Replication of Coliphage M-13¹

I. Effects on Host Cells After Synchronized Infection

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Techniques have been described for synchronization of bacteriophage M-13 infection of host cells. The latent period in infected cells was 10 min, and no appreciable number of intracellular phage was observed. Phage production proceeded in three phases after release of the starvation block: an initial rapid exponential rate of progeny phage release without cell lysis, a period of rate transition accompanying the resumption of host cell division, and a second, slower exponential rate of phage production which paralleled the rate of host cell division. The size of infected cells was not affected by infection, but the generation time was increased by 25%. Starved infected cells exhibited a much longer lag in attaining an exponential rate of growth upon the addition of nutrients than did an uninfected control culture.

Some of the growth characteristics of cells infected with small filamentous coliphages have been reported (12, 14, 20). These phages all exhibit the unusual property of release from the host cell without lysis while cell growth continues (2, 4, 9, 12, 14, 16, 18). Optical density measurements of cells infected with coliphage have indicated a lowered growth rate when compared to measurements of an uninfected culture (9, 12, 17, 20). Similar results have been reported for *Vibrio* and *Pseudomonas* filamentous phages (10, 11). The magnitude of the decrease in growth rate varies with the multiplicity of infection (10, 12), the phage, the host cell, and the medium employed. Ray, Bscheider, and Hofschneider (13) have reported an increase in generation time from about 36 or 38 min to 70 min after infection of *Escherichia coli* K-12 W945F⁺ growing in a supplemented minimal medium.

Adsorption of the filamentous phages to host cells is slow (8, 17), and the proportion of infected cells is small during the initial stages of infection (12). This small number of infected cells may account for the failure of some investigators to observe larger growth rate differences.

The purpose of this paper is to: (i) describe a method for synchronizing the infection with M-13 by starvation, (ii) to determine the effect of such an infection on the growth rate of infected cells

maintained at low cell density, and (iii) to assess the effect of infection on cell size.

MATERIALS AND METHODS

Phage and bacteria. Coliphage M-13 was obtained from David Pratt, University of Wisconsin, Madison.

E. coli strain S-26, originally described by Garen and Siddiqui (6), was furnished by R. L. Sinsheimer, California Institute of Technology, Pasadena.

Media. KC broth, top agar, and bottom agar have been described previously (5). The dilution buffer contained 7 g of NaHPO₄, 3 g of KH₂PO₄, and 4 g of NaCl per liter of distilled water. The starvation buffer contained 4 g of (NH₄)₂HPO₄, 2.3 g of NH₄H₂PO₄, and 4.67 g of NaCl per liter of water, adjusted to pH 6.8. Media and buffer used in electronic counting were filtered through membrane filters (0.80, 0.45, and 0.22 μ m pore size; Millipore Corp., Bedford, Mass.) to remove particulate matter.

Preparation of purified M-13. *E. coli* S-26 was grown with aeration to a concentration of about 3×10^8 viable cells per ml in KC broth at 37 C. A portion of the culture was added to melted top agar at 46 C to give a final concentration of approximately 3.5×10^7 viable cells per ml, and M-13 bacteriophage was added immediately to a final concentration 10^8 plaque-forming units per ml. A Cornwall pipette was used to rapidly dispense 2.5 ml of the top agar onto previously poured bottom-agar plates. These agar-overlay plates were allowed to stand for 10 min at room temperature, and were then incubated overnight at 37 C.

After incubation, the top agar layers were removed with a sterile spatula, and the plates were washed with 3 to 4 ml of KC broth. The top agar and washings were combined and gently agitated on a Vortex mixer. The mixture was then centrifuged at $23,000 \times g$ in a Sorvall refrigerated centrifuge at 4 C for 40 min.

