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Effects of Temperature and Host Cell Growth Phase on Replication of F-Specific RNA Coliphage Qβ

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Human enteric viruses have been found in groundwater in the absence of fecal coliforms. Because detection of human enteric viruses is costly, time-consuming, and lacking in sensitivity, F-specific RNA (FRNA) coliphages, which infect *Escherichia coli* by attachment to F pili, are being examined for suitability as indicators of human enteric viruses in groundwater. Temperatures and host cell growth conditions that constrain F-pilus expression will limit FRNA coliphage replication in groundwater and wastewater, as is desirable in an indicator. Below 25°C F-pilus synthesis ceases; FRNA coliphage Qβ did not replicate below this temperature in batch cultures. One-step replication studies indicated that the replicative cycle is prolonged and that fewer progeny are released as the temperature decreases. The decreases in phage replication observed in the one-step replication studies were a consequence of fewer cells infected as the temperature was lowered or as host cells entered stationary phase. The numbers of phage particles released from infected cells did not change. The minimum temperature for replication of Qβ, 25°C, is not maintained in wastewater and does not occur in Wisconsin groundwater. On the basis of temperature and host cell growth phase, we have concluded that extensive replication of FRNA coliphages does not occur in wastewater and groundwater in Wisconsin and areas with similar cool climates.

Groundwater is an important source of drinking water in the United States. As it is often used without treatment, groundwater is a major vehicle of waterborne illness, including viral disease (15, 20, 22). During the period from 1986 to 1992, 112 outbreaks (defined by the U.S. Centers for Disease Control and Prevention as two or more similar human illnesses related, as shown by epidemiologic investigation, to the consumption of a common source of water intended for drinking) comprising 48,213 illnesses were reported in the United States. Sixty-nine (61.6%) of these outbreaks, comprising 18,522 individual illnesses, were attributed to groundwater consumption. These included 4 outbreaks (44 illnesses) of hepatitis A, 3 outbreaks (1,374 illnesses) of Norwalk virus-like gastroenteritis, and 47 outbreaks (12,023 illnesses) of acute or chronic gastrointestinal illness, some of which may well have been caused by viruses. During 1989 and 1990, hepatitis A was implicated in two outbreaks associated with the use of well water (15). A Norwalk virus-like agent was implicated in a third outbreak, which involved about 900 people, and was caused by contamination of well water with effluent from a sewage treatment facility at a resort.

Human enteric viruses can migrate into groundwater from on-site wastewater disposal sites (2, 17). Human enteric viruses have been found in groundwater in the absence of fecal coliforms (2, 32), and the numbers of human enteric viruses in water have failed to correlate reliably with the numbers of coliforms (32). The persistence of viruses and *Escherichia coli* in groundwater, wastewater, and sterile wastewater was concluded to be too disparate for bacteria to serve as index organisms for viruses in these aquatic environments (23). Therefore, bacteria are not suitable indicators of human enteric viruses in groundwater.

There is general agreement that direct examination of water for enteric viruses is not practical: the tests are time-consuming, expensive, and difficult to perform (28). Direct detection is further complicated by the lack of appropriate host cell cultures for some epidemiologically significant viruses, including hepatitis A virus, Norwalk virus, and rotaviruses.

An indicator of human enteric virus contamination is needed for monitoring the safety of groundwater. This indicator should be of human fecal origin, should not reproduce in groundwater, and should be present in groundwater in numbers representative of human enteric virus contamination (28). Additionally, it should be similar to human enteric viruses with respect to physical properties influencing behavior in groundwater, nonpathogenic, and easily and rapidly detected.

Coliphages, bacteriophages of *E. coli*, potentially fulfill these and other requirements for an indicator of human enteric viruses in groundwater (9). Coliphages occur in sewage (8) and in groundwater (32). Coliphages behave similarly to enteroviruses in groundwater (27) and during wastewater treatment (5, 18). Coliphages and human enteric viruses, therefore, appear to have similar potentials to enter groundwater systems.

As human enteric viruses are of fecal origin, an indicator coliphage should likewise be associated with human feces or sewage. Some coliphages are more likely than others to be of human fecal origin. F-specific RNA (FRNA) coliphages (e.g., MS2, R17, and Qβ) have been recommended for modeling viral behavior in water (11). Infection by these phages of F^+ *E. coli* host cells is initiated by attachment to the F pili, which are synthesized only under certain conditions of growth of the host. F pili are not produced below 25°C and are maximally produced at 37°C (24). F pili are synthesized by exponentially growing host cells, with maximum piliation occurring in late logarithmic growth phase (30). Pili are lost as cells progress into stationary phase. Thus, the dependence of FRNA coli-

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phages on temperature- and temporally regulated F pili restricts the replication of these phages.

