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Assembly Site of Bacteriophage f1 Corresponds to Adhesion Zones between the Inner and Outer Membranes of the Host Cell

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Morphogenesis of the filamentous bacteriophage f1 occurred at adhesion zones between the inner and outer membranes of the host cell. Quantitation of adhesion zones in cells infected with mutant phage strains suggested that the phage gene I protein may be involved in the formation of adhesion zones for phage assembly.

The filamentous virus f1 specifically infects strains of *Escherichia coli* harboring F plasmids. Its life cycle does not involve lysis of the host; rather, progeny phage are released through the cell envelope without impairing cell viability. Assembly of the phage particle itself appears to occur entirely at the cell membrane, as all the phage coat proteins and gene products involved in morphogenesis are believed to be membrane proteins (4, 17). Because the newly synthesized single-stranded DNA viral genome must cross the three components of the *E. coli* cell envelope (the inner membrane, the peptidoglycan layer, and the outer membrane) as it is assembled with the coat proteins, the phage assembly site might correspond to adhesion zones or areas of close contact or fusion between the inner and outer membranes of *E. coli* (1, 2). These regions appear to play an important role in the incorporation of various macromolecules into the bacterial outer membrane (2, 3, 6, 16), and there is evidence to suggest that F pili are assembled at adhesion zones (2). We present in this communication evidence from electron microscopy that f1 extrusion occurs at sites where the inner and outer membranes of the cell are joined closely and resemble adhesion zones. Comparisons of the frequency of adhesion zones in cells under various conditions suggest that the f1 gene I protein may be involved in the formation of adhesion zones for phage assembly.

E. coli K-12 VL386 [F′ (*pro lac*)/Δ(*pro lac*) *ara thi rpsL* ϕ(*fimD-lac*) λp1(209)] (5) was used as the host for the growth of phage f1 for electron microscopy studies. This strain produces no type I pili, owing to inactivation of the *fimD* gene by the insertion of λp1(209). After infection and removal of excess unadsorbed phage, 25 min was allowed for phage production to begin, and the cells were then plasmolyzed by the addition of an equal volume of prewarmed medium containing 40% (wt/wt) sucrose. After 4 min, the cells were fixed by the addition of glutaraldehyde to 1% (wt/wt) and formaldehyde to 0.6% (wt/wt) and by incubation for 15 min at 37°C and then for 1 h at 4°C. All subsequent steps were carried out at 4°C.

The cells were washed in 20% sucrose–50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 7.2), reduced by incubation with 25 mM $NaBH_4$ for 15 min, washed, and suspended in sucrose-HEPES buffer plus 20 mg of bovine serum albumin per ml. Thirty micrograms of rabbit immunoglobulin G (IgG) raised against whole phage or of preimmune rabbit IgG was added, and the sample was incubated at 4°C for 4 h. The cells were washed, resuspended in the same buffer, and incubated with 100 μg of goat antirabbit IgG overnight at 4°C. The cells were postfixed with 1.5% OsO_4, treated with 1% (wt/wt) tannic acid in sucrose-HEPES buffer for 45 min, and stained with 0.5% (wt/wt) uranyl acetate in aqueous 20% (wt/wt) sucrose. The stained pellets were dehydrated in ethanol and embedded in Epon. Thin sections with an average thickness of 50 nm were examined in a JEOL-100C electron microscope at 60 kV.

Figure 1 shows thin sections of uninfected bacteria treated as described above to visualize adhesion zones but without the antibody addition. Cell envelope features that closely resemble previously described adhesion zones (1, 2) may be observed. These are sites where the outer membrane is pulled in to meet with the inner membrane or, conversely, sites where the retracted inner membrane is stretched out to join with the outer membrane. At a higher magnification (Fig. 1B), the double-track profile of the outer membrane, the single peptidoglycan layer, and the double track profile of the cytoplasmic membrane can be discerned. The inner and outer membranes are apposed very closely at these sites and may be continuous. Three examples of nascent phage being extruded from plasmolyzed infected cells are shown in Fig. 2. The thickness of the decorated nascent phage ranged from approximately 20 to 40 nm, as expected for phage (diameter of 8 nm) decorated with single (rabbit antiphage IgG) or double (rabbit antiphage IgG plus goat antirabbit IgG) layers of antibody. More than 90% of the 529 phage particles observed appeared to originate in adhesion zones on the cell surface. The membranes were very closely joined and appeared to be continuous at the site of phage extrusion, and no sites were observed where separate sections of inner and outer membranes were linked by an intervening portion of a phage filament. The small percentage of phage which did not appear to originate in adhesion zones might be accounted for by an accidental rupture of the adhesion zone during preparation of the sample. Given the average width of an adhesion zone (about 30 nm) and the average number of adhesion zones seen on a section through an infected cell (29 adhesion zones for a section 2.5 μm in circumference), adhesion zones represent approximately 1.2% of the circumference of a cell section. Therefore, it is extremely unlikely that the observed phage distribution at adhesion zones was random.

