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Phospholipase Activity in Bacteriophage-Infected *Escherichia coli*

III. Phospholipase A Involvement in Lysis of T4-Infected Cells

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Bacteriophage studies with *Escherichia coli* K-12 (λ)DR⁻DS⁻, a mutant lacking the major known fatty acyl hydrolases (phospholipases), and its wild-type parent showed equivalent phage infection with regard to phage production and time of phage release. Further examination of the DR⁻DS⁻ mutant, however, revealed that the progeny bacteriophage were released without complete dissolution of the host cell. Prolonged cell integrity of the infected mutant was noted by spectrophotometry and supported by direct microscope examination. The phage release occurred at normal "lysis" time with phage yields comparable to that of the wild-type bacteria. Inner membrane degradation was indicated by the release of β-galactosidase, a cytoplasmic enzyme, and of trichloroacetic acid-precipitable RNA. Thus, outer membrane degradation is required for dissolution of phage-infected cells, and this degradation is at least partly dependent on activation of host phospholipases.

The infection of *Escherichia coli* with virulent T4 phage normally results in the lysis of cells accompanied by the release of progeny phage. Lysis is usually defined by the dissolution of the infected cells with a concurrent decrease in turbidity of the culture (1). The requirement of phage-specific endolysin in the lytic process is indicated by the demonstration that T4*e* mutants can infect *E. coli*, but lysis does not occur (13). Since endolysin is made significantly before lysis, it is likely that the inner membrane prevents its escape and thereby protects the mucopeptide from endolytic degradation. Thus it is possible that inner membrane degradation is also required for phage-induced lysis. Under some conditions, however, mucopeptide degradation is not required. Emrich (10) demonstrated that infection of *E. coli* with T4*es* resulted in lysis of the host cells. This mutant is deficient in both endolysin production (*e* gene) and resistance to lysis from without (*s* gene). The *s* gene product confers resistance to lysis from without, presumably by altering the cell envelope in such a way as to make it impervious to damage by superinfecting phage added at high multiplicities (10, 19). Only about 10% of the progeny phage of T4*es*-infected bacteria was released, the remainder presumably being entrained in the intact mucopeptide (10), but this indicates that membrane changes occurring in cells infected with T4*s* are sufficient to account for host cell lysis. We have previously reported that infection of *E. coli* with T4*s* results in deacylation of phospholipids prior to lysis (6), similar to the report by Bennett et al. (3), with T4rII mutants. Both T4*s* and T4*r* mutants are incapable of lysis inhibition. Although phospholipase activation is suggested by these reports, it is not clear whether it is the primary cause of early lysis. It is known that various kinds of membrane damage are sufficient to activate host phospholipase. In the experiments reported here we have examined the effects of T4*s* infection of *E. coli* K-12 (λ) DR⁻DS⁻, a host which is deficient in phospholipase A (9, 15). Although this strain of *E. coli* is not completely devoid of phospholipase activity (15), it was reasoned that if phospholipase activation is required, then abnormal phage infection might be observed. We could not use T4*r*II mutants, also incapable of lysis inhibition (8) and capable of early free fatty acid (FFA) production (3), because they are restricted in *E. coli* strains lysogenized by lambda phage, and in strains cured of the prophage the *r* gene defect of T4 is innocuous.

MATERIALS AND METHODS

Bacteria and phage. *E. coli* strain K-12 (λ)DR⁻DS⁻ and its wild-type parent were obtained from S. Nojima. This mutant is deficient in both

detergent-resistant (DR) phospholipase A and detergent-sensitive (DS) phospholipase A (9, 15). *E. coli* K-12 (λ)IYMel and wild-type T4 phage were obtained from L. Astrachan. T4B*s*, a mutant deficient in the ability to cause resistance to lysis from without (10), was obtained from J. Emrich. *E. coli* cells infected with T4B*s* do not demonstrate lysis inhibition upon superinfection (10), and in this respect they phenotypically resemble T4*r* mutants. High-titer phage stocks were prepared by differential centrifugation of phage lysates of *E. coli* K-12 (λ)IYMel, and phage were assayed by the methods of Adams (2).