It is not certain where FRNA coliphages replicate. There are reports regarding replication in water (13, 25). FRNA coliphages have been found in wastewater, including sewage from households, and in the feces of warm-blooded animals (8). Of the four serogroups of FRNA coliphages, serogroups II and III are associated with wastewater from human sources, whereas groups I and IV are associated with other warm-blooded animals. FRNA coliphages have propagated in the intestines of gnotobiotic mice established with F^+ *E. coli* but not in germfree mice or in gnotobiotic mice established with F^- *E. coli* (3). These data suggest that FRNA coliphages naturally replicate in the gut. Low titers in feces seem to be related to the lack of host cells in the gut which are fully derepressed for F-pilus synthesis (14). An unexamined possibility is that secreted antibodies neutralize phage, resulting in lower titers in feces. Replication in sewage apparently may occur, although how it occurs is unknown (12). Where on-site treatment of wastewater is a concern, the possibility of replication in a septic tank becomes significant.

Factors affecting F-pilus synthesis will determine where FRNA coliphages replicate. Neither wastewater nor groundwater in northern climates is expected to sustain the temperatures required for F-pilus synthesis, nor are many host cells expected to be in the physiological state conducive to F-pilus synthesis. The objective of the present study was to examine in detail the effects of temperature and host cell growth phase or rate on the replication of Qβ, an FRNA coliphage, in order to determine in which environments these coliphages are most likely to replicate.

MATERIALS AND METHODS

Sources of bacteriophages, host bacteria, media, and reagents. Generally, Luria-Bertani (LB) broth (10 g of Bacto Tryptone [Difco Laboratories, Detroit, Mich.], 5 g of yeast extract [Difco], 5 g of NaCl in 1 liter of water [pH 7.2 with added NaOH] [21]) was used for the phage diluent and culture growth medium. Nutrient agar (Difco) was used to determine the numbers of CFU and PFU per milliliter. Dulbecco's phosphate-buffered saline (PBS with phenol red and without Ca^{2+} or Mg^{2+}; Sigma Chemical Co., St. Louis, Mo.) was used as the diluent when plating to determine the number of CFU per milliliter. Gelatin in saline (0.1% [wt/vol] gelatin and 0.5 g of NaCl in 100 ml of water, autoclaved) was used as the phage and antiserum diluent for determination of the numbers of infected cells as a function of host cell growth phase.

FRNA coliphage Qβ and its host, *E. coli* K-12 Hfr, were obtained from the American Type Culture Collection (23631-B1 and 23631, respectively). Phage stocks were produced by inoculating LB broth with 10^5 PFU and 10^5 CFU of phage and host, respectively, per ml and incubating the cultures for 6 to 8 h at 37°C. Cells were removed by centrifugation at 10,000 × *g* for 20 min at 4°C. The filter-sterilized culture supernatant fluid was reduced to 1/10 of its original volume by ultrafiltration through 100,000-molecular-weight-porosity membrane (YM100, 90-mm diameter; Amicon, W. R. Grace Co., Danvers, Mass.). The fluid in the ultrafiltration cell was reconstituted to its original volume with fresh LB broth and was reduced again to 1/10 of its original volume. The reconstitution-reduction procedure was repeated three times. After the final reduction, the phage extract was filter sterilized through a 0.2-μm-porosity Acrodisk (Gelman Sciences, Inc., Ann Arbor, Mich.) and stored at 4°C, with little drop in the phage concentration (10^{11} PFU/ml) over several months.

Production of antiserum to FRNA coliphage Qβ. Production of rabbit polyclonal antibodies to phage Qβ was done by the Animal Care Unit of the University of Wisconsin—Madison Medical School. The *K* value, a measure of the immune serum's ability to neutralize phage, was 844 (1). The antiserum diluted 1:1,840 neutralized >99.9% of Qβ PFU in 10 min.

General method for phage replication studies in batch cultures. Fifty milliliters of LB broth, equilibrated to the experimental temperature in a 125-ml DeLong flask, was inoculated with overnight cultures of bacteria and high-titer stocks of FRNA coliphage Qβ. Bacteria and phage were diluted in LB broth to initial concentrations of 10^5 CFU/ml and 10^5 PFU/ml, respectively. After being mixed and at the indicated times, 2 ml of the culture was reserved on ice. The remainder of the culture was incubated at the indicated temperature, with shaking to promote aeration. Samples were assayed for CFU and PFU per milliliter, as described below.

Plaque assay. The plaque assay was done according to the method described by Adams (1). Samples for the phage assay were mixed with a few drops of chloroform to halt bacterial growth and phage replication. *E. coli* was grown to mid-logarithmic phase (3×10^8 to 6×10^8 CFU/ml) in LB broth at 37°C with aeration and was kept on ice for the duration of the assay (≤2 h). Serial decimal dilutions of the samples were prepared in LB broth just before assay. Triplicate plates were prepared of each dilution of batch culture samples tested. Plaques were counted 6 to 24 h later.

Replication of Qβ in anaerobic cultures. Anaerobic culture tubes were prepared according to the Hungate techniques (10), by using CO_2 gas to remove dissolved air from LB broth that had cysteine (50 mg/100 ml; Sigma) as a reducing agent. Fresh overnight cultures of *E. coli* and stocks of Qβ were diluted in anaerobic broth dilution blanks before inoculation of the replication culture.

