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Lysis of Lysis-Inhibited Bacteriophage T4-Infected Cells

STEPHEN T. ABEDON

Department of Microbiology and Immunology, Arizona Health Sciences Center, University of Arizona, Tucson, Arizona 85724

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T4 bacteriophage (phage)-infected cells show a marked increase in latent-period length, called lysis inhibition, upon adsorption of additional T4 phages (secondary adsorption). Lysis inhibition is a complex phenotype requiring the activity of at least six T4 genes. Two basic mysteries surround our understanding of the expression of lysis inhibition: (i) the mechanism of initiation (i.e., how secondary adsorption leads to the expression of lysis inhibition) and (ii) the mechanism of lysis (i.e., how this signal not to lyse is reversed). This study first covers the basic biology of the expression of lysis inhibition and lysis of T4-infected cells at high culture densities. Then evidence is presented which implies that, as with the initiation of lysis inhibition, sudden, lysis-associated clearing of these cultures is likely caused by T4 secondary adsorption. For example, such clearing is often observed for lysis-inhibited T4-infected cells grown in batch culture during T4 stock preparation. The significance of this secondary adsorption-induced lysis to wild T4 populations is discussed. The study concludes with a logical argument suggesting that the lytic nature of the T4 phage particle evolved as a novel mechanism of phage-induced lysis.

Despite the degree to which bacteriophage (phage) T4 is otherwise understood molecularly, physiologically, and genetically, there is a real gap in our understanding of the mechanism of T4 lysis. In contrast, the lysis of such phages as lambda, φX174, and MS2 has been well characterized molecularly (15). One could argue that the dearth of effort made toward understanding T4 lysis over the past 20 years is a consequence of its complexity and, in particular, the existence of a phenomenon known as lysis inhibition (LIN) (reviewed in references 2 and 15).

LIN is an extension of the T4-infected-cell latent-period length and a magnification of the phage T4 burst size. LIN is induced by the adsorption of a second T4 phage and requires at least six T4 gene functions (i.e., genes *rI*, *rIIA*, *rIIB*, *rIII*, *rIV*, and *rV* [9]). Thus, T4-infected cells may lyse at the end of a normal latent period, when LIN is not induced, in a way that is probably similar to that of phage lambda-infected cells (15). Given additional T4 adsorptions (secondary adsorptions), however, this lysis occurs at the end of a significantly longer LIN latent period.

A further complication on T4 lysis is the lytic nature of T4 particles (reviewed in reference 15). Multiple T4 adsorptions lead to the lysis from without of not previously T4-infected cells and, with greater multiplicities, T4-infected cells as well. Traditionally, lysis from without is measured as a dramatic drop in culture turbidity occurring soon after high-multiplicity phage addition (e.g., see references 5 and 11). Multiple T4 secondary adsorptions can also lead to a loss of plaque-forming ability (secondary trauma). Surprisingly, lysis from without and secondary trauma are distinguishable genetically. That is, different T4 genes code for the bulk of resistance to each phenomenon, gene *imm* for resistance to secondary trauma and gene *sp* for resistance to lysis from without (5).

Two basic mysteries shroud our understanding of the expression of LIN. First, there is the mechanism by which secondary adsorption induces LIN (2, 4, 6, 12, 15). Second, there is a no less important but mostly ignored gap in our understanding of how, once the LIN state is induced, it is reversed, thus allowing for the lysis of T4-infected cells expressing LIN. High-density batch cultures consisting of T4-infected cells in the LIN state (LIN cultures) are often observed to clear rapidly (LIN collapse), implying a similarly fast reversal of the induction of LIN across a population of lysis-inhibited T4-infected cells. One may speculate that the mechanism inducing this apparently synchronized population lysis involves some sort of precisely timed and executed reversal of whatever mechanisms are involved in the delay of lysis in individual lysis-inhibited T4-infected cells. If such a reversal is effected intracellularly, it would be likely that the control of LIN collapse is as complex as the control of lysis of T4-infected cells not expressing LIN (15). Have T4 phages, then, twice evolved the capacity to control precisely and internally the timing of their own lysis? In this study it is shown that, as with the induction of the LIN state, LIN collapse is associated with secondary T4 adsorption. This association suggests a mechanism of extracellular induction of LIN collapse perhaps related to lysis from without or secondary trauma. First, however, an experimental overview of the basic biology of T4 growth and lysis in LIN culture is presented.

Additionally, the potentially multifaceted T4 colonic life history is reviewed, and the significance of secondary adsorption-induced LIN collapse to T4 growth in such a setting is discussed. With this broad approach to understanding T4 growth characteristics, a logical argument is presented which suggests that the lytic nature of the T4 particle evolved in T4 populations as a useful and novel mechanism of phage-induced cell lysis.

MATERIALS AND METHODS

Abbreviations. Note the following abbreviations: MOI (multiplicity of infection) and MOSI (multiplicity of superinfection).

Phage and bacterial strains. All bacterial and phage strains were obtained from laboratory stocks. Unless otherwise noted, experiments were done using *Escherichia coli* S/6/5 host cells and phage T4D (wild type). Phage stocks were grown from isolated plaques by the bottle lysate technique (3).

Media. Unless otherwise noted, the medium employed

was H broth (13) and growth temperature was held constant at 37°C. Casamino Acid- or yeast extract-enriched H broth consists of 1 or 2 parts (of 10 parts) of 6% Bacto vitamin assay Casamino Acids or Bacto yeast extract–0.5% NaCl solution pH adjusted to 7.2 and added to 8 or 9 parts of H broth. Medium containing twice the concentration of all H broth ingredients except NaCl is called 2× H broth. Phages were allowed to form plaques by using the soft-agar overlay method (3) and EH soft and hard agar (13).

Lysis profile assay. Cultures were initiated with 0.05 ml or less of cells overnight per 10 ml of medium to ensure that cells entered the exponential growth phase before phage addition. To promote aeration, cultures were grown in loosely capped 250-ml sidearm Erlenmeyer flasks in a shaker water bath. To measure culture turbidity a Klett-Summerson photoelectric colorimeter (Klett Manufacturing Co., Inc., New York, N.Y.) was used. To estimate the initial MOI, the total cell count was found, per unit of turbidity, in a few experiments using a Petroff-Hausser cell-counting chamber (Hausser Scientific).

RESULTS

T4 lysis profile. One simple means of studying LIN is monitoring the lysis profiles (15) of T4-infected cell cultures. Lysis profile assays consist of growing a cell culture to a given concentration, adding phages at a given MOI, and then doing repeated measurements of culture turbidity. LIN is induced in such an experiment merely by ensuring that the infected-cell concentration is sufficiently high that cell turbidity may be readily measured. Under these conditions it is the lysis of part of the infected cell population that supplies the secondary phages necessary for LIN induction.

The lysis profile of T4-infected cells expressing LIN has only been minimally characterized (e.g., see reference 6). To ease understanding of the T4 lysis profiles presented throughout this study, note the following characteristics of the T4 lysis profiles shown in Fig. 1.

(a) Time zero is the point of phage addition. (a to b) Following phage addition, especially noticeable in the lysis profile of cultures infected with a MOI of 0.1 (MOI 0.1 cultures), a rise in culture turbidity is typically observed. This rise in turbidity is associated with an increase in cell number. This increase presumably results from the division of uninfected cells, as observed following lysis profiles using phase-contrast light microscopy (data presented below). (c) Lysis of T4-infected cells occurs after approximately 25 min under one-step conditions (reference 7 and experiments not presented). At this point a decrease in culture turbidity for MOI 1 to 20 LIN cultures is observed. However, this first lysis turbidity decline is not observed in MOI 0.1 LIN cultures. This discrepancy may be interpreted as follows. The first lysis turbidity decline is probably associated with the lysis of a fraction of the infected cell population at the end of their normal (not lysis-inhibited) latent period. In higher-MOI cultures this lysis is visible as a decline in culture turbidity because a significant fraction (MOI = 1) or a great majority (MOI > 1) of cells in these cultures were infected soon after phage addition. In MOI 0.1 LIN cultures, on the other hand, only about 5% of the cells should be infected just before the end of the normal T4 latent period (MOI of 0.1 plus a doubling of uninfected cells over the course of a normal T4 latent-period length). Thus, a first lysis turbidity decline may not be observed in MOI 0.1 cultures simply because even the simultaneous lysis of all the infected cells in the culture may lead to only about a 5%

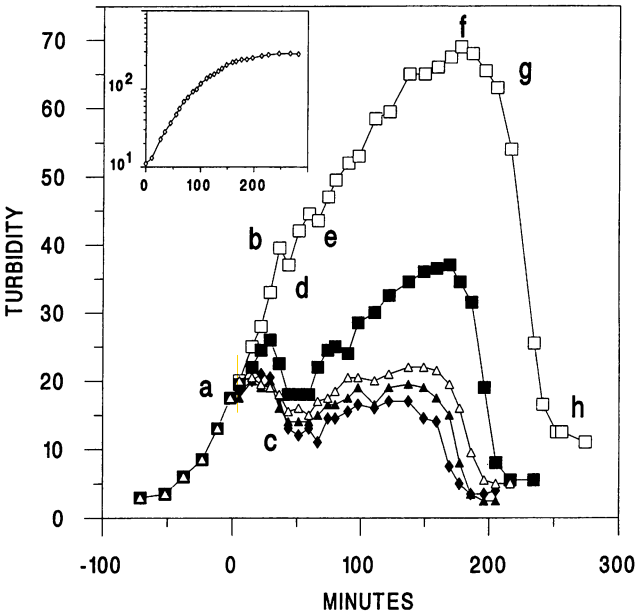

FIG. 1. Lysis profiles with a MOI varying from 0.1 to 20. Primary phages at a given MOI were added at time zero to bacterial suspensions when culture turbidity reached 20: MOI of 0.1 (open squares), MOI of 1 (closed squares), MOI of 5 (closed diamonds), MOI of 10 (closed triangles), and MOI of 20 (open triangles). Different features of curves are shown as follows: (a) phage addition, (a to b) cell division, (c) first lysis turbidity decline, (d) phage adsorption turbidity decline, (e) second lysis turbidity decline, (e to f) LIN turbidity rise, (f) maximum turbidity, (f to g) initial LIN turbidity decline, (g to h) LIN collapse, (h and on) turbidity stabilization, and (inset) *E. coli* S/6/5 growth curve.

decrease in culture turbidity. Adsorption and infection of uninfected cells and secondary adsorption of cells that are infected but not yet lysed presumably occur because of first lysis and progeny release. (d) A decline or stabilization of culture turbidity for MOI 0.1 LIN cultures is often observed soon after the first lysis turbidity decline seen in higher-MOI LIN cultures. This phage adsorption turbidity decline is presumably a consequence of the adsorption by progeny phages released at first lysis (6). (e) At a point about two one-step latent-period lengths following phage addition in MOI 0.1 culture lysis profiles, a second decline or stabilization of culture turbidity is observed. Often, though, there is no apparent turbidity rise between the phage adsorption turbidity decline and this second lysis turbidity decline. Lysis of at least some cells adsorbed during phage adsorption turbidity decline presumably occurs during the second lysis turbidity decline. Given a sufficiently large burst size, the resulting progeny release should lead to the secondary adsorption of most of the remaining phage-infected cells. (e to f) A rise in culture turbidity is seen at the end of the turbidity declines described above. The explanation for this LIN turbidity rise is not known. However, it presumably represents an increase in the turbidity associated with individual infected cells. For instance, as dealt with in more detail below, LIN culture turbidity continues to rise after the majority of cells are presumably infected, while cell concentration remains constant or declines. Operationally, the LIN turbidity rise may be considered to begin with an uninterrupted turbidity rise following the first or second lysis turbidity decline (depending on the initial MOI) and end

when culture turbidity reaches a maximum. (f) Maximum turbidity is the peak in the lysis profile. Roughly, the LIN latent-period length is the period beginning with the infection of most of the cells in a LIN culture and ending when the LIN culture has reached maximum turbidity. (f to g) Following maximum turbidity, LIN cultures begin a slow initial LIN turbidity decline. (g to h) This initial LIN turbidity decline is followed by a steeper drop in turbidity: LIN collapse. It is LIN collapse, representing the lysis of most of the T4-infected cells in a LIN culture, which is analogous to the rapid clearing of bottle lysate LIN cultures often seen during T4 stock preparation. (h and on) After LIN collapse, culture turbidity stabilizes and, over time, often rises. This turbidity stabilization marks the culmination of cell lysis observable by culture turbidity measurement.

