial genes *tolQ*, *tolR*, and *tolA*. pIII, a three-domain (D1, D2, and D3) protein [5,6], is a minor coat protein that is located at one end of the extended phage particle [7,8]. The infecting phage particle adsorbs to the tip of the bacterial conjugative F-pilus via the D2 domain of pIII. The pilus is an elongated helical array of protein subunits that extends out from the cell wall, and the pilus appears to resorb into the plasma membrane of the host, drawing the phage particle into contact with the cell [9].

Penetration of the viral DNA into the host cytoplasm depends on the integrity of D1 (the N-terminal domain of pIII) and particularly tolR, tolQ, and tolA [6,11]. The tolA product is essential for infection by filamentous phages [11]. The absence of any one of the tolR, tolQ, or tolA proteins abrogates productive infection by filamentous phages, even though the phages can bind to the pili and the bacterium is capable of producing progeny phages when transformed with phage DNA [10,12,13]. The tolQ, tolR, and tolA proteins are also required for the insertion of pVIII into the membrane during infection [12]. These proteins have at least one integral part (TolA and TolR, one; TolQ, three), the tolQRA complex probably spans the entire periplasmic space [14] and is associated with adhesion between the inner and outer membranes [15].

BIO-phages were biotinylated mainly in the pVIII protein and were detected by electron microscopy as thick filaments or clusters covered with reaction products. The results also demonstrated that cells infected with BIO-phages can be detected clearly. The periplasmic spaces in many of these stained cells were expanded and filled with reaction products. These findings indicate that the infecting phages affected adhesion of the inner and outer bacterial membranes during infection.

As for the expansion of the periplasm, several possible models can be proposed. One may originate from interaction of pIII with tolQRA. Binding of the phage protein pIII to the bacterial product tolA may alter the function of the tolQRA complex. Alternatively, the interaction between tolA and the D1 domain of phage gene 3 product may locally disrupt the outer membrane structure to facilitate passage of viral mate-

rial (i.e. DNA and coat proteins). Indeed, expression of the D1 domain in the host cell periplasm causes excessive leakage of periplasmic proteins into the medium [16], possibly due to the interaction of D1 with tolA. Mutations in *tolQ*, *tolR*, *tolA*, *tolB*, or *pal* also cause bacteria to leak periplasmic proteins into the culture medium as well as to become extremely sensitive to detergents such as deoxycholate [17–19]. Binding of D1 domain pIII product to tolA might cause functional and/or conformational changes in the tolQRA complex and related molecules, followed by separation of the outer and inner membranes, their increased permeability, and periplasmic expansion. The increased permeability might lead to the stable location of pVIII into the periplasm and might be part of the driving force for translocating the phage DNA into the cytoplasm as suggested by Webster [20].

In the previous reports, the major coat protein, pVIII, is inserted into the inner membrane [21,22], and the pVIII from the infecting phage and that newly synthesized in the host cell are assembled into progeny phages [21,23]. In our study, BIO-phage-derived protein was detected not only in the inner membrane but also in the outer membrane and periplasm. In light of our findings, we proposed a following process for the integration of phage major coat proteins into the cell. During the second step of infection, the coat proteins of infecting phages are first integrated into the outer membrane, because some cells showed outer membrane-specific staining without periplasmic and inner membrane staining, and no cells showed staining of the inner membrane and periplasm without outer membrane staining. After their integration into the outer membrane, we postulate that some of the phage coat proteins were moved to and retained in the periplasm via binding to some unknown ligands, e.g. proteins and/or peptidoglycans, and, some of the proteins are integrated into the inner membrane. A wave-like pattern of staining (Fig. 3a and 5e,f) was observed in the periplasmic space of infected cells and might show the movements of the phage coat proteins.