From thin-section data like those in Fig. 1, the number of adhesion zones per cell section was plotted against the cell section circumference, and the linear regression coefficient was determined (Fig. 3). The slope of this line gives the average number of adhesion zones per unit length of a thin section 50 nm thick. Assuming that *E. coli* is a cylinder 2.0

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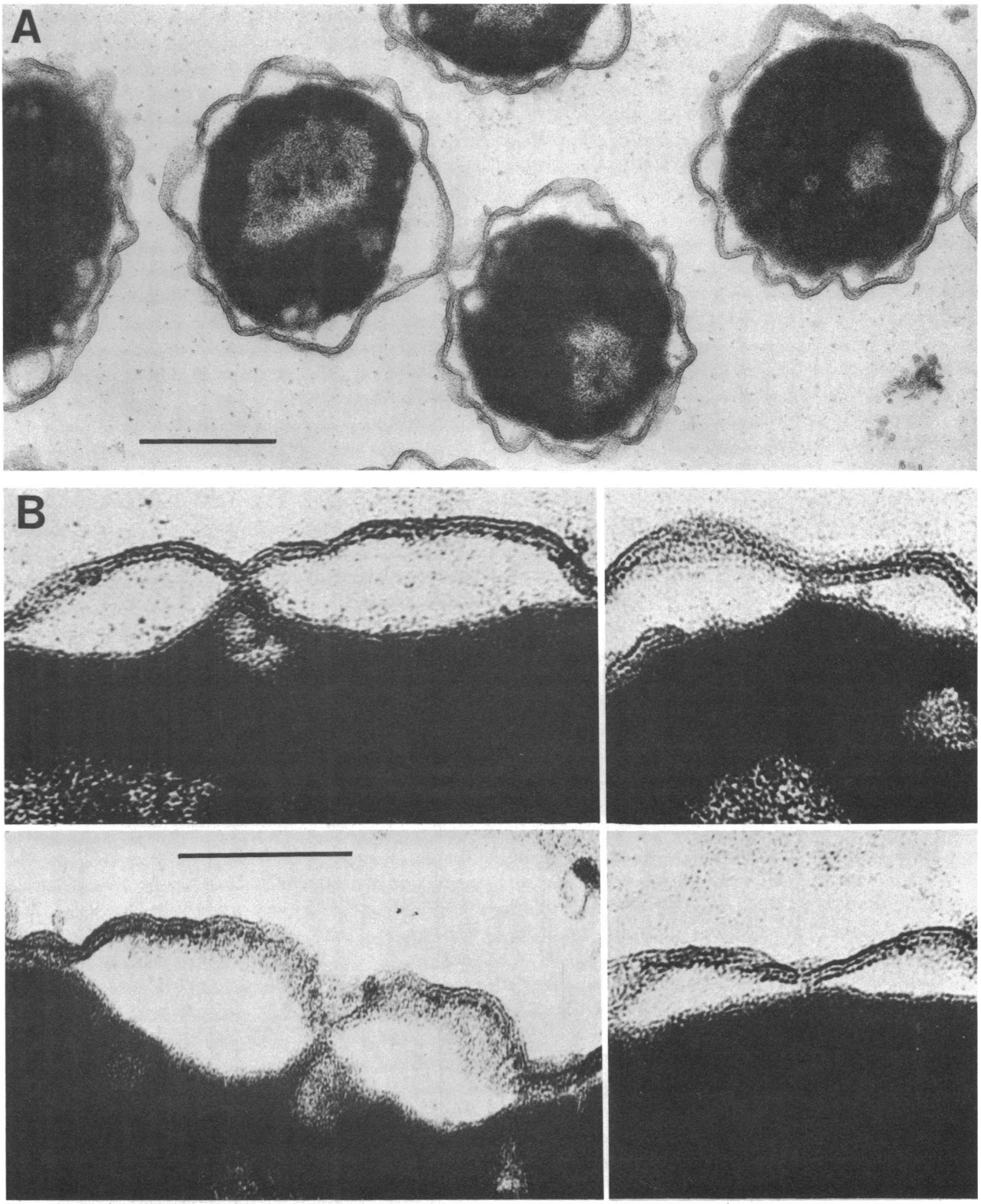

FIG. 1. Visualization of adhesion zones in thin sections of uninfected bacterial cells. Cells of strain VL386 were plasmolyzed and prepared for electron microscopy as described in the text. (A) Bar, 0.3 μm; (B) bar, 0.15 μm.