Varying initial MOI. In Fig. 1, just described, lysis profiles associated with LIN cultures initiated with MOIs ranging from 0.1 to 20 are compared. This is done as a first step in defining what parameters must be strictly controlled to render lysis profiles highly reproducible and conveniently revealed. Note the following observations: (i) lysis profiles can be of varying shape, (ii) MOI 5 through 20 culture lysis profile shapes cluster about each other, (iii) LIN latent-period lengths are of similar duration for all five MOIs tested, and (iv) the MOI 0.1 LIN culture shows the most dramatic LIN turbidity rise and LIN collapse.

A comparison of lysis profiles when MOIs range from 0.05 to 0.4 has also been done (not shown). MOI 0.1 and 0.2 culture lysis profile shapes are similar. The LIN latent-period lengths of MOI 0.1 through 0.4 LIN cultures are also similar. The MOI 0.05 LIN culture latent-period length, in contrast, was often found to be longer than that seen with the MOI 0.1 through 0.4 LIN cultures. Thus, MOI 0.1 or 0.2 LIN cultures give consistent LIN latent-period lengths and lysis profile shapes, plus dramatic LIN collapse.

Varying culture turbidity. When the initial MOI was kept constant, LIN culture latent-period length did not differ greatly when phage addition was made at culture turbidities ranging from 5 to 40 (not shown). However, LIN cultures allowed to reach higher turbidities before phage addition (especially 30 and greater) showed considerable lengthening of the period of initial LIN turbidity decline. This lengthening may be tied to changes in culture physiology at these turbidities. In particular, with uninfected-cell growth curves a turbidity of approximately 100 defines the end of exponential *E. coli* S/6/5 growth (Fig. 1). Thus, under the conditions employed, initial cell turbidities ranging from 10 to 20 have the experimental advantage of (i) short periods of initial LIN turbidity decline, (ii) dramatic LIN collapse, (iii) potentially less complex infected-cell physiologies, and (iv) relatively short total experimental duration.

Varying MOSI. LIN is initiated by secondary adsorption, and it is of interest to determine what influence explicitly adding secondary phages to LIN cultures has on lysis profile shape. For these experiments an initial MOI of five was used. Fifteen minutes after primary phage addition a second phage aliquot was added. For consistency with Bode (4), here the term MOSI will be used to describe the multiplicity of these secondary phages. Still, it is understood, since T4 phages express superinfection exclusion (references 5 and 14 and references cited therein), that MOSI more accurately refers to the multiplicity of secondary adsorption. In the experiments presented in Fig. 2, the MOSI is varied from 5 to 40. As can be seen, LIN latent-period lengths tend to be longer when MOSIs are greater than zero. In addition, MOSI 5 through 20 culture lysis profile shapes routinely cluster,

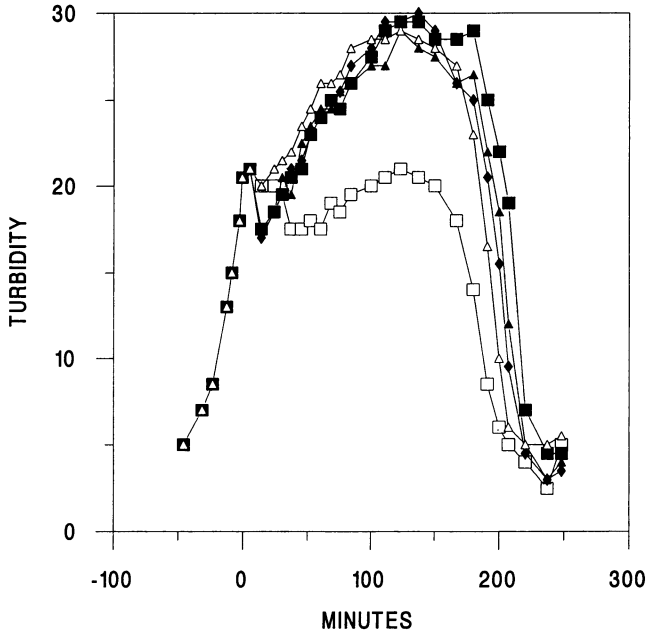

FIG. 2. Lysis profiles with a MOSI varying from 0 to 40. A single, parental LIN culture was initiated with a MOI of five at a turbidity of 20 (time zero). This culture was divided, and T4D secondary phages at a given MOSI were added to each resulting LIN culture 15 min after the initiation of the parental LIN culture: MOSI of 0 (open squares), MOSI of 5 (closed squares), MOSI of 10 (closed diamonds), MOSI of 20 (closed triangles), and MOSI of 40 (open triangles).

and LIN latent-period lengths are of similar duration for all MOSI non-0 culture lysis profiles. MOSI 40 LIN cultures, however, often failed to reach as high a turbidity as the MOI 5 through 20 culture lysis profiles (i.e., in repeat experiments not presented).

Synchronized lysis. In the experiments already presented, exploring the basic biology of T4 growth and lysis at high culture densities, T4 lysis profile shape is shown to be influenced by conditions found at the beginning of phage infection: MOI, MOSI, and culture turbidity. Do culture conditions toward the end of the LIN latent period also influence lysis profile shape? To explore this question, two bacterial cultures were initiated 40 min apart (denoted early and late LIN cultures in the legend to Fig. 3). MOI 0.2 infections were established, also approximately 40 min apart. The two cultures were then mixed after the occurrence of the second lysis turbidity decline in the late LIN culture. If the culture conditions toward the end of the LIN latent period have no influence on lysis profile shape, then the lysis of these mixed LIN cultures should proceed as follows: (i) LIN collapse should begin simultaneously in both the mixed and early LIN cultures, (ii) the mixed LIN culture turbidity should temporarily stabilize at an intermediate turbidity, reflecting a 40-min delay in the lysis of the infected cells derived from the late LIN culture, (iii) this turbidity should stay stable or rise until the time of LIN turbidity decline of the late LIN culture, and (iv) the late LIN culture and the mixed LIN culture lysis profiles should then overlap with the occurrence of late culture LIN collapse. However, as presented in Fig. 3, mixed LIN cultures show a timing of LIN collapse intermediate to that seen with either pure LIN culture. In addition, no intermediate turbidity stabilization

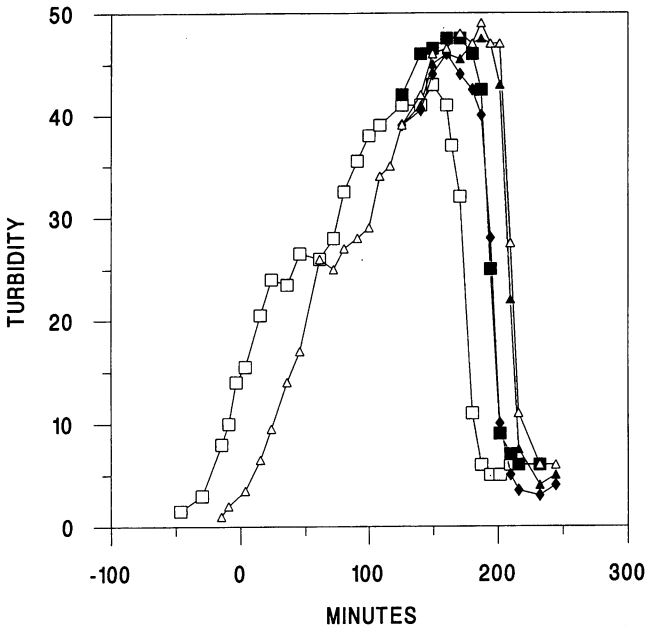

FIG. 3. Lysis profiles of mixed LIN cultures. Phages were added at a MOI of 0.2 at a culture turbidity of 15 to two bacterial suspensions inoculated with cells 40 min apart (time zero is at phage addition to the early LIN culture). After the second lysis turbidity decline of the late LIN culture, early and late LIN cultures were mixed with the following ratios (early volume/late volume): 1:0 (open squares), 3:1 (closed squares), 1:1 (closed diamonds), 1:3 (closed triangles), and 0:1 (open triangles).

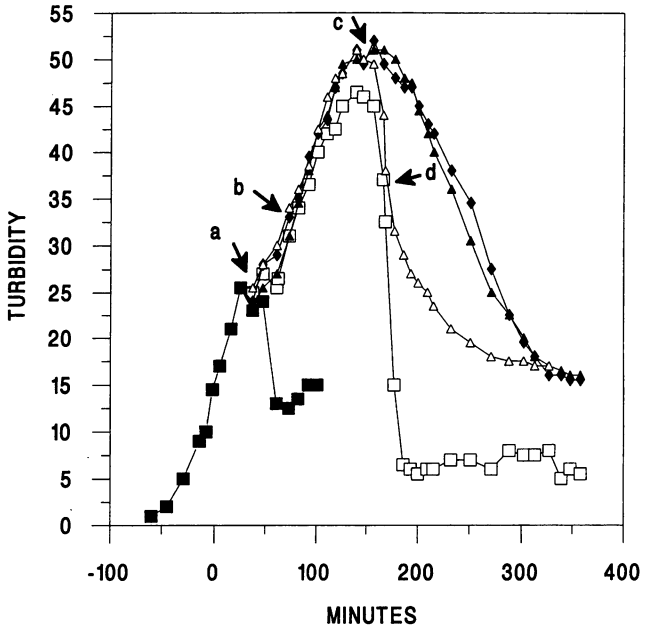

FIG. 4. Lysis profiles following anti-T4D addition. A single LIN culture was initiated with a MOI of 0.2 when turbidity reached 15 (time zero). Individual LIN cultures were drawn from this parental LIN culture, and then anti-T4D was added with a 10^{-2} final dilution at various points in the lysis profile, as shown by arrows and as follows: parental LIN culture with no anti-T4D added (open squares), addition during phage adsorption and second lysis turbidity decline (closed squares; arrow a), addition early in LIN turbidity rise (closed diamonds; arrow b), addition late in LIN turbidity rise (closed triangles; arrow c), and addition during LIN collapse (open triangles; arrow d).

occurred. Thus, the mixing of two LIN cultures appears to result in a synchronization of their LIN collapse.

Inactivating antiserum addition. In reference 2 it is argued that secondary phage adsorption can serve as a signal between lysing cells and infected cells found in the same environment. If this secondary adsorption signal synchronizes LIN collapse (in unmixed as well as mixed cultures), then it may be predicted that addition of sufficient T4-inactivating antiserum (rabbit anti-T4D) to a LIN culture will inhibit that synchronization. This prediction was tested, and the results are presented in Fig. 4. Addition before the occurrence of much second lysis (arrow a) resulted in an abrupt drop in culture turbidity, suggesting that the quantity of anti-T4D added was sufficient to interfere with the induction of the LIN state. Addition of anti-T4D after the second lysis turbidity decline (arrow b) had no immediate effect on culture turbidity. Nonetheless, it and anti-T4D addition just before maximum turbidity (arrow c) resulted in both a delay in the onset of LIN turbidity decline and a reduced rate of LIN collapse. Anti-T4D addition during LIN collapse (arrow d) also resulted in an inhibition of LIN collapse. The rate (i.e., steepness) of LIN collapse is presumably a measure of the degree of synchronization of LIN collapse. Thus, these results suggest that, when not inhibited by added anti-T4D, secondary adsorption occurring during LIN turbidity decline plays a role in the synchronization of LIN collapse.

Similar results were seen with *E. coli* B, B/5, CR63, CR63 lambda, and K594 lambda (data not shown), although it should be noted that some of these strains (i.e., B/5, CR63, and K594 lambda) showed much shallower LIN collapse than seen here with the *E. coli* S/6/5 host. This problem is perhaps similar to that seen with T4-infected LIN culture lysis profiles by Doermann (6). When Casamino Acid-enriched H broth rather than unenriched H broth was used as the growth medium, LIN cultures hosted by these *E. coli* strains displayed more pronounced LIN collapse. It was under these nutrient-enriched conditions that anti-T4D interference with the LIN collapse while *E. coli* B/5, CR63, and K594 lambda hosts were used was observed (experiments not presented).

Adsorption-incompetent progeny phages. The anti-T4D experiments presented above suggest that secondary adsorption synchronizes LIN collapse. Consistently, one would predict that LIN cultures producing progeny phages that are structurally incapable of adsorbing to unlysed cells also should display aberrant and delayed lysis. Phages containing an amber mutation in gene *37* [*amN91*(37)] infecting an *E. coli* S/6/5 host (amber suppressor minus) produce progeny phages lacking functional long tail fibers required for T4 adsorption. However, *amN91*(37) phage stocks were grown using an *E. coli* CR63 host (amber suppressor plus) to ensure the adsorption competence of those primary (i.e., initially infecting) and secondary *amN91*(37) phages added to cultures. Initiation of infections with a MOI of five followed by a MOSI of 10 at 15-min intervals allows *amN91*(37) phage-infected cells to express LIN (i.e., as in Fig. 2). Adsorption-competent *amN91*(37) secondary phages were used (rather than T4D) to ensure that *amN91*(37) primary phage-infected cells could not receive a functional gene *37* from secondary phages. For consistency, T4D primary phage-infected cells were adsorbed with secondary phages drawn from the same *amN91*(37) stock.

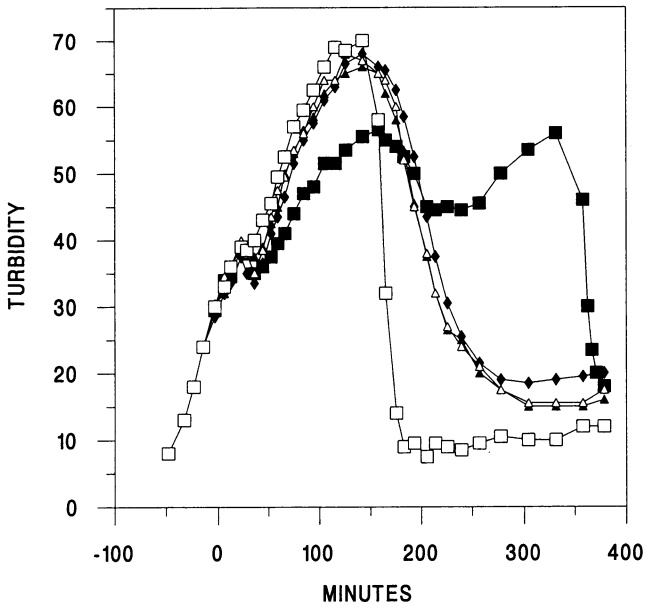

FIG. 5. Lysis profiles of cells infected with *amN91*(37) phage. Phages were added at a MOI of five to five bacterial cultures at a turbidity of 20 (time zero). Secondary phages were added at 15-min intervals following LIN culture initiation. Primary phages were either T4D or *amN91*(37), but all secondary phages were *amN91*(37) [added *amN91*(37) stocks were grown with an *E. coli* CR63 amber suppressor-plus host]. Curves consist of the following: (i) T4D primary phage plus 15-min *amN91*(37) secondary phage addition (open squares), (ii) *amN91*(37) primary phage plus 15-min secondary phage addition (closed squares), (iii) LIN culture ii plus 30-min secondary phage addition (closed diamonds), (iv) LIN culture iii plus 45-min secondary phage addition (closed triangles), and (v) LIN culture iv plus 60-min secondary phage addition (open triangles).

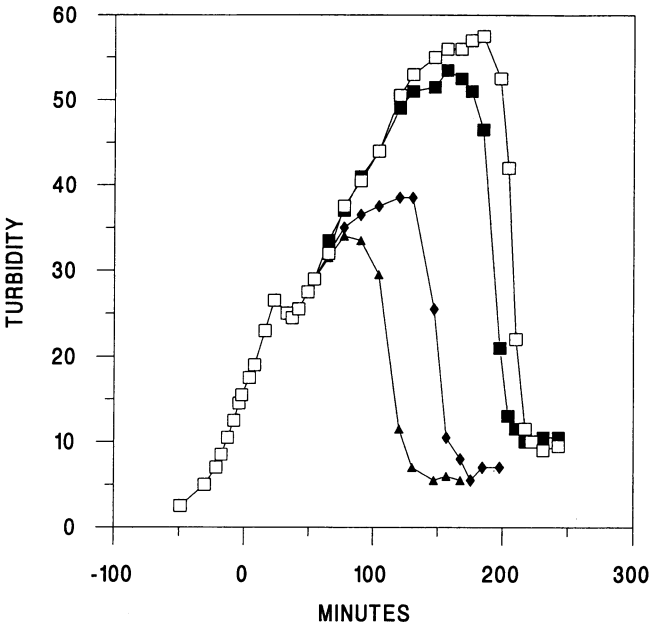

FIG. 6. Lysis profiles with high-MOSI secondary phage addition. A parental LIN culture was initiated with a MOI of 0.2 when turbidity reached 15 (time zero). Secondary phages were added approximately 30 min after the beginning of the LIN turbidity rise period: parental LIN culture with a MOSI of zero (open squares), MOSI of 30 (closed squares), MOSI of 50 (closed diamonds), and MOSI of 70 (closed triangles).

As shown in Fig. 5, although the cultures initiated with *amN91*(37) primary phages reach maximum turbidity only slightly later than the T4D-infected control, *amN91*(37) phage-infected LIN cultures undergo a much shallower LIN collapse. More striking, *amN91*(37) phage-infected LIN cultures receiving only one MOSI 10 secondary adsorption reliably showed a recovery in turbidity rise following what appeared to be the beginning of LIN collapse. This second turbidity rise could represent a continued increase in turbidity of the infected cell population not sufficiently damaged by secondary adsorption to lyse with the rest of the phage population. However, this explanation fails to address why this LIN culture should then undergo such a steep second LIN collapse. Nevertheless, the fact that *amN91*(37) phage-infected LIN cultures lyse at all, along with the anti-T4D experiments presented in Fig. 4, suggests that secondary adsorption toward the end of the LIN latent period is not required for LIN collapse. Notwithstanding, both the *amN91*(37) phage and anti-T4D experiments suggest that secondary adsorption occurring early during the LIN latent period influences the timing of LIN collapse.

High-MOSI adsorption. If early secondary adsorption influences the timing of lysis of LIN cultures, then secondary adsorption of a sufficient MOSI during LIN turbidity rise may result in a premature induction of maximum turbidity and LIN turbidity decline. In this experiment, to the extent that infected-cell lysis occurring in LIN cultures leads to secondary adsorption before the explicit addition of secondary phages to these cultures, such explicit secondary adsorptions may be considered tertiary adsorptions. As presented in Fig. 6, secondary phages were added, at MOSIs of 30, 50, and 70, approximately 30 min after the start of the period of LIN turbidity rise. Addition of secondary phages at a MOSI of 70 resulted in turbidity stabilization followed by early induction of LIN turbidity decline. Secondary adsorptions with MOSIs of 30 and 50 also resulted in a truncation of the period of LIN turbidity rise. These latter LIN cultures, especially, took sufficiently long to reach LIN turbidity decline that it is not likely that the secondary phages added simply induced lysis from without (e.g., see reference 5). Thus, just as blocking secondary adsorption appears to delay LIN collapse (Fig. 4 and 5), artificially enhancing the MOSI apparently accelerates LIN collapse; both results are consistent with secondary adsorption limiting LIN latent-period length.

Determination of lysis profile by total cell count. Lysis profiles were established with both cell total count and turbidity measurements. As shown in Fig. 7, the total cell count declines throughout the LIN latent period. Thus, during the LIN turbidity rise the turbidity associated with individual T4-infected cells must increase more rapidly than that of the culture as a whole (nevertheless, no indication of significant infected-cell lengthening occurring over the course of LIN lysis profiles was observed). This decline in total cell count suggests that T4-infected-cell lysis, progeny phage liberation, and therefore secondary adsorption occur in LIN cultures throughout the LIN latent period.

Testing alternative hypotheses. Besides the effect of secondary adsorption on the induction of LIN turbidity decline, it also may be speculated that environmental change could play a role in LIN culture lysis, especially given the caveats

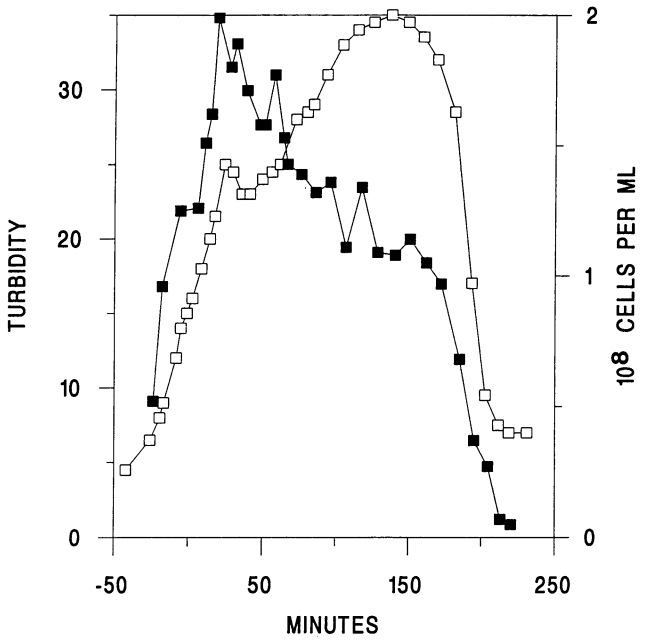

FIG. 7. Lysis profiles using both turbidity and total cell number measurements. A single LIN culture was initiated at a MOI of 0.2 when turbidity reached 15 (time zero) and was monitored by measurements of either turbidity (open squares) or total cell count (closed squares).

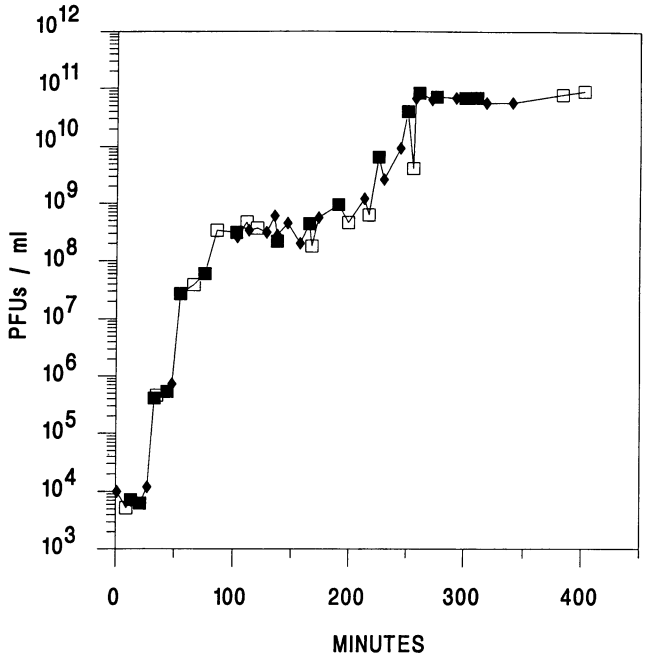

FIG. 8. Phage titers during phage growth in a bottle lysate. Cells were grown with bubbling for aeration and to effect mixing in a 200-ml volume until they reached a concentration of approximately 10^7/ml. At this time (time zero) sufficient phages to give a final concentration of approximately 10^4/ml were added. Phage titers were found by the soft-agar overlay method. Shown are results of three independent experiments.

associated with Fig. 4 and 5, discussed above. Such environmental changes include (i) oxygen limitation, (ii) nutrient limitation, and (iii) buildup of metabolic poisons. An attempt to avoid oxygen limitation was made throughout these experiments as described in Materials and Methods. While it is possible that these conditions were not sufficient to ensure reliable oxygenation of LIN cultures, the following observations suggest that LIN turbidity decline is not a consequence solely, if at all, of oxygen starvation: (i) the LIN latent-period length is highly reproducible between flasks (in particular, see reference 2), (ii) the LIN latent-period length is similar between LIN cultures of different turbidities (discussed above and data not shown), and (iii) longer rather than shorter initial LIN turbidity decline periods are seen with LIN cultures of higher turbidity. Indeed, nutrient decline, buildup of metabolic poisons, and oxygen limitations should all be accentuated at higher infected-cell concentrations. Therefore, points ii and iii also argue against the merits of nutrient limitation or buildup of metabolic poisons as an adequate explanation for the induction of LIN turbidity decline. Further evidence that nutrient limitation does not play a significant role in the initiation of LIN collapse is the observation that the lysis of LIN cultures grown in H broth media supplemented with an excess of H broth, Casamino Acids, or yeast extract showed relatively small variation in LIN latent-period length (not shown). In addition, pH changes little over the course of a lysis profile, dropping 0.1 pH unit or less during the LIN latent period (data not presented).

Bottle lysate lysis profile. This study began with the observation that T4 bottle lysates show a steep decline in turbidity apparently upon phage lysis (2). In the experiments presented, this LIN collapse was defined under more controlled and measurable conditions. To the extent that relatively uncontrolled bottle or flask lysate T4 growth mimics the growth characteristics of T4 phages in the wild (reviewed in references 1 and 2 and below), it is the kinetics of the release of progeny phages that define the relevance of LIN collapse. In Fig. 8 a T4 bottle lysate monitored by counting PFU is presented. Note (i) the exponential rise in PFU until approximately 90 min into growth, (ii) a leveling of PFU presumably associated with the expression of LIN by these cultures, (iii) a slow and then accelerated rise in PFU presumably representing the lysis of infected cells (see also reference 6), and (iv) a leveling of PFU presumably associated with a resulting dearth of cells, infected and uninfected, to which progeny phages may adsorb.

DISCUSSION

Fortunately, for the experimental observation of LIN, lysis profiles observed with a given T4 population initiated over a range of MOIs tend to be of consistent shape (Fig. 1 and, especially, low-MOI experiments described but not presented). Similarly, the induction of LIN by the addition of exogenous secondary phages also results in consistent lysis profile shape over a range of MOSIs (Fig. 2). LIN collapse during these experiments is very rapid, implying a synchronized lysis of infected cells in LIN culture. This synchronized LIN collapse implies that individual infected cells "know" when to lyse such that all or most of the infected cells in a culture lyse at approximately the same time. In a synchronously infected phage culture, such rapid culture lysis is expected, given no expression of LIN (7), because phages tend to have non-lysis-inhibited latent-period lengths of a given, intracellularly defined duration.

Simplistically, then, one may suppose that lysis-inhibited infected cells also have an intracellularly defined latent-period length and therefore synchronized LIN collapse occurs because each infected cell intrinsically knows when to lyse. However, by this reasoning T4 phages must have evolved two intracellular mechanisms timing their lysis, one used when LIN is not induced and the other used when LIN is induced. Indeed, it is likely that two such mechanisms exist but unlikely that the mechanism of synchronized LIN collapse is controlled solely intracellularly.

For example, the synchronized LIN collapse of a mixture of two LIN cultures initiated at different times (Fig. 3) suggests that the timing of the lysis of individual infected cells expressing LIN is under the control of some factor in the extracellular environment. The ability of experimental protocols designed to interfere with secondary adsorption to also interfere with LIN collapse (Fig. 4 and 5) suggests that this extracellular factor is secondary adsorption. Similarly, premature LIN collapse may be induced by the explicit tertiary addition of a high MOSI of phages (Fig. 6). Normally such tertiary phages are likely made available in LIN cultures by the gradual lysis of some fraction of the infected cell population (Fig. 7 and 8). Thus, the lysis of a fraction of infected cells releases progeny phages that initially induce LIN but ultimately induce LIN collapse. Below is an exploration of the ecological context in which synchronized LIN collapse evolved plus a more detailed model of what may be occurring mechanistically during synchronized LIN collapse.

T4 colonic life history. LIN and the shorter T4 latent-period length when LIN is not expressed are probably both adaptations to growth in the presence of high concentrations of host cells (1, 2). Particularly, the chemical adsorption requirements of T4 phages are consistent with their natural growth environment being the colonic lumen of animals (2a). There, high *E. coli* concentrations are typically found. On the other hand, the chemical adsorption requirements of T4 phages are inconsistent with their adsorption in extracolonic environments such as sewage. Also, high extracolonic *E. coli* concentrations are rarely found.

A hypothetical life history of T4 phages is graphically presented in Fig. 9. There, six phases of T4 growth in the colonic lumen of animals are highlighted: (up to a) pregrowth search (1), (a to b) exponential growth (1), (b to c) lysis inhibition (2), (c to d) LIN collapse (this study), (d to e) dispersal (2a), and (e and on) phage-cell equilibrium (8). The pregrowth search begins when, either via passage from the mouth or through host range mutation, a phage particle with a novel host range enters the colonic lumen. Only when the cell concentration is sufficiently high is it likely that cell adsorption occurs. Given successful adsorption, exponential T4 growth may follow. Once most of the cells in the environment have been phage infected, exponential T4 growth ceases and, if the infected-cell concentration is sufficiently high, LIN is induced. LIN collapse is a decline in the infected-cell concentration and an associated rise in the free-phage concentration. Dispersal of free phages to extracolonic environments should occur with some rapidity through peristaltic action. Rather than completely dispersing out of the colonic lumen, phages and phage-sensitive cells might establish long-term cocultures (phage-cell equilibria), as they often do in chemostats.

Synchronized LIN collapse. Superinfection exclusion is a mechanism by which secondary phages are allowed to adsorb irreversibly (suicidally!) to T4-infected cells but are prevented from contributing genetically to the progeny

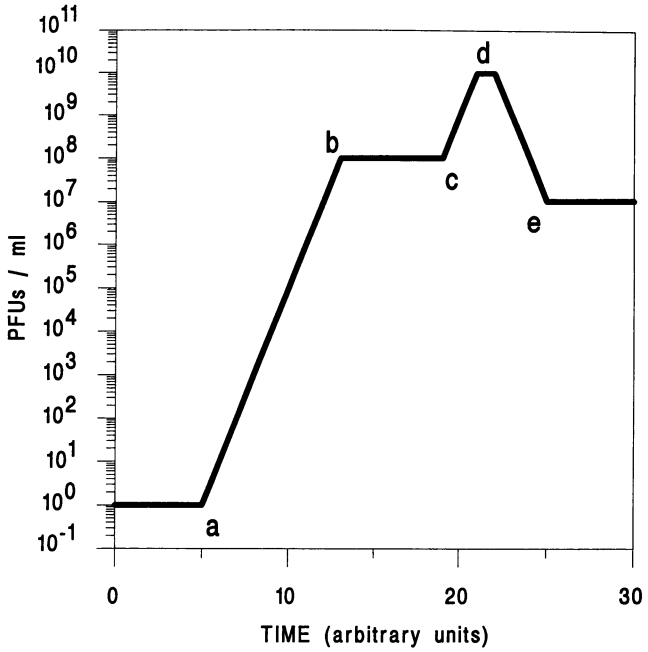

FIG. 9. T4 phage colonic lumen life history. Summary of T4 growth in the colonic lumen of animals with various hypothetical phases of T4 growth highlighted as described in the text: (until a) pregrowth search, (a to b) exponential growth, (b to c) LIN, (c to d) LIN collapse, (d to e) dispersal, and (e and on) phage-cell equilibrium.

phage burst (i.e., as measured by the exclusion of secondary phage markers from the primary phage progeny burst, wild-type primary phages exclude 98.6% ± 0.6% of secondary phages after only 4 min of infected-cell metabolism [2a; see also reference 10 and references cited therein]). At the high infected-cell concentrations at which LIN is expressed, as in the experiments presented here, the probability that a free progeny phage will adsorb to an infected cell is high. Thus, LIN infected cells that lyse early, compared with the rest of the infected cell population, expose their progeny to superinfection exclusion and, consequently, to a high probability of death. In contrast, an infected cell that delays lysis until the infected-cell concentration is low should show a much higher probability of progeny survival. This is because, after delayed lysis, infected cells expressing superinfection exclusion should be scarce (2). Nonetheless, delaying lysis indefinitely precludes the dispersal of progeny phages. Ideally, then, a T4-infected cell could monitor its environment sufficiently that lysis may be delayed past, but not too far past, the time neighboring cells have lysed. Environmental monitoring implies some degree of sensory awareness. Detection of the occurrence of secondary adsorption is an obvious, precedented (2), and perhaps inescapable mechanism by which environmental monitoring might occur.

In T4's colonic lumen growth environment, high host cell concentrations lead to a high probability of secondary adsorption as uninfected cells become scarce. This secondary adsorption induces LIN. Because T4-infected-cell concentrations are high during the expression of LIN, selection favors maintenance of the LIN state whether or not infected cells can still produce progeny phages intracellularly. For reasons not fully understood (e.g., reference 4 and references cited therein), some infected cells are not able to

maintain this LIN state and these cells lyse. The progeny from these lysing infected cells adsorb to infected cells still present in the environment. This cumulative secondary adsorption weakens the cell envelope of these infected cells, despite the expression of T4 genes *imm* and *sp*, which normally protect the infected-cell envelope from secondary adsorption-induced damage. Eventually, secondary adsorption weakens the cell envelope sufficiently that additional secondary adsorptions lead to lysis from without. Alternatively or additionally, secondary adsorption occurring early in the LIN latent period may cause or initiate damages that by themselves lead to cell envelope instability later in the infection, with or without additional secondary adsorptions. Furthermore, to the extent that secondary phages might bypass superinfection exclusion during the expression of LIN, it might also be consistent with the data that phenotypic expression by superinfecting phage DNA plays a role in LIN collapse.

These envelope instabilities may lead to an interruption of infected-cell metabolism (trauma?) and the induction of premature lysis such as that seen when T4-infected cells are treated with KCN (reviewed by Young [15]). The secondary adsorption load experienced by infected cells residing in the same environment should be similar such that secondary adsorption-induced lysis becomes significantly more likely at approximately the same time across the T4-infected cell population. Increasing cell lysis leads to an upsurge in secondary adsorption-induced lysis, resulting in a synchronized lysis of the T4-infected cell population. These progeny phages are released into an environment containing lowered numbers of infected cells expressing superinfection exclusion. Thus, synchronized LIN collapse may act to increase the probability that any given infected cell produces progeny that both are released in a timely manner and have higher likelihoods of not secondarily adsorbing other T4-infected cells.

To some degree it is a function of suicidal secondary phages, rather than infecting primary phages, which induces this synchronized lysis. Therefore, as far as synchronized lysis represents an evolved trait it may also represent evolved cooperation between the progeny phages produced by a single infected cell (especially in a poorly mixed environment) or the clonally related T4 phage-infected cells replicating in a given LIN culture. Thus, one may postulate that the lytic nature of the T4 virion particle evolved in full or in part to assist in the lysis of lysis-inhibited, T4-infected cells to which they adsorb.

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