The location of the end of the infecting phages in the section could be categorized according to at least two types. In

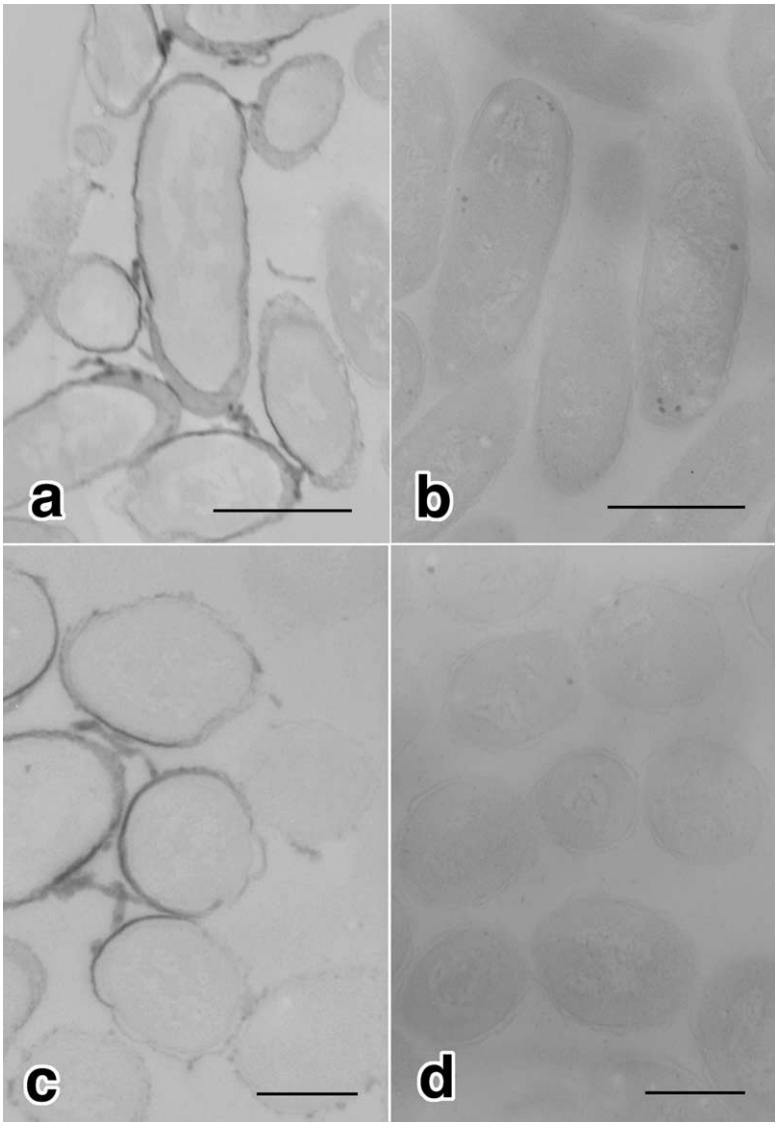

Fig. 4. Periplasmic expansion of infected *E. coli*. In many *E. coli* infected with BIO-phages both long-axis (a) and short-axis (c) sections showed expanded periplasmic spaces, which were filled with positive reaction products. The expansion was not observed in non-infected cells (b,d). Original magnifications: a,b, ×10 000; c, ×10 000; d, ×24 000. Bars: a–d, 1.0 μm.