Media and reagents. Tryptone broth, containing 1% tryptone (Difco) and 0.1 M NaCl, was used as growth media. Soft and hard agar for plating phage and bacteria contained tryptone broth and 0.6 and 1.5% agar (Difco), respectively. Isopropyl-β-D-thiogalactopyranoside (IPTG) and *o*-nitrophenyl-β-D-galactoside (ONPG) were obtained from Sigma Chemical Co. (St. Louis, Mo.). [2-^{14}C]uracil (50 mCi/mmol) was obtained from New England Nuclear Corp. (Boston, Mass.). Aquasol was obtained from New England Nuclear Corp. and used as scintillation fluid.

Assay for lysis and phage release. Overnight cultures of *E. coli* were diluted 1:100 into fresh tryptone broth and incubated with aeration at 37 C until the density reached 2×10^8 cells/ml. Cells were then infected with T4B*s* at an input multiplicity of 5, and incubation was continued. At 5 min after the primary infection, the cells were superinfected with the same phage, again at an input multiplicity of 5. At 10 min after primary infection a 10^7-fold dilution of the infected culture was prepared, and incubation and aeration of both the diluted and undiluted samples was continued. At various times samples were removed from both and after appropriate dilution plated in overlay assays (2) to determine release of progeny phage. Lysis, or decrease in turbidity, was monitored by determination of absorbancy at 600 nm of samples removed from the undiluted culture of T4B*s*-infected cells.

Release of β-galactosidase. The time course for the degradation of the inner membrane of infected cells was monitored by the appearance of β-galactosidase in filtrates. Cultures of *E. coli* were prepared as described above, with the exception that isopropyl-β-D-thiogalactopyranoside (final concentration of 1.1×10^{-4} M) was added when absorbancy at 600 nm reached 0.3. Incubation was continued until cell density reached 2×10^8 cells/ml. At various times after infection with T4B*s*, 1.0-ml samples were removed and filtered through membrane filters (Millipore; pore size, 0.45 μm). The filtrates were then assayed for β-galactosidase activity using a colorimetric assay based upon the enzymatic hydrolysis of *o*-nitrophenyl-β-galactoside (18).

Release of trichloroacetic acid-insoluble [^{14}C]uracil-labeled polymers. *E. coli* cells were labeled prior to infection by growth in media containing 0.05 μCi of [^{14}C]uracil/ml. When cell density reached 2×10^8/ml, the cells were collected by centrifugation and washed two times with growth media to remove unincorporated label. After resuspension in tryptone broth and a 15-min period of incubation, the bacteria were infected with T4B*s* phage. At various times after infection samples were removed for centrifugation (2,000 × *g* for 15 min), and trichloroacetic acid (final concentration equal to 10%) was added to the supernatant and precipitate fractions were obtained. The acid-insoluble material in each fraction was collected on membrane filters (Millipore; 0.45 μm), and after washing with 5% trichloroacetic acid and ethanol the filters were transferred to vials for scintillation spectrometry. The filtrates and the wash fluid were also assayed to determine the acid-soluble radioactivity.

RESULTS

It is known the *E. coli* cells infected with T4B*s* mutants lyse early (10) and that FFA accumulates prior to lysis (6). In cells infected with wild-type T4, FFA formation, indicative of phospholipid hydrolysis, is not observed until just prior to and during lysis (7, 12). To determine if phospholipase activation leading to FFA accumulation is required for lysis, we compared the lysis times of *E. coli* K-12 (λ)DR$^-$DS$^-$ and its wild-type parent after infection with T4*s*. Figure 1 indicates that lysis, measured by decreased absorbancy, is extensively delayed in the DR$^-$DS$^-$ host, whereas the wild-type host was lysed at the normal time. Since the DR$^-$DS$^-$ mutant is deficient in phospholipase A, experiments of this type suggested that phospholipid deacylation has a role in phage-induced lysis of *E. coli*.