One-step phage replication experiments. The effects of temperature and growth phase on the length of the Qβ replication cycle were determined in phage one-step replication studies (6). In these experiments, a culture of *E. coli* was grown at 37°C. When the desired stage of growth was reached, as determined by A_{650}, the culture was set on ice for <30 min while final preparations were made. The diluent was equilibrated to the experimental temperature. A mid-logarithmic-phase host cell culture, A_{650} of 0.3 to 0.37, was made ready for assay of unadsorbed or released phage in replication tube samples. At time zero, 0.9 ml of the host culture was infected with 0.1 ml of 10^8 PFU of Qβ per ml, and incubation was begun. At 4 min, 0.1 ml of the infected culture was diluted in 9.9 ml of LB broth (cumulative dilution, 10^{-2}). At 4.5 min, a tube with a cumulative dilution of 10^{-4} was produced by serial dilution of the first dilution. At 5 min, 2 ml of the 10^{-4} dilution was treated with chloroform and kept on ice until unadsorbed phage could be assayed. At 7 min, a tube with a cumulative dilution of 10^{-6} was produced by serial dilution of the second. The 10^{-4} and 10^{-6} dilutions were incubated for the duration of the experiment, and 0.1 ml of samples of each dilution were periodically assayed to determine when release of progeny phage began and its duration. At the conclusion of the experiment, multiple plaque assay plates were prepared from the two dilutions and the chloroform-treated portion of the 10^{-4} dilution prepared earlier. Single plates were prepared for intermediate-time samples, as preparation of duplicate plates was difficult.

To study growth phase effects on the one-step replication of Qβ, 50 ml of LB broth in a 300-ml nephelo-type culture flask was inoculated with 0.2 ml of a fresh overnight culture of *E. coli* and incubated at 37°C with gentle shaking. The approximate host density was monitored by determining the A_{610} of the culture. At indicated times, a 2-ml sample was removed from the flask and set on ice. Portions of the sample were plated for determination of the number of CFU per milliliter, and the remainder was used for the one-step replication experiments described above and for infected cell experiments (described below). Qβ and antiserum raised against Qβ were diluted in a gelatin solution instead of LB broth in these assays. Five plates of the initial and final samples of the one-step replication experiments were prepared (intermediate-time samples from the one-step replication experiments were not prepared). Phage replication was assumed to be complete in 80 min.

Determination of numbers of infected cells. A host culture was prepared and infected as described for the one-step replication study (1). The infected culture was incubated for 10 min at the indicated temperature, and 0.1 ml was combined with 0.9 ml of anti-Qβ serum, which had been diluted 1:1,840 in gelatin (or in LB broth for temperature effect studies). After 10 min, the neutralized culture was set on ice to prevent the lysis of infected cells before samples could be serially diluted in chilled LB broth. The number of infected cells was determined by plating with a host cell culture, as in a plaque assay. Preliminary studies indicated that infected cells did not significantly decrease in number during 2 h of storage on ice before assay.

Determination of burst sizes. A culture of *E. coli* was prepared, infected, and incubated as described for the infected cell assays (1). At 10 min, 0.1 ml of the infected culture was treated with 0.9 ml of Qβ antiserum, which had been diluted 1:1,840 in LB broth. This step was omitted when the unadsorbed phage could be essentially eliminated by dilution. After neutralization of unadsorbed phage, but before release of progeny could begin, the infected culture was diluted (typically 10^{-6} to 10^{-7}) in LB broth so that there was less than one infected cell per ml, and 0.5-ml samples of this last dilution were distributed into sterile tubes. LB broth was chilled on ice to slow phage replication in 37°C experiments, to ensure that the diluted culture could be distributed before release of progeny could begin. The tubes were incubated at the same temperature as the infected culture. When sufficient time had elapsed for complete lysis of infected cells, 0.1 ml from each tube was assayed for progeny phage. Samples were stored at 4°C to allow dilution and reassay of samples that had produced more than 300 plaques.

Mathematical analysis of single-cell burst size analyses. When the average number of infected cells per tube is less than 1, the distribution of infected cells in the tubes assumes a Poisson distribution (1). The number of infected cells in the undiluted infected culture can be estimated from the dilution factor and the number of infected cells distributed among the tubes. To determine the number of infective progeny phage released from an infected cell, the numbers of plaques on the plates are summed, multiplied by 5 (0.1 ml of each 0.5-ml sample is plated), and divided by the total number of infected cells distributed among the tubes. Experiments were repeated to permit statistical analysis of the results.