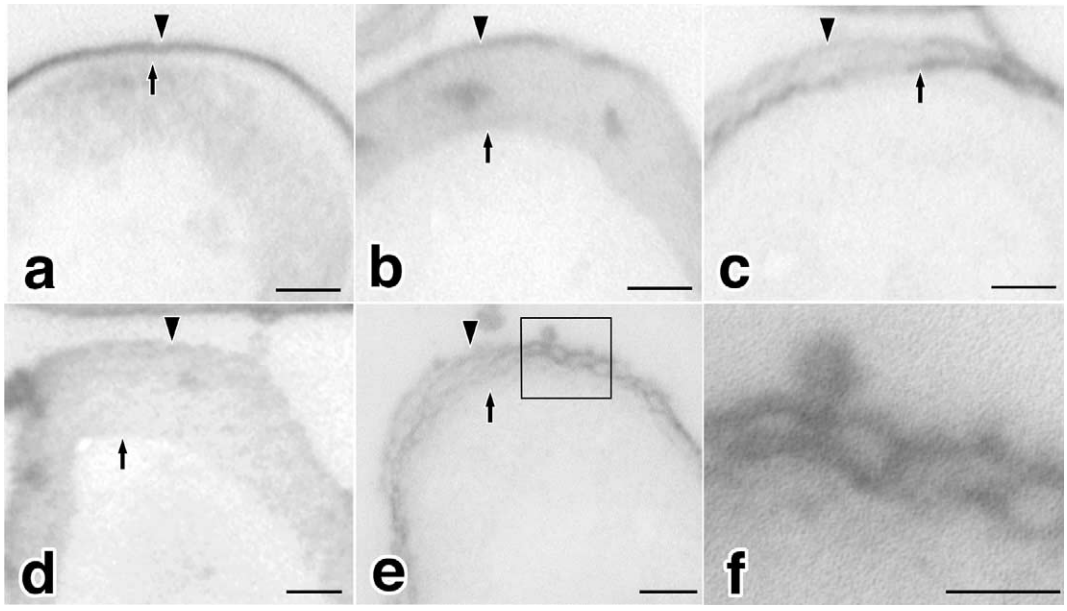

Fig. 5. Staining patterns of BIO-phage-derived proteins in *E. coli*. BIO-phage-derived proteins were detected on the outer (arrowheads) and inner (arrows) membranes and periplasm of *E. coli*, and the infected cells showed various staining patterns. a: Some cells showed staining that was restricted to the outer membrane (outer membrane-specific staining). The others showed diffuse-type staining, with staining of outer and inner membranes and the periplasmic space (b–d). Diffuse staining comprises several patterns: outer membrane-dominant (b), inner membrane-dominant (c), and non-specific, which showed equivalent staining of the outer and inner membranes (d). Some cells showed a wavy line of staining in the periplasmic space (e,f). f: Close-up of the boxed region in e. Original magnifications: a–c, ×12 000; d, ×10 000; e,f, ×42 000. Bar: a–e, 100 nm; f, 50 nm.

one, the end was located on the surface of the cell (Fig. 3a), and in the other, the end of the phage was located in the periplasmic space, near the inner membrane (Fig. 3b). These findings can be explained by dividing phage infection into two phases. Some cells with surface-located phage ends showed outer membrane-specific staining without remarkable periplasmic expansion (Figs. 5a), suggesting initial phase of infection. During this phase, the end of the phage would not cross the outer membrane completely, and the coat proteins would be integrated only into the outer membrane. In comparison, observations of the phage end in the expanded periplasm indicate a later phase of infection.

On the basis of observations reported here, the following hypothesis for the process by which phages infect the host cell can be proposed (Fig. 7): during initial phase of infection, phage coat proteins first integrate into the outer membrane of the host cell, then into the periplasmic space, and finally the inner membrane. Due to major coat proteins in the periplasm, the space is expanded, during later phase of infection, the expansion of the periplasm and its increased permeability (due to initial infection) enable the phage to enter the periplasm via interactions with some proteins and/or other molecules, including tolQRA, after which the coat proteins might be integrated into the inner membrane. Elucidation of molecules that can interact with pVIII in the periplasm would be intriguing for further discussion of the infection mechanism. To obtain time-dependent events during infection we evaluated the cells at 5 min and 1 h after infection, however, the findings at 1 h were similar to those at 5 min (Fig. 6). Because infection usually takes place in less than 10 s, observing the cells at intervals of just several seconds after infection would be required. It should be noticed that every pVIII pro-