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The sediment, which contained less than 1% of the total phage, was discarded. The supernatant fluid was centrifuged in a Spinco no. 30 rotor for 16 to 18 hr at 28,000 rev/min at 5°C. The supernatant fluid containing >10% of the total phage infectivity was removed and discarded. The pellets were suspended in a small volume of the dilution buffer and combined. The cell debris and residual agar were removed by centrifugation at $12,100 \times g$ for 15 min. The bluish, highly viscous supernatant fluid was removed, and solid CsCl (Harshaw optical grade) was added (0.46 g/g of solution) to a final mean density of 1.29 ± 0.3 g/ml. This material was then placed in a Spinco SW-39 rotor and spun for 44 hr at 37,000 rev/min at 5°C. The density gradient showed a bluish gelatinous phage band located in the center of the gradient. The gradient was collected (30 drops per fraction into 1 ml of dilution buffer) by use of the Beckman density gradient fractionating system. Most (95 to 99%) of the infectious phage particles were found in one or two fractions. These peak fractions were combined and further purified as desired in additional equilibrium density gradients. The phage were then dialyzed against four changes (>100 volumes) of dilution buffer.

A preparation of 100 agar-overlay plates routinely yielded 10^{15} to 2×10^{15} purified infectious M-13 bacteriophage, or about 10^{13} phage per plate.

Starvation synchronization of infection. *E. coli* S-26 was grown in KC broth to 10^9 cells per ml at 37°C with light aeration to assure maximal piliation. Cells were then centrifuged, washed twice with starvation buffer, resuspended, and incubated in 0.1 volume of starvation buffer at 37°C for 90 min. M-13 coliphage was subsequently added at an input multiplicity of infection of 10 and allowed to adsorb for 90 min. The cells were centrifuged at $23,000 \times g$ for 10 min, washed with 10 ml of starvation buffer three times, and suspended in ice-cold KC broth. A dilution into prewarmed KC broth initiated phage development.

Intracellular phage assay. An 0.1-ml sample was added to a tube containing 0.8 ml of 0.033 M tris(hydroxymethyl)aminomethane, pH 8.1, and 0.1 ml of a solution (2 mg/ml) of lysozyme. After 5 min, 1 ml of 0.8% ethylenediaminetetraacetic acid was added, and the mixture was allowed to stand for 10 min. The cells were then alternately frozen in a dry-ice acetone bath and thawed in a water bath (37°C) four to seven times. The samples were then placed in an ice bath and were later assayed for viable phage.

Phage assays. Viable phage or infective centers were assayed on agar-overlay plates as described by Adams (1). The plating bacteria was *E. coli* S-26, grown to a cell density of approximately 3×10^8 cells/ml in KC broth at 37°C, used at 0.3 ml of culture per plate.

Electronic measurement of cell size and estimation of particle concentration. Measurements of cell size and particle concentration were made with the apparatus described by Harvey and Marr (7). The transducer, glassware, and pore were manufactured by Particle Data, Ellenhurst, Ill. Polyvinyltoluene latex spheres (nominal diameter, 1.305 μ m) used for calibration were obtained from Dow Chemical Co., Midland,

Mich. All measurements were made at 1 ma. A 30- μ m drilled ruby pore was used in all experiments. Appropriate dilutions were made to insure count rates and distributions that were not distorted by coincidence. All counts were of 0.02 ml.

Viable cell counts. Viable cell counts were made on pour plates (2 ml top agar) employing the same media as used for plating viable phage.

RESULTS

Synchronization of phage growth. Results of typical experiments made by the method for starvation synchronization of infection are illustrated in Fig. 1. At the time of the initiation of phage development, 95 to 99% of the phage were in eclipse. A comparison of electronic counts to viable counts showed 88% of the infected cells still viable after the 180-min starvation period. The ratio of infective centers to viable cells was used to determine the proportion of infected viable cells. The average ratio of nine separate experiments was 0.71, with a range of 0.61 to 0.81. A latent period of 10 min was observed after release of the starvation block, and the subsequent rise in intracellular plaque counts was accompanied by a simultaneous increase in infective centers. This indicated that the number of intracellular phage accumulated per cell was very small. Phage production 10 min after the release of the starvation block approached an exponential rate and continued for the next 30 to 40 min. This initial phase was followed by a period of rate transition, and a second, slower exponential rate of phage synthesis accompanied the resumption of host-cell multiplication.