Statistical analysis. Statisticians at the College of Agricultural and Life Sciences, University of Wisconsin—Madison, were consulted for appropriate sta-

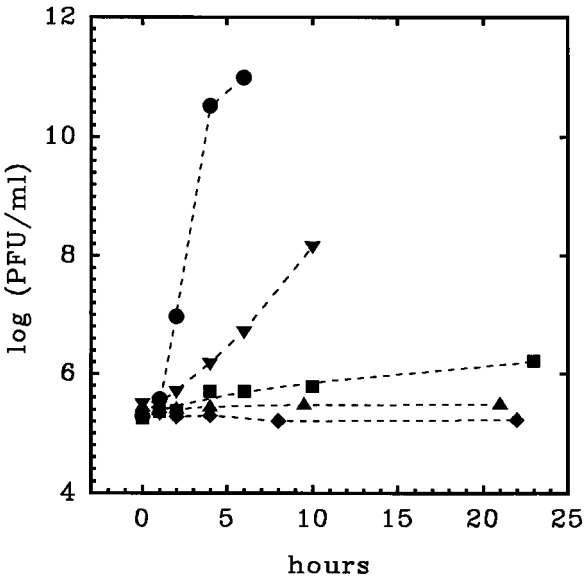

FIG. 1. Effect of temperature on replication of phage Qβ on *E. coli* in LB broth. The temperatures studied were 37°C (●), 30°C (▼), 25°C (■), 22°C (▲), and 20°C (◆). Inocula for the experiments were grown at 37°C.

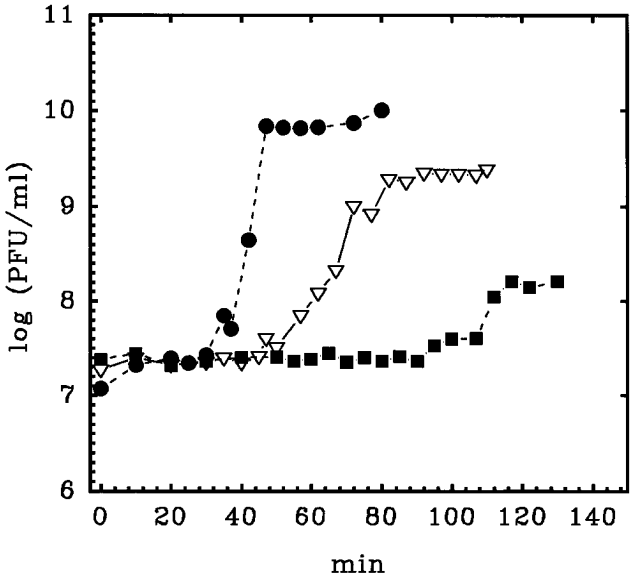

FIG. 2. One-step replication of Qβ on *E. coli* as a function of temperature. Cultures of host cells were grown into mid-logarithmic phase at 37°C, infected with Qβ, and incubated at 37°C (●), 30°C (▽), or 25°C (■).

tistical tests. Data from some batch culture, infected cell, single-cell burst size, and one-step replication experiments were analyzed by Student's *t* test, in which the variances were not assumed to be equal, or by one-way analysis of variance (26). Untransformed data were usually analyzed in these tests. In some batch experiments, particularly those that had durations shorter than 22 to 24 h, the logarithmic transformations of the mean values (PFU per milliliter) could be modeled as straight lines and were therefore analyzed by linear regression analysis and by using correlation coefficients. Linear regression analysis tested the probability of the lack of significant differences between the computed slope and zero. Overlap of confidence intervals between two regression slopes indicated that the two slopes were probably the same. Unless a probability is given, the likelihood of type I (α) error may be assumed to be less than 0.05.

One-step replication experiments were modeled as four-parameter logistic curves from logarithmically transformed data, with the nonlinear curve-fitting feature of SigmaPlot 5.0 (Jandel Scientific, Sausalito, Calif. [16]). The parameters are *a*, asymptotic maximum number of PFU per milliliter; *b*, the slope parameter, in which $b > 0$ indicates a negative slope; *c*, the time to inflection of the slope; and *d*, asymptotic minimum number of PFU per milliliter. The significance of the difference in phage yields ($a - d$) in two one-step replication experiments was assessed with an adaptation of the *t* test. Differences in time elapsed to the inflection points (*c*) of replication curves were compared by one-way analysis of variance.

RESULTS

Effects of temperature. (i) Batch experiments with host cell inocula grown at 37°C. Qβ replicated most rapidly and to the greatest extent at 37°C (Fig. 1). The replication rate at 37°C is estimated from the regression slopes to be about four times greater than at 30°C. At 4 and 6 h, the concentrations of phage in the 30°C culture were approximately 10^4 times less than in the 37°C culture. Significant phage replication occurred in the 25°C culture, but Qβ concentrations increased only 10-fold in 24 h. No phage replication occurred at 22 and 20°C, as regression slopes were not significantly different from zero, although host cell growth occurred at these temperatures.

(ii) Batch experiments with inocula grown at 30 and 25°C. At lower temperatures, host cells can retain F pili synthesized during growth at 37°C (24); it was thought that the low level of Qβ replication at 25°C was due to F pili or subunits conserved from host cell growth at 37°C. When host cell inocula were grown at 30, 25, or 20°C, the level of Qβ replication at 25°C was essentially the same as that for an inoculum grown at 37°C: the slopes determined by regression analysis were similar, and the 95% confidence intervals overlapped (data not presented).