Whereas it is known that in T4*s*-infected *E. coli* lysis inhibition normally cannot occur (10), and FFA accumulation is observed early in the latent period (6), these results (Fig. 1) seemed

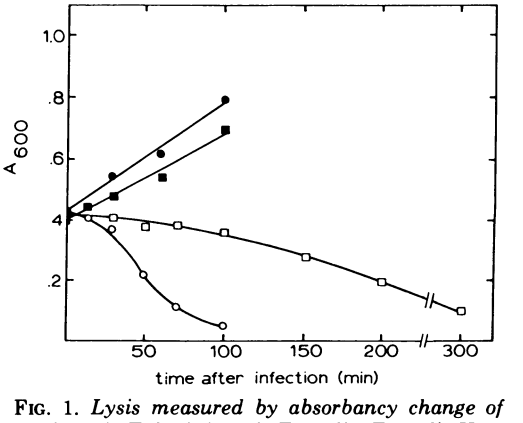

FIG. 1. *Lysis measured by absorbancy change of control and T4Bs-infected E. coli. E. coli K-12 (λ)DR$^-$DS$^-$ and wild type were grown and infected with T4Bs as described. Symbols:* □, *DR$^-$DS$^-$, infected;* ○, *wild type, infected;* ■, *DR$^-$DS$^-$, uninfected;* ●, *wild type, uninfected.*

to confirm a suggestion that phospholipase activity is required to terminate the latent period and to institute lysis. Such an interpretation, however, is weakened by the results of experiments shown in Fig. 2. In these experiments the time of release of progeny from T4Bs-infected DR⁻DS⁻ and its wild-type parent was assayed. To simulate conditions which normally lead to lysis inhibition, the T4*s*-infected cells were superinfected with the same phage, at the same multiplicity. Additionally, since it has been reported that lysis inhibition cannot be demonstrated if infected cells are diluted extensively after superinfection (5), the time of release of progeny from both undiluted and diluted cultures of infected cells was compared. To our surprise, these experiments indicated that the release of phage progeny occurred at the same time, regardless of the presence of host phospholipase, and at a time significantly before lysis was observed in the experiment shown in Fig. 1. Furthermore, the release of phage progeny is initiated by about 30 min, confirming the report that T4*s* phage, even when superinfected, cannot give lysis inhibition (10). These results, together with those shown in Fig. 1, indicate that phage-induced lysis and the release of progeny phage are separable events.

The difference in lysis time of T4Bs-infected DR⁻DS⁻ and its wild-type parent (Fig. 1) was confirmed by direct counts of intact, infected cells. At various times after infection samples were removed, and after dilution the number of remaining intact cells was determined by use of hemocytometer chambers. Table 1 indicates that by 30 min after infection only 37% of the wild-type infected cells remained structurally intact, whereas almost 90% of the infected DR⁻DS⁻ cells could still be counted. By 60 min all of the wild-type cells had apparently lysed, whereas 35% of the phospholipase-deficient cells remained visible. Whereas the difference in lysis times was not as dramatic as indicated by changes in absorbancy (Fig. 1), these results are in agreement with the suggestion that phospholipase is involved in the lytic event. We have observed that phage-infected DR⁻DS⁻ cells are quite fragile in late stages of the latent period, and the manipulations involved in their transfer to counting chambers may have accelerated their dissolution.

Bacterial viruses such as the filamentous M13 and f_d DNA phage (17) mature in the membrane, and lysis of the host cell is not required for their release. The virulent phage of the T series, however, mature in the cytoplasm, and their release could be expected to require extensive damage to the inner membrane. Furthermore, if phage are released by such a process it could be expected that cytoplasmic phage-specific endolysin also escapes, resulting in mucopeptide degradation and lysis of the host cell. Since lysis occurs after phage release in DR⁻DS⁻ cells infected with T4*s*, a phage capable of producing endolysin, the possibility that