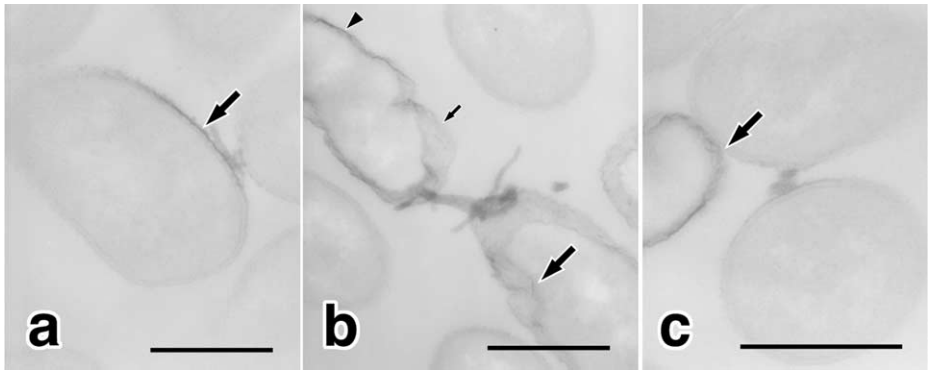

Fig. 6. Observation of *E. coli* infected with BIO-phages after 5 min of their mixing. After 5 min of the mixture *E. coli* and BIO-phages, the cells were treated and observed. In the cells, outer membrane-specific staining (a; arrow), diffuse-type staining: outer membrane-dominant (b; arrowheads), inner membrane-dominant (b; arrow), and non-specific (b; small arrows) and a wavy line of staining in the periplasmic space (c; arrow) were observed. Original magnifications: a,b, ×36 000; c, ×48 000. Bars: a–c, 0.5 μm.

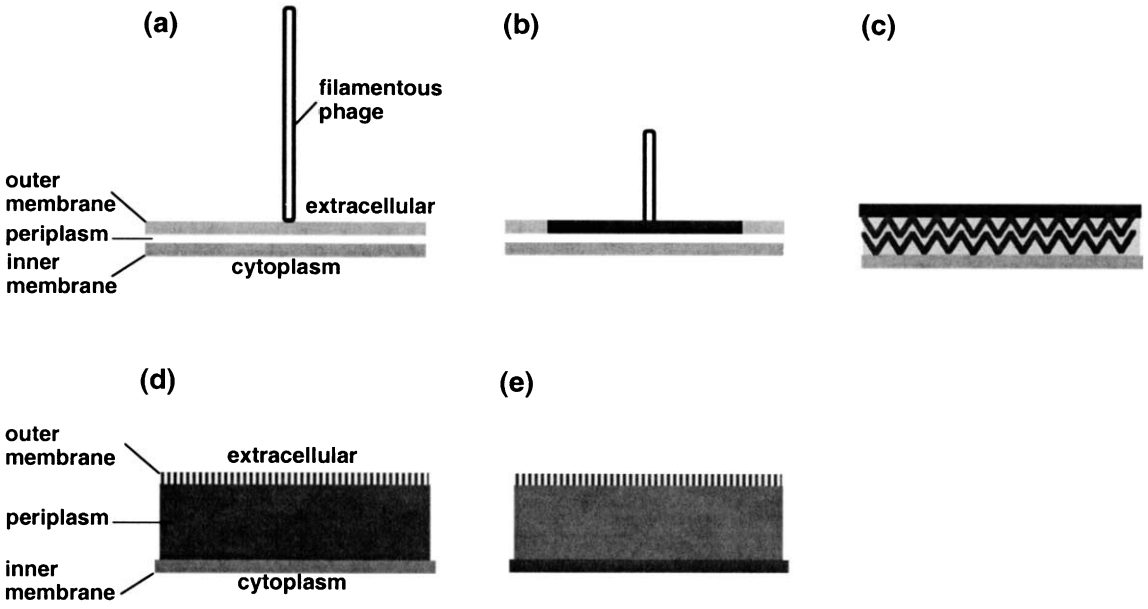

Fig. 7. Model of infection of *E. coli* by filamentous phages. During initial infection (a–e), phage coat proteins integrate into the outer membrane of the host cell first (b), then into periplasmic space (c,d), and finally into the inner membrane (e). After this phase, the periplasm is expanded, and phage-derived proteins move from the outer membrane to the inner membrane, causing the wave-like staining pattern (c). During later phase of infection, the expanded periplasm increases in permeability (d,e), and translocation of phage DNA into the cytoplasm (e).

tein on the phage is not biotinylated. Therefore, the possibilities of other infection route of the biotinylated pVIII cannot be denied merely from the results. Further investigations would be required for examining the infection mechanism proposed here.

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