One-step replication of Qβ as a function of temperature. One-step replication experiments (6) were used to study the effects of temperature on the duration of the phage replication cycle and the relative amounts of progeny phage released. Host cell cultures grown at 37°C were infected with Qβ, and incubation of the infected cultures was begun at the indicated temperatures. Five plates each of initial and final samples were prepared.

The amount of Qβ released from infected cultures decreased as the temperature was reduced (Fig. 2). Significantly more phage was released at 37 and 30°C than at 25°C. The yields at 37 and 30°C, however, are not significantly different. As the temperature was reduced from 37 to 25°C, the amount of Qβ released by infected cultures decreased approximately 100-fold.

The lengths of the replication cycles, as determined by the slope parameters of the curves fitted to the data, increased as the temperature was reduced (Table 1). The slope parameters of the curves shown in Fig. 2 are typical of those observed in other experiments. The mean slope parameters at 37 and 30°C are significantly different ($P < 0.001$) from the mean slope parameter at 25°C (109 min) but are not significantly different from each other.

TABLE 1. Parameter values of logistic equation fit to one-step replication data for temperature effect experiments

Temp of culture (°C)	Mean (SE)		
	Minimum[a] $\log_{10}$ PFU/ml	Time to inflection (min)	Maximum[a] $\log_{10}$ PFU/ml
37	7.41 (0.071)	41.6 (0.573)	9.91 (0.0068)
30	7.38 (0.037)	65.7 (0.841)	9.39 (0.048)
25	7.39 (0.0019)	109 (1.36)	8.24 (0.063)

[a] Calculated as asymptotes. The calculated parameters for slope are not reported because differences were not significant.

TABLE 2. Infection by Qβ as a function of temperature

Temp (°C)	Single-cell burst size analysis		Infected cell assay IC/ml[c]
	PFU/IC[a]	IC/ml[b]	
37	776 ± 161	$(1.9 \pm 0.8) \times 10^7$	$(1.3 \pm 0.13) \times 10^7$
30	1,144 ± 346	$(5.6 \pm 3.2) \times 10^6$	$(6.9 \pm 1.3) \times 10^6$
25	899 ± 270	$(4.4 \pm 1.7) \times 10^5$	$(1.9 \pm 0.2) \times 10^{6d}$

[a] Mean number of PFU (± standard deviation) released by a single infected cell (IC). One-way analysis of variance indicated that the means are not significantly different at $\alpha = 0.05$ ($F = 2.43$; $P = 0.124$). Means were determined from five experiments at 37 and 30°C and seven experiments at 25°C.

[b] Mean number of infected cells per milliliter (± standard deviation) in undiluted infected cultures.

[c] Mean number of infected cells per milliliter (± standard deviation) as determined by the infected cell assay. Means were determined from five plates.

[d] Significantly different ($P < 0.001$) from the result determined by single-cell burst size analysis.

Effects of temperature on the number of infected cells. Infected cell studies were done to determine the effect of temperature. As the temperature was decreased from 37 to 25°C, the number of infected cells per milliliter was reduced by a factor of 10 (Table 2). One-way analysis of variance indicated that significant differences exist among the mean numbers of infected cells per milliliter ($P < 0.001$).

The mean number of infected cells per milliliter can also be estimated from the single-cell burst size analysis. The mean numbers of infected cells per milliliter determined from the single-cell burst size analysis experiments were similar to those determined from the infected cell assays at 37 and 30°C ($P > 0.2$); however, differences between the values obtained from the two assays at 25°C were significant ($P < 0.001$).

Effect of temperature on PFU released from infected cells. The individual determinations of PFU per infected cell for each temperature varied greatly (Table 2). Because the standard deviations of the PFU-per-infected-cell values were large, no dependency of burst sizes on temperature could be observed. One-way analysis of variance indicated that the mean numbers of PFU per infected cell did not differ significantly ($P = 0.106$). Temperature had little effect on the number of infective progeny phage released from a single infected cell.

Effect of host cell growth phase on one-step replication of phage Qβ. In a preliminary experiment, less phage replication appeared to occur as host cells progressed from mid-logarithmic to late logarithmic phase, but the length of the phage replication cycle did not change (Fig. 3). Qβ replication was essentially abolished, as expected, in the stationary-phase culture. The slight increase in the final phage concentrations can be attributed to derepression of cell growth resulting from dilution into fresh medium.

Effects of growth phase on numbers of infected cells and one-step replication of phage Qβ. Three separate series of infected cell assays were done (Fig. 4A). The numbers of infected cells at the indicated times were analyzed for significant differences. The number of infected cells reached a maximum in mid-logarithmic-phase cells and decreased by 10-fold when the host cell culture reached stationary phase. The infected cells at no time comprised more than 10% of the total number of CFUs. The greatest differences in the numbers of infected cells occurred in the 4-h (mid-logarithmic phase) and 20-h (stationary phase) samples or the 4-h and 2.5-h samples from the mid-logarithmic phase culture. Significant differences ($P < 0.05$) also existed between the 4-h and the 6- to 9-h samples.