Growth of cells infected with M-13. In experiments to determine the effect of infection on the host-cell growth rate, an attempt was made to have cells in the same physiological state. For this reason, cultures were maintained at low cell density ($<10^7$ cells/ml) by periodic dilution for 72 hr. The cells were then allowed to grow to 10^9 cells/ml (Fig. 2), and a portion of the culture was removed and starved as previously described for starvation synchronization of phage infection. This culture was divided and one portion was infected. Figure 3 illustrates the growth of infected and uninfected cultures after they have been resuspended in warmed KC broth.

After an initial lag phase, the starved, uninfected cells resumed the same specific growth rate ($K = 1.58 \text{ hr}^{-1}$) exhibited by the culture prior to starvation, with a cell generation time of 44 min. Infected cells, after an extended lag period, resumed exponential growth with a lower specific growth rate ($K = 1.28 \text{ hr}^{-1}$) and a generation time of 30 min. A comparison of infective centers to viable counts indicated that about 70% of the

FIG. 1. Synchronous infection with M-13. Intracellular and infective centers were assayed at various times after resuspension of starved, M-13-infected *E. coli* S-26 in KC broth at 37 C. Symbols: Δ , infective center assay; $\circ$, intracellular phage assay; $\bullet$, bacteria per ml measured by electronic counts; $\square$, bacteria per ml measured by viable counts.

cells were infected initially, whereas 85 to 95% of the cells were infected after several hours. The 25% increase in generation time was retained in a portion of the infected cells which were diluted when the infected culture was at a cell density of approximately 3×10^7 cells/ml. This dilute, infected culture continued to grow at the same exponential rate.

Determination of cell size. The size distributions of uninfected and infected cells (Fig. 4) were determined on the same samples used for determination of cell numbers. The mean cell size of the uninfected cells (corrected for background) in a steady state at 37 C was $1.2 \mu\text{m}^3$. This size decreased to $0.822 \mu\text{m}^3$ when the cell density reached approximately 10^8 cells/ml. After starvation, the mean was $0.58 \mu\text{m}^3$. Changes in the mean and the mode of the cell size distribution for infected and uninfected cells at various times after infection are depicted in Fig. 5. The increases in cell size of the infected and uninfected cultures were identical for the first 2.5 hr. At this time, the uninfected cells reached a cell density of approximately 10^7 to 3×10^7 , and the cell size began to diminish. The infected culture did not reach this cell density until about 3.5 hr after infection, owing to its

slower rate of division, at which time a decrease in cell size was noted.

Estimation of cell mass. Changes in cell mass obtained by multiplying cell number times mean cell volume are illustrated in Fig. 6. The uninfected culture, after an initial lag, showed an exponential increase in mass for several cell generations. The infected culture showed a longer lag in mass production, but, after about 3 hr, an exponential rate of mass synthesis was obtained. The rate was maintained upon dilution.

DISCUSSION

The rapid rise in intracellular phage production at 10 min after release of the starvation block was used as an index of the success of the synchronization technique. In infections that were poorly synchronized, the increase in phage was more gradual. The release of phage from the host cells during the first few minutes of infection approached an exponential rate. At later times, the analysis was complicated by the resumption of host cell division. This complication prohibits a direct comparison with data obtained by Denhardt and Sinsheimer for ϕX174 (3).

FIG. 2. Normal growth of *E. coli* S-26 in KC broth at 37 C. A culture was maintained at low cell density for 72 hr by periodic dilution and then allowed to grow to stationary phase. Mean cell-size calculations from population size distributions indicated a change from the steady-state mean cell size at a cell density of 10^7 to 3×10^7 cells/ml.

FIG. 3. Growth of *E. coli* S-26 infected with M-13. Cells were infected as described. Growth of the infected and uninfected cultures was followed by electronic measurement of cell numbers. Symbols: $\circ$, uninfected cells; $\bullet$, infected cells; $\blacksquare$, viable infected cells.