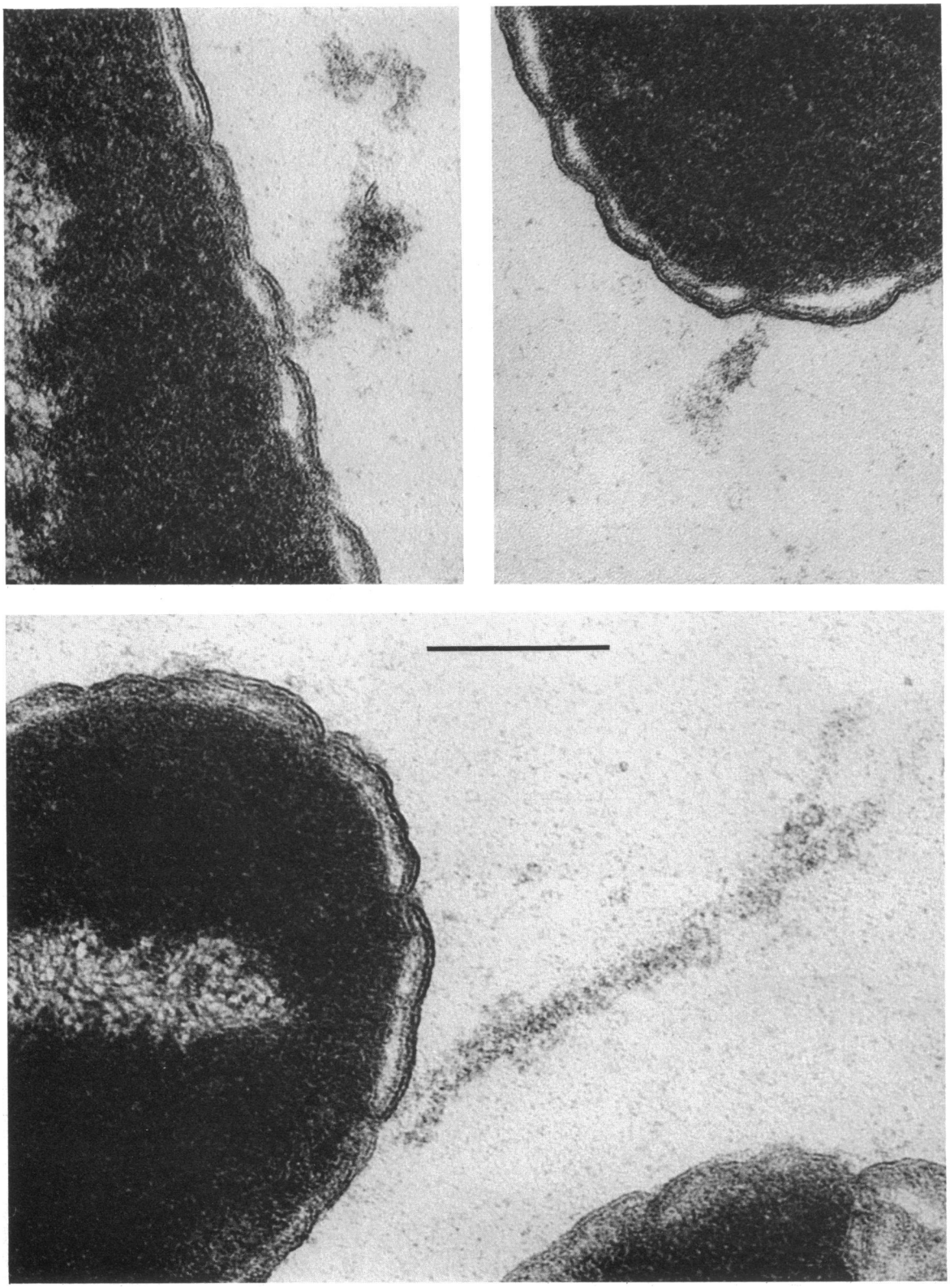

FIG. 2. Nascent phage at adhesion zones. Cells were infected with f1, plasmolyzed, fixed, decorated with rabbit antiphage IgG and goat antirabbit IgG, and prepared for electron microscopy as described in the text. Bar, 0.15 μm.