One-step replication experiments were done in conjunction with the infected cell assays. Five plates each of the initial and final (82 min of growth) samples were prepared. Intermediate-time samples were not plated. The apparent phage yields in the one-step replication studies paralleled the infected cell study results: there was a maximum yield during exponential growth of the cells, and apparent yields diminished as the host cell cultures grew into stationary phase at 18 to 20 h (Fig. 4B). The phage yields at 3 h were significantly different ($P < 0.05$) from those at 14 h or later.

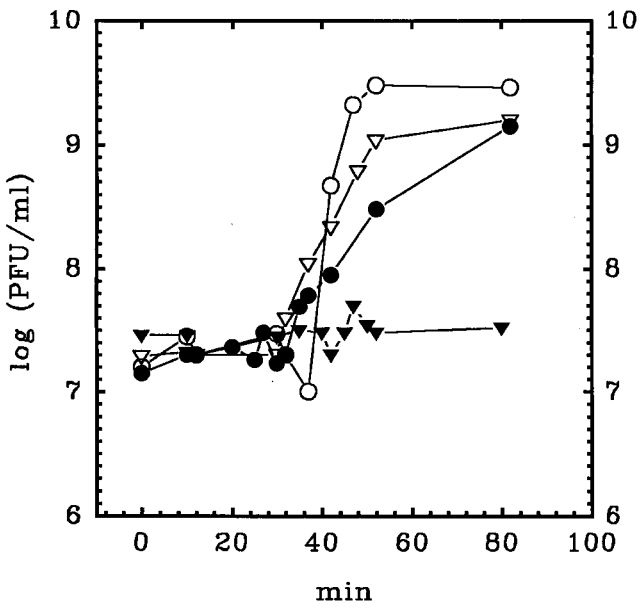

FIG. 3. Effect of host cell growth phase on the replication of Qβ. Two separate experiments were done. In one experiment, mid- (○) and late (●)-logarithmic-phase cultures were used for the one-step replication experiments. Cultures contained 2.4×10^8 and 2.4×10^9 CFU/ml, respectively. In the other experiment, mid-logarithmic (▽)- and stationary (▼)-phase cultures were used for one-step replication experiments. Cultures contained 1.45×10^8 and 5.8×10^9 CFU/ml, respectively.

Effects of host cell growth phase on single-cell burst size of Qβ. Although the apparent decrease in phage yields in the one-step replication experiments (Fig. 4B) paralleled the decrease in the numbers of infected cells (Fig. 4A), it seemed unlikely that the single-cell burst size of Qβ would change as the host cell culture progressed from logarithmic to stationary phase. To test this hypothesis, single-cell burst size analyses were done, with the following procedural changes to prevent host cells in late logarithmic phase from derepressing growth when the cells were diluted into fresh LB broth: (i) antiserum and Qβ were both diluted in 0.1% gelatin with 0.5 g of NaCl per 100 ml, and (ii) nalidixic acid was added to the LB broth (20 μg/ml, final concentration) used to dilute the infected culture to inhibit cell division. A trial experiment in which nalidixic acid was added to the diluent used in a single-cell burst size experiment with a mid-logarithmic-phase culture indicated that the antibiotic did not adversely affect the assay. Qβ burst sizes did not change as a function of host cell growth phase, although the numbers of infected cells decreased significantly (Table 3).

Effects of anaerobiosis on replication of Qβ. The dynamics of Qβ replication under anaerobic conditions was different from that under aerobic conditions (Fig. 5). Under anaerobiosis, phage replication was essentially complete at 4 h, whereas Qβ replication was complete at 10 h in cultures incubated under aerobic conditions. Qβ concentrations in anaerobic cul-

