Growth Inhibition and Appearance of the Membranous Structure in *Escherichia coli* Infected with Bacteriophage fd

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Thirty-six mutants of fd, a virus that infects but does not kill *Escherichia coli*, were isolated; 35 mutants were categorized into six complementation groups. Abortive infection with mutants in genes 1, 3, 4, 5, and 6, but not in gene 2, produced a cessation of host cell growth, generally linked to low burst size and to the formation of aberrant intracytoplasmic membranous structures. The membranous structure was studied during infection with various phage and hosts. Appearance of the membranous structure was linked specifically to incomplete phage maturation at the cell membrane, rather than solely to the inhibition of host cell growth or to infection with mutant phage, since (i) in one host, cell growth was inhibited, but no membranous structure developed; and (ii) when antibody against virus was added to cells infected with wild-type phage, phage extrusion was inhibited, cell growth stopped, and the membranous structure once again developed.

Several very similar filamentous bacterial viruses infect a number of bacteria, including *Escherichia coli* (see review in reference 16). Unlike other bacterial viruses, wild-type fd phage are released from growing and dividing cells without killing or lysing the host cell (9, 10, 16). Cell number and colony-formers continue to increase in cultures infected with wild-type phage; however, growth slows (3, 8, 11, 17-19, 24).

In contrast to the relatively mild effect of wild-type phage, infection of a nonpermissive host by mutant phage can result in cessation of growth (23) and the development of an aberrant membranous structure at the poles of the cell (25).

A previous study of filamentous phage interaction with the cell membrane suggested that 1 phage development somehow involves the cell membrane (25). However, it remained unclear what relationship holds between aberrant membrane formation, inhibition of host macromolecule synthesis, and the specific reduction of phage development.

Here we have examined the degree of correlation of growth inhibition and the appearance of internal membranous structure in cells infected with fd amber mutants. One host was found in

which membrane formation was uncoupled from the cessation of host cell growth. Aberrant membrane formation seems to be more closely related to severe inhibition of phage development.

MATERIALS AND METHODS

Bacterial strains and phage strains. Bacterial strains used are listed in Table 1. Wild-type fd phage (15), M13 amber mutants (23), and f1 amber mutants (25) were kindly supplied by T. Tokunaga (National Health Institute in Tokyo), D. Pratt (University of Wisconsin), and N. D. Zinder (Rockefeller University), respectively.

Media. N broth was composed of 1% (w/v) peptone (Daigo Eiyo Kagaku K.K., Osaka), 0.5% (w/v) extract of bonite (Katayama Kagaku K.K., Osaka), and 0.3% (w/v) NaCl, adjusted to pH 7.2. N agar was prepared by the addition of 1.5% (w/v) agar to N broth. Soft agar was prepared by the addition of 0.7% (w/v) agar to N broth. Dilution fluid contains 0.145 M NaCl, 0.005 M MgCl₂, and 0.005 M MgSO₄.

Selection of streptomycin-resistant mutants. A log-phase culture of *E. coli* S26R1d suII in N broth was centrifuged, suspended in dilution fluid, and irradiated by ultraviolet light (National 15-w germicidal lamp) for 1 min at a distance of 38 cm. The treated bacteria were shaken in N broth at 37 C for 2 hr to allow the segregation of mutant cells and plated on N agar containing 200 µg of streptomycin per ml. After overnight incubation at 37 C, streptomycin-resistant mutants were isolated.

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TABLE 1. *Bacterial strains used*

<i>E. coli</i> strain	Streptomycin ^a	Permissiveness	Source
K-10	s	—	L. Cavalli
S26R1d	s	+	A. Garen
DUS1	r	—	From S26R1d
DUS2	r	+	From S26R1d

^a Abbreviations: r = resistance; s = sensitivity.

Chemicals. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was purchased from K & K Laboratories, Jamaica, N.Y. Streptomycin sulfate and dihydrostreptomycin were from Meiji Seika, Ltd., Japan.

Mutagenesis of fd phage: nitrous acid. The fd phage were suspended in 0.1 M sodium acetate-acetic acid buffer (pH 4.5) containing 0.5 M KNO₂. After 45 min at 37 C, treated phages were diluted and plated on N agar with the indicator strain *E. coli* S26 Rle sul. Phage survival was approximately 10⁻⁵.

Mutagenesis of fd phage: nitrosoguanidine. Wild-type fd was adsorbed to strain S26 Rle at a multiplicity of 5. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was added to a final concentration of 30 to 50 µg per ml of N broth. After 2 to 3 hr at 37 C, bacteria were centrifuged out by centrifugation at 2,000 × *g* for 10 min, and the phage in the supernatant were diluted and plated.

Mutagenesis of fd phage: hydroxylamine. Wild-type fd phage were treated in vitro at 37 C with 1 or 1.2 M NH₂OH according to the procedure of Freese, Bautz, and Freese (7).

Isolation of amber mutants. Samples of single plaques arising from stocks of mutagenized phage were tested for growth on permissive and nonpermissive hosts. Where multiplication occurred only on the permissive host, an isolated plaque was picked from the permissive host plate, diluted, and plated with each host. After another single plaque isolation from the plate of the permissive host, an isolated plaque was picked to make a mutant stock.

Genetic complementation test. Standard complementation tests by the method of Pratt, Tzagoloff, and Erdahl (23) were carried out at 37 C for 10 min, with K10 in N broth as the nonpermissive host. M13 and f1 amber mutants in known genes were used to determine the gene loci of unknown fd amber mutants. Mutants were obtained in six of the eight complementation groups reported for the filamentous phage M13 (22).

Measurement of burst size in liquid culture. Log-phase cultures of a strain of host cells were centrifuged, suspended in 0.1 M NaCl-0.01 M tris(hydroxymethyl)-aminomethane (Tris; pH 7.5)-0.005 M MgCl₂-0.005 M MgSO₄, infected at a multiplicity of 0.05, and incubated at 37 C for 15 min to allow phage adsorption. Cells were washed with cold 0.1 M NaCl-0.01 M Tris (pH 7.5)-0.005 M MgCl₂-0.005 M MgSO₄ and diluted in N broth (time zero). After these infected bacteria were incubated at 37 C for 60 min, the plaque-formers in the extracellular fluid were titrated on S26 Rld. The average burst size was obtained by dividing the total

number of progeny phage at time 60 min by the total number of infective centers at time zero. By that time, the membranous structures are observed in most cells after infection with amber mutants (*in preparation*).

Electron microscopy. Cells growing logarithmically in N broth were infected with fd phages. At the indicated times, samples were fixed with 1/10 volume of 1% (w/v) osmium tetroxide in Veronal acetate buffer for about 20 min at room temperature (13). The pellet after centrifugation was fixed in 1% osmium tetroxide in Veronal acetate buffer overnight at room temperature. After fixation, the cells were treated for 2 hr in uranyl acetate solution, dehydrated with series of increasing ethanol concentrations, and embedded in Epon 812 (14). Sections were made with a Sorvall Porter-Blum MT-2 ultramicrotome and stained with lead citrate. Observations were made on a JEM7 electron microscope at 80 kv. To estimate the frequency of cells with aberrant membrane formation, 100 to 200 cells cut longitudinally were observed at each time indicated.

RESULTS

Amber mutants isolated. Thirty-six amber mutants were isolated from 10,000 survivors of mutagenized phage stocks. Amber mutants with reversion frequencies of 2 × 10⁻⁴ or less were isolated; a few hundred with higher reversion rates were discarded.

A summary of the assignment of fd amber mutants to genes is shown in Table 2. Of the 36 mutants, 1 was a double mutant in genes 4 and 6.

Growth inhibition of K-10 (nonpermissive) bacteria infected with amber mutant and appearance of the membranous structure. To investigate the extent of correlation of host inhibition and membrane development, both cells and phage were varied in a number of trials. Three lots of log-phase cultures of *E. coli* K-10 in N broth were infected with wild-type fd, fd am PN2 (gene 3), and fd am PN29 (gene 3), respec-

TABLE 2. *Assignment of amber mutants to fd genes*

Gene	No. of mutants ^a	Mutagen used ^b
1	3	NG, HA
2	4	HA
3	10	PN, HA
4	9	NG, HA
5	6	HA
6	3	HA
Double mutant	1	

^a Thirty-six amber mutants were isolated, and genes were determined by complementation tests as described in Materials and Methods.

^b NG, HA, and PN are abbreviations for nitrosoguanidine, hydroxylamine, and potassium nitrite, respectively.

tively, at a multiplicity of 35 to 90, and the optical density at 660 nm was read at 15- or 30-min intervals with a Hitachi EPO-B photoelectric photometer. The growth inhibition of host bacteria infected with wild-type fd and the cessation of growth of K-10 infected with fd amber mutants was observed about 60 min after infection as shown previously (16, 23). The stoppage in this latter case was accompanied by a loss of colony-forming ability. Permissive host S26 R1d su II, infected with any kind of fd phage, continued to grow at 37°C, though the growth rate was lower than that of the uninfected culture.

Figure 1 shows electron micrographs of thin sections of K-10 infected with wild-type fd. In this section, no membranous structure is evident in the cytoplasm. Bradley and Dewar showed intracellular membranes in bacteria infected with wild-type ZJ/2 (2). But in that case, the growth curve leveled off temporarily for about 1 hr. The sections of K-10 infected with fd am PN2 (gene 3) or HA81 (gene 4) show the characteristic membranous structure (Fig. 2, 3). The membranous structure seems to be continuous with the cytoplasmic membrane (Fig. 2) and concentrated at the pole or poles of the bacteria and at the junction of dividing cells.

Growth inhibition and the appearance of membranous structure in K-10 nonpermissive bacteria were scored after infection with fd amber mutants belonging to genes 1 to 6. The nonpermissive cells infected with fd amber mutants (except gene 2) stopped growing and showed the membranous structure 2 hr after infection. These results are in good agreement with the previous report (25).

Aberrant membrane synthesis in streptomycin-resistant strains and the effect of streptomycin. Because streptomycin interacts with membranes (1, 4, 5) as well as ribosomes (6, 27, 28), its effects were tested in two streptomycin-resistant hosts. The strains used were derivatives of a host permissive for growth of amber phage. They proved to have unusual properties during fd infection, especially in presence of streptomycin.

When infected with wild-type phage, one streptomycin-resistant strain (DUS2) showed about the same burst size as its parental strain, but the other (DUS1) showed a much lower burst size (Table 3). Also unexpected was the death of both these hosts when they were infected in the presence of streptomycin. Each grew well during infection without antibiotic or when uninfected in the presence of antibiotic. But infection rendered these resistant cells phenotypically sensitive (Table 3); growth stopped and the cells lost viability. However, growth inhibition

in these strains, in contrast to infection by amber mutants, was accompanied by considerable phage production and little if any aberrant membrane formation (Table 3).

The difference seen with these hosts extended to infection with am PN2 (gene 3) phage. Each strain contains an amber suppressor, but one streptomycin-resistant allele (DUS1) restricts the function of the suppressor, as is the case with T4 and λ amber mutants, whereas the other (DUS2) is much less restrictive. Accordingly, burst sizes of 0.2 and 508, respectively, were observed for the amber mutants (Table 3). In the presence of phage, both "streptomycin-resistant" strains were again rendered phenotypically sensitive (Table 3). Once again, growth inhibition was not associated with membrane formation in a case where the phage yield remained high (DUS2, Table 3), but low phage yield (DUS1, Table 3) was correlated with extensive membrane formation.

In all cases, growth inhibition per se was not associated with membrane proliferation. Rather, the results suggested that a special type of growth inhibition associated with low phage yields was concomitant with aberrant membrane formation.

Membrane formation in cells treated with antiphage serum. Pratt et al. (23) reported that if phage-specific antiserum is added after phage infection, cell growth stops. To try to test the possibility that aberrant membrane formation was more directly linked to a block in phage formation, we examined S26R1d (permissive) infected with wild-type fd in N broth containing anti-fd serum. Again membranous structures were observed (Fig. 4). This observation suggests that the blockage of fd phage extrusion by antiserum outside of cells is coupled with the appearance of aberrant membranes.

DISCUSSION

In contrast to many other phages, wild-type fd usually does not affect cell growth or multiplication; but phage mutants in all but one gene stop cell growth and kill the bacteria (23). Growth inhibition, formation of aberrant cytoplasmic membranes, and variable burst size can all be observed.

Generally, growth inhibition, formation of aberrant intracytoplasmic membranes, and low burst size were all correlated. However, in some cases where growth inhibition was observed, phage yields remained considerable, and little or no aberrant membrane formation was observed (Table 3).

Since low phage production seems especially closely associated with membrane formation, it

may be that a block of phage production can itself induce inhibition of cell growth and membrane proliferation. Thus, with lesser inhibition of phage production by host mutation (Table 3) or action of low levels of antiserum, cell death

occurs; with more extensive inhibition of phage maturation, membrane formation is observed (Table 3).

The notion that abortive maturation of phage at the cell membrane can inhibit cell growth is

FIG. 1. Section of *E. coli* K-10 infected with wild-type fd for 2 hr. $\times 39,000$.

FIG. 2. Membranous structure in *E. coli* K-10 infected with fd am PN2 (gene 3) for 2 hr; it can be seen to be an extension of the cytoplasmic membrane. $\times 42,000$.

FIG. 3. Section of *E. coli* K-10 infected with fd am HA81 (gene 4) for 2.5 hr. $\times 44,000$.

FIG. 4. Section of *E. coli* S26 R1d infected with wild-type fd in N broth containing anti-fd serum. $\times 52,000$.

TABLE 3. Summary of host growth inhibition, appearance of membranous structure, and burst size of fd and am PN2

Phage	<i>E. coli</i> strain	Streptomycin ^a	Burst size ^b	Host growth inhibition ^c	Membranous structure ^d
fd	K-10	—	521	—	—
	S26R1d	—	326	—	—
	DUS1	—	3	—	—
		+	31	+	±
	DUS2	—	237	—	—
		+	98	+	—
am PN2	K-10	—	0.8	+	++
	S26R1d	—	205	—	—
	DUS1	—	0.2	+	+
		+	4	+	++
	DUS2	—	508	—	—
		+	263	+	—

^a Streptomycin (200 µg/ml) was added in N broth.

^b Burst size was measured as described in Materials and Methods.

^c The presence (+) or absence (—) of growth inhibition was determined from the growth curve of bacterial strains infected with phages.

^d Thin sections were made from each infected culture to score for membranous structures. The symbols —, ±, +, and ++ indicate 0%, less than 20%, 20 to 60%, and more than 60% of cells, respectively, showing membranous structures, among 100 cells scored.

not a strange one: action of many colicins on the cell envelope, even at a level of one per cell (12, 20), do just that. However, the resultant proliferation of intracellular membranes is certainly caused by the special association of fd development with the cell membrane (see review in reference 16).

Significant change in membrane structure during fd infection is consistent with the induced sensitivity of "resistant" cells to streptomycin (Table 3). These cells, containing streptomycin-resistant ribosomes, are made phenotypically sensitive. The mechanism of lethality is presumably not via ribosomes and may well be a consequence of binding of the drug to the modified membrane.

Another hint that inhibition of phage production is directly linked to membrane formation comes from the fact that mutants in gene 2 show no accumulation of membranes. The gene 2 product is involved in viral deoxyribonucleic acid replication (21), a process often believed to take place at the cell membrane (26).

It seems reasonable to imagine that the pro-

cess for net production and release of phage requires a modification of the cell membrane involving a local lesion at the poles of the cell. When only incomplete virus is formed or extrusion is blocked by antiserum, the exit system is unbalanced so that the lesion of the membrane leads to invagination and proliferation of the cell membrane from the poles of the cell into the cytoplasm.

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