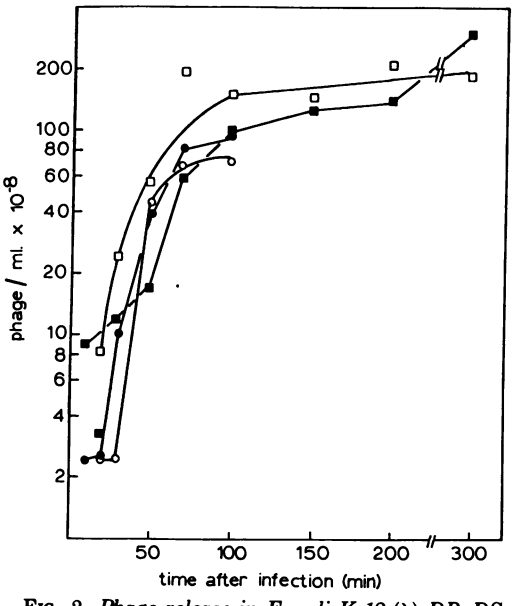

FIG. 2. *Phage release in E. coli K-12 (λ) DR⁻DS⁻ and wild type. Cells were grown and infected, and portions were diluted and assayed for phage released as described. Symbols:* □, *DR⁻DS⁻, diluted;* ○, *wild type, diluted;* ■, *DR⁻DS⁻, undiluted;* ● *wild type, undiluted.*

TABLE 1. *Hemocytometer study of intact cells*[a]

Time post-infection (min)	Cells observed (%)			
	K-12 (λ)DR⁺DS⁺		K-12 (λ)DR⁻DS⁻	
	Infected	Control	Infected	Control
0		100		100
15	72		94	
30	37	152	89	156
60	0		35	

[a] Both cells were grown in tryptone broth to a density of 2×10^8 cells/ml, at which time T4 Bs was added at an input multiplicity of 3. At the indicated times, samples were removed and diluted 40-fold for observation in a hemocytometer under phase contrast. This dilution gave approximately 100 bacteria at control times. The number of bacteria counted at each time was compared to the original uninfected number for that cell type.

membrane degradation is not necessary for phage release was examined. Figure 3 indicates that β-galactosidase, synthesized prior to infection, is found in the supernatants of wild-type and DR⁻DS⁻ cells after infection. With the wild type the time of release was in agreement with the time of phage release. In DR⁻DS⁻ the release was somewhat delayed, but it nevertheless occurred substantially before the time of lysis (Fig. 1).

Table 2 also illustrates that inner membrane degradation occurs at the time of phage release. Cells were labeled prior to infection by growth in media containing [^{14}C]uracil. After infection the extent of release of cellular components containing the label was assayed, and the amount released as macromolecules (or fragments of macromolecules) was estimated by the amount of trichloroacetic acid-precipitable label. Both the T4*s*-infected phospholipase-deficient host and its wild-type parent released the label, much of it being acid precipitable, again indicating that extensive membrane degradation occurred. We did not attempt to identify the classes of RNA represented in the release. The decrease in amount of acid-precipitable RNA in the wild type at 60 min presumably is a reflection of nuclease digestion, since the total (soluble plus precipitable) in the supernatant was not significantly changed. Whereas the results indicated a significant release of acid-precipitable material by 20 min after infection,

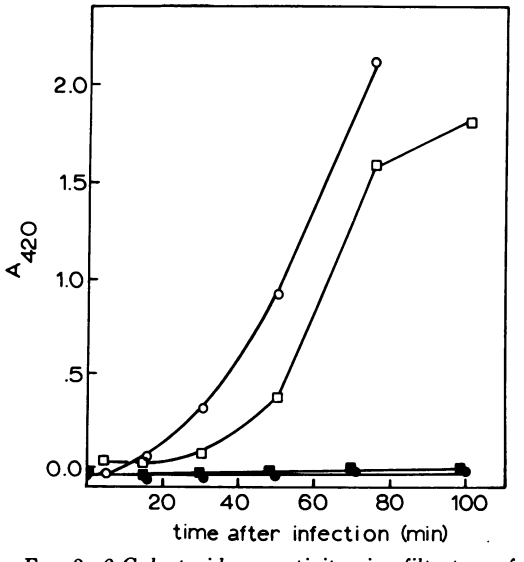

FIG. 3. *β-Galactosidase activity in filtrates of T4Bs-infected and uninfected E. coli. Cells were grown and infected as described. □, DR⁻DS⁻, infected; ○, wild type, infected; ■, DR⁻DS⁻, uninfected; ●, wild type, uninfected.*

TABLE 2. *Release of [^{14}C]uracil*[a]

Time post-infection (min)	Counts/min in acid-precipitated supernatant (%)[b]			
	K-12 (λ)DR⁺DS⁺		K-12 (λ)DR⁻DS⁻	
	Infected	Control	Infected	Control
20	42 (73)[c]	4 (6)	40 (62)	9 (15)
40	74 (96)	3 (5)	58 (79)	12 (16)
60	46 (92)	2 (4)	52 (90)	18 (22)

[a] Growth of bacteria, incorporation of label, and assay were as described in Materials and Methods.