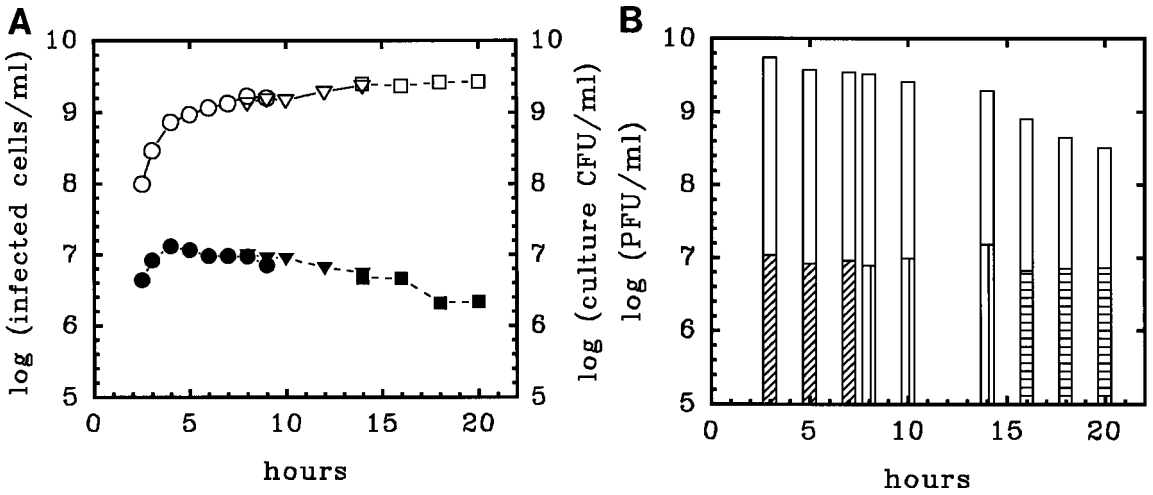

FIG. 4. Effects of host cell growth phase on numbers of infected cells and one-step growth of Qβ. (A) Three separate experiments (○ and ●, ▽ and ▼, □ and ■) were done to determine the effects on the numbers of infected cells. Open symbols denote the log average number of CFU per milliliter of the host cell culture at the indicated sampling times, and the closed symbol denotes the log average number of CFU per milliliter in the culture samples which became infected. Data are averages for five plates. Error bars were calculated for the third series of experiments to demonstrate that at 18 to 20 h the host cell culture had entered stationary phase but were not included because they were obscured by the symbols. (B) One-step replication experiments were done with portions of samples taken at 2- to 4-h intervals from cultures of host cells incubated at 37°C. Initial (filled bars) and final (unfilled bars) phage concentrations for a one-step replication experiment at each sampling time are shown. Three experiments are presented: 3 to 7 h (mid- to late logarithmic phase) (diagonal fill); 8 to 14 h (late logarithmic to stationary phase) (vertical fill); and 16 to 20 h (stationary phase) (horizontal fill).

tures increased only 100- to 1,000-fold times over initial concentrations, compared with 10^6- to 10^7-fold in the aerobic culture. The differences in Qβ replication between aerobic and anaerobic cultures were large and highly significant ($t = 39$; 10 degrees of freedom). Cessation of Qβ replication in the anaerobic cultures coincided with the decrease in the number of host cells. The decrease in the number of CFU per milliliter was reproducible and significant ($P < 0.001$). It was not observed in cultures without phage or in the aerobic cultures.

DISCUSSION

Dependence of FRNA coliphage replication on temperature and growth phase of host cells. Replication of FRNA coliphage Qβ decreases as temperature decreases, as a consequence of fewer host cells being infected rather than the release of fewer infectious phage particles. The numbers of infected cells determined by single-cell burst size experiments and infected cell assays are statistically similar at 37 and 30°C. Fewer infected cells were detected in the single-cell burst size experiments than in the infected cell assays at 25°C. The reason for the discrepancy is not known. The lower estimate for the single-cell burst size experiment suggests that abortive infections may have occurred in the burst size experiments.

Phage replication was significantly slower in cells incubated at 25°C and ceased below 25°C. The low rate of Qβ replication at 25°C suggests that very few host cells had F pili. Cessation of Qβ replication below 25°C is consistent with reports of temperature regulation of the F pilus (19, 24, 31). A previous study

TABLE 3. Infection with Qβ as a function of host cell growth phase

Growth phase of host cells	Cell concn (CFU/ml)[a]	Single-cell burst size analysis	
		IC/ml[b]	PFU/IC[c]
Mid logarithmic	$(4.9 \pm 0.8) \times 10^8$	$(1.9 \pm 0.8) \times 10^7$	776 ± 161
Late logarithmic	$(7.3 \pm 1.6) \times 10^8$	$(5.5 \pm 1.8) \times 10^6$	655 ± 17

[a] Mean number of CFU ± standard deviation of phage host *E. coli*, determined by dilution plating on nutrient agar. Mid-logarithmic-phase cultures were generally grown for 3 to 3.5 h at 37°C. Late-logarithmic-phase cultures were grown for 5 to 6 h at 37°C. Nalidixic acid was added to the LB broth (20 μg/ml, final concentration) used to dilute the infected culture samples.

[b] Mean number of infected cells (IC) per milliliter (± standard deviation) in an undiluted infected culture. Student's *t* test indicated that the means were significantly different at $\alpha = 0.05$ ($t = 3.43$, 4 df, $P = 0.027$).

[c] Mean number of PFU (± standard deviation) released by a single infected cell. Means were determined from five experiments with mid-logarithmic-phase cultures and two experiments with cultures in late logarithmic phase. One-way analysis of variance indicated that the mean number of PFU per infected cell in the late-logarithmic-phase culture was not significantly different from the mid-logarithmic-phase values at 37, 30, and 25°C.