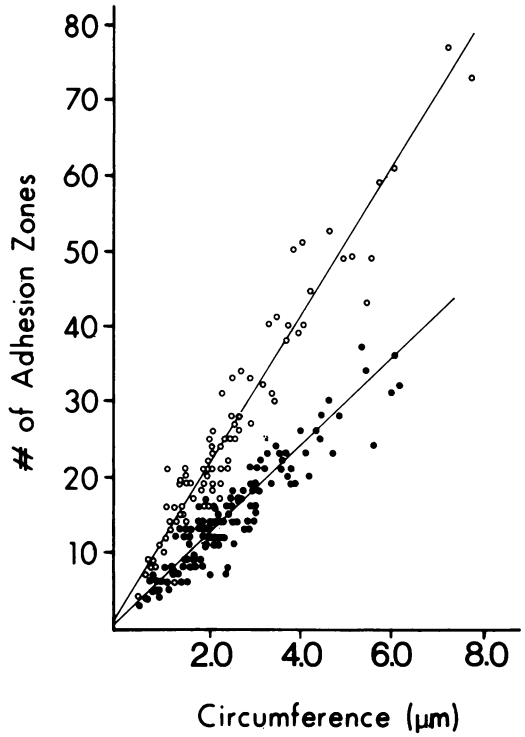

FIG. 3. Number of adhesion zones in infected and noninfected cells. The number of adhesion zones per cell cross section was determined and plotted against the circumference of the cross section. Symbols: ●, noninfected cells; ○, cells infected with f1. The slopes of the best-fit lines were determined by linear regression analysis: for noninfected cells, the slope = 5.75 adhesion zones per μm (r = 0.94; $P < 0.001$); for infected cells, the slope = 9.82 adhesion zones per μm (r = 0.96; $P < 0.001$).

μm in cross-section circumference, it can be calculated from the slope that a 50-nm-thick cross section from uninfected VL386 would contain 11.5 adhesion zones. Given an average length of 1.5 μm for such a cell, 30 such sections would cover the length of the cell, adding up to 345 adhesion zones per bacterium. This value is in excellent agreement with that of 250 to 400 adhesion zones per cell published by Bayer (2).

The number of adhesion zones per cell in strain VL386 increased approximately 70% by 30 min after infection with wild-type phage f1, rising to approximately 590 adhesion zones per cell (Fig. 3 and Table 1). Similar results have been observed with three different preparations of f1-infected VL386 cells. To determine whether any phage gene products were involved in the formation of new adhesion zones in strain VL386, we performed the same quantitation experiments with VL386 infected with different amber mutant strains of f1 (Table 1). An increase similar to that observed with wild-type phage was produced by infection with mutants with amber mutations in most phage genes. The only exception was observed with R3, a mutant with a nonpolar amber mutation in gene I. Infection with R3 produced no increase in adhesion zones over the number seen in uninfected cells, suggesting that the product of gene I (pI) may play a role in the formation of adhesion zones during f1 morphogenesis. Similar results were obtained in two separate experiments. The observed differences in the number of adhesion zones per cell cannot be attributed to changes in cell size after infection with a wild-type or amber mutant phage, as measurements of cell section circumferences revealed no differences in the average sizes of the cells.

TABLE 1. Effect of f1 amber mutations on the number of adhesion zones in infected cells of strain VL386

Phage	Gene	No. of cell sections measured	Slope[a]	No. of adhesion zones per cell[b]
None (not infected)		136	5.75	345
Wild type f1		91	9.82	589
R3	I	96	5.75	345
R158	III	89	8.92	535
R12	IV	72	10.00	600
R148	V	105	9.64	578
R5	VI	76	10.36	622
R100	VII	155	8.86	532
8H-1	VIII	90	9.70	582
N-18	IX	108	8.86	532

[a] Slope (given as the number of adhesion zones per micrometer) of the best-fit line in a plot of the number of adhesion zones against the circumference of the cell section. The regression coefficients were all greater than 0.94 and indicated significant linear correlations at the $P = 0.001$ level.

[b] Estimated from the slope as described in the text.

pI has been shown to be required for morphogenesis of the phage (9, 11, 12). Genetic evidence has shown that the function of pI in assembly requires the interaction of pI with a bacterial protein (13, 14) which has recently been identified as thioredoxin (7, 15). Localization studies have demonstrated that thioredoxin is at the periphery of the cell in an osmotically sensitive compartment thought to correspond to an adhesion zone (8, 10). The data presented here suggest that pI plays a role in the formation or induction of the formation of specialized adhesion zones at which f1 assembly and extrusion take place. All of these observations suggest that pI could be an integral membrane protein located at the adhesion zone. Recent fractionation studies on cells carrying a cloned copy of f1 gene I have shown that pI is tightly associated with the bacterial envelope and that overproduction of pI in the absence of other phage proteins rapidly results in death of the bacteria (J. I. Horabin and R. E. Webster, personal communication). We have analyzed the number of adhesion zones in bacteria containing the cloned gene *I* but have found no difference from cells not containing the plasmid (data not shown). Whether this result implies that a number of other phage proteins are required in addition to pI for adhesion zone formation or merely reflects the deleterious effect of pI on the cells is uncertain.

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