The latent period for phage production in starvation-synchronized cells is in good agreement with the latent period reported by Hoffmann-Berling et al. (8), when cells are infected normally with the similar phage fd. The fact that extracel-

FIG. 4. Cell size distribution of M-13-infected and uninfected *E. coli* S-26. Population size distribution of infected cells 180 min after resuspending the starvation-blocked cells in KC broth at 37 C and of an uninfected control 165 min after resuspension. Size distribution corrected for background.

FIG. 5. Changes in cell size of infected and uninfected cultures. The mean and mode of the population cell-size distributions were calculated from the infected and uninfected cultures shown in Fig. 3. Polyvinyltoluene latex spheres (nominal diameter 1.305 μ m) were used to calibrate the electronic volume measurements.

lular phage was detected early in infection can be explained if phage are excreted as rapidly as they are produced. If this is the case, the exponential rate of phage release early in infection could reflect an intracellular exponential rate-limiting step in "early" M-13 replication.

The assay of intracellular phage was complicated by the sensitivity of M-13 to sodium dodecyl

FIG. 6. Effect of M-13 replication on the production of cell mass. The mass of the infected and uninfected cultures was determined by multiplication of the number of cells per milliliter by the mean cell size at the various times shown in Fig. 3. Symbols: $\circ$, mean cell volume (μm^3) times cells per milliliter of the uninfected culture; $\bullet$, mean cell volume (μm^3) times cells per milliliter of the infected culture.

sulfate, as reported for another filamentous phage, ZJ/2 (19), and by the failure of chloroform to give reproducible results. Therefore, a freeze-thaw technique was used which was found to be satisfactory. This technique required cells to be rapidly frozen and thawed, since phage were inactivated when lysates remained frozen for a long period of time.

The number of phage produced during the exponential phase of cell growth corresponded to that reported by Hoffmann-Berling and Maze (9) for fd, but it was considerably less than the 1,000 phage per cell per cell generation reported by Ray, Bschieider, and Hofschneider (13) for *E. coli* K-12 W945F⁺. However, we noted variations in phage production with different host strains and different media. The suggestion by Panter and Symons (12) that AE2-infected *E. coli* cells growing at a slower rate produce more phage per cell per cell generation, and the proposal of Salivar et al. (14) that there is a negative correlation between growth rate and phage production in infected cells in the same culture were not studied.

Changes in the growth rate of cells infected with filamentous phages have been noted by numerous authors. The 25% increase in generation time which we found for M-13-infected cells

was in agreement with the change in growth rate reported for fd-infected cells by Hoffmann-Berling and Maze (9). The increase in generation time was retained at least four to five generations after they were diluted into fresh medium. It was also evident that M-13-infected cells acquired an exponential growth rate much slower than an uninfected control.

When the growth rate of a culture changes, differences are often observed in the size distribution of a cell population. However, we did not observe any significant differences in size distributions between infected and uninfected cells. The infected culture reached a mean cell size equivalent to that of an uninfected steady-state population. Further, the infected culture attained this size as rapidly as the uninfected culture. Recent studies have indicated that filamentous phages produce changes in the cell membranes (15). If this is true for M-13-infected cells under our conditions, these membranous changes probably do not affect the separation of daughter cells, since this would be reflected in a change in the cell size distribution and the mean cell size. The uninfected culture increased in mass after a shorter lag period than the infected culture, and the exponential rate of mass increase was greater than that of infected cells. Since the size of infected and uninfected cells was identical, this difference in the rate of mass synthesis was probably a reflection of a slower rate of cell division in the infected culture.

The calculation of total phage mass produced per cell per cell generation relative to host cell synthesis has been made by Salivar et al. (14) for M-13 and by Hoffmann-Berling and Maze for fd (9). The calculations indicate that the total phage mass synthesized should not account for the decrease in growth rate. Their calculations, however, include neither the amount of intracellular phage-directed synthesis of components not incorporated into mature phage particles, nor material in noninfective phage particles. Until accurate data are available on these factors, the validity of such calculations cannot be assessed.

It may be concluded that one of the major effects of M-13 infection of host cells was an increase in generation time resulting in a reduced growth rate. There was little effect on cell size.

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