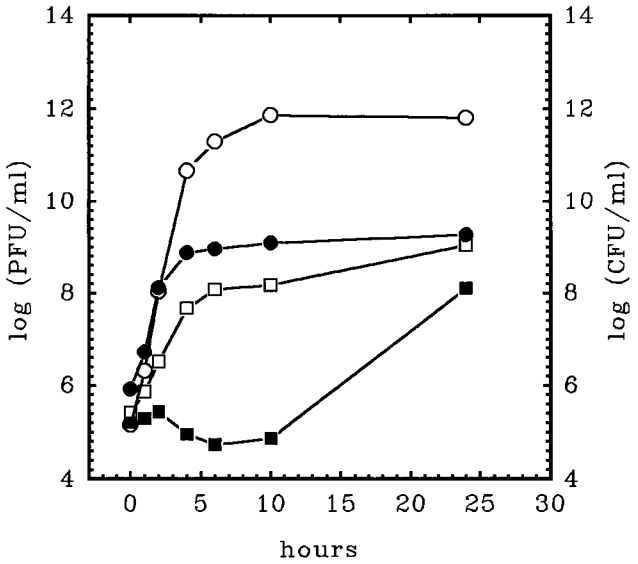

FIG. 5. Replication of Qβ at 37°C in LB broth incubated under aerobic (open symbols) and anaerobic (closed symbols) conditions. Circles, Qβ concentrations; squares, host cell concentrations.

has indicated that FRNA coliphage fr does not plaque below 24°C and that host cells cultured at 18°C do not adsorb phage (19). Novotny and Lavin (24) reported that the number of F pili per cell varied with the growth temperature but that the average length of pili remained about the same. A low level of F piliation was detectable in cultures grown at 25°C by inhibition of F-specific phage plaque formation by antiserum specific for F pili. Cells grown at 37°C, homogenized at high speeds to shear off F pili, and resuspended in medium at 25°C produced pili, suggesting that F pili are assembled from pools of subunits.

There is one report that F-pilus subunits are not synthesized below 30°C, and therefore, cells are not F piliated below this temperature (13). This claim is not consistent with the results of temperature dependence studies (23) and our data. Possibly, the low level of Qβ replication observed at 25°C resulted from F pili assembled from a pool of subunits produced when the inoculum was grown at 37°C. To deplete or prevent the synthesis of F pilins, inocula were grown at 30, 25, and 20°C. If F-pilin synthesis ceases below 30°C, then host cell cultures grown at temperatures below 30°C from inocula likewise incubated at temperatures below 30°C could not produce F pili and FRNA phage replication could not occur. However, not only did inocula grown at 20 to 30°C permit Qβ replication to occur at 25°C but also the extent of replication was indistinguishable from that observed when the inoculum was incubated at 37°C.

It has been claimed that FRNA coliphages replicate in wastewater or groundwater at 20°C (13). FRNA coliphage GA was used in the studies of FRNA coliphage replication. This and other serogroup II FRNA coliphages are reported to replicate at a maximum rate at 30°C and can replicate at 20°C (8). The phage replication experiments reported by Havelaar and Pot-Hogeboom (13) model a specific case in which the host cell is infected at 37°C and is shed into wastewater or groundwater environments at lower temperatures. Serogroup II FRNA coliphages may continue to replicate at 20°C but could not be expected to infect cells at this temperature as host cells do not produce F pili. Preliminary experiments with Qβ (a serogroup III FRNA coliphage) indicated that while Qβ replicates at 25°C when cells are diluted into rich medium, replication may not continue when the infected cells are diluted into nutrient-poor liquids, such as PBS.

FRNA coliphage replication diminished as cells approached stationary phase, as expected (1). F piliation of a culture reaches a maximum in late logarithmic phase and then diminishes as the culture progresses into stationary phase (30). Burst size analysis indicated that decreased Qβ replication in late-logarithmic-phase cultures results from fewer infected cells rather than reduced burst sizes. The small amount of phage replication observed in stationary phase cultures was probably due to derepression of cell growth when infected cultures were diluted into fresh medium in the course of the experiments and probably cannot be entirely eliminated. It is not certain what effect the addition of nalidixic acid to the diluent in single-cell burst size analyses had, but the antibiotic was certain to stop cell multiplication (4). In any event, fewer cells are infected as host cultures exit late logarithmic phase. Because of the detection limits in the infected cell assay, the decrease of the number of infected cells to 0 as cells progressed into stationary phase could not be observed. A 100-fold dilution of the infected culture was needed to abolish the effects of antiserum on detection of infected cells.

Qβ replication under anaerobic conditions was examined to determine the effects of anaerobiosis and the decrease in the host cell growth rate. Groundwater can be aerobic even at great depth (7). The number of F pili per cell and the length of F pili increase in cultures that are incubated anaerobically (28). The anaerobic LB broth had a pH of approximately 5, which would cause some inactivation of Qβ and inhibit multiplication of the host cells. The decrease in the number of CFU after 2 h of incubation under anaerobic conditions was unexpected but was reproducible and statistically significant. This decrease