[b] Percent of total cell-bound counts (ca. 30,000 counts/min) at zero time.

[c] Numbers in parentheses are percentages of total counts found in supernatant fraction (acid precipitable and soluble).

i.e., somewhat prior to phage release, we believe this was probably due to fragility occurring as a consequence of centrifugation at 4 C. In the preceding experiment (Fig. 3), this difficulty was obviated by assaying filtrates instead of supernatants.

DISCUSSION

Normally the infection of *E. coli* by virulent T4 phage is terminated by lysis of the host cell with concomitant release of progeny phage. In this communication we refer to lysis as the dissolution of cell structure, as monitored by a decrease in turbidity of the infected culture. The data presented here indicate that the release of progeny phage is not necessarily dependent upon the lysis or dissolution of the host cell. These two events are separable in T4*s*-infected *E. coli* mutants which are deficient in phospholipase A.

At this time we have evidence which directly implicates the outer membrane as the structure which remains intact and continues to contribute to the turbidity after the release of progeny phage. That the maintenance of turbidity can be observed only in DR⁻DS⁻ cells indicates that phospholipids have a direct role. In *E. coli* phospholipids are components of both the inner and the outer membranes. We can eliminate the inner membrane as the responsible structure, since its degradation was indicated by the release of β-galactosidase, a cytoplasmic enzyme, and of acid-precipitable RNA. Furthermore, since T4 phage mature in the cytoplasm and not the inner membrane, it can be expected that this membrane is degraded to permit their release. Since phage escape and presumably inner membrane degradation can occur in both T4*s*-infected wild type and DR⁻DS⁻ hosts, we

may also conclude that neither the DR nor the DS phospholipase is required. The involvement of other phospholipase, however, is not precluded, since it is known that the DR⁻DS⁻ mutant contains some additional but uncharacterized phospholipase activity (15), and it has been demonstrated that a phage-specific phospholipase may be formed (14).

The outer membrane of gram-negative enterobacteria is comprised of lipoproteins, lipopolysaccharides, and phospholipids held together in a trilaminar array (16) at least in part by hydrophobic interactions. There is some evidence which indicates that it may augment the mucopeptide in its structure-maintaining role. This was indicated by experiments which demonstrated that spherical forms of *E. coli* can be generated if the cells are treated with lysozyme alone and then diluted from sucrose into water (4). Usually EDTA, which is known to release lipopolysaccharides and other outer membrane components (4), is used in conjunction with lysozyme for either lysis or spheroplast formation. Additionally, lysozyme degradation of mucopeptide without the generation of spherical forms has been demonstrated (11). Since the DR phospholipase, compartmentalized in the outer membrane, has the ability to deacylate the major phospholipids of *E. coli*, we believe that its activation in normal infections could diminish the hydrophobic interactions between the phospholipids and other lipid-containing components of the outer membrane, resulting in their dissociation. In its absence, as in DR⁻DS⁻ mutants, the hydrophobic interactions could remain intact, thus permitting maintenance of outer membrane integrity and thus turbidity beyond the time of release of phage progeny.

The maintenance of structure of bacteria is normally attributed mainly to the mucopeptide. We did not examine the mucopeptide to determine that it too was degraded. However, since T4B*s* phage can direct the synthesis of endolysin, and since it is a small enzyme which could be expected to escape at the same time β-galactosidase and RNA was released, we believe it likely that the mucopeptide did not remain intact. Additionally, it has been demonstrated that *E. coli* cells infected with T4*es*, a mutant unable to cause endolytic cleavage of the mucopeptide, release only about 10% of the progeny phage, the remainder being entrained in the intact mucopeptide (10). In our experiments the number of phage progeny released from the mutant host was approximately the same as from wild-type hosts, implying that mucopeptide degradation had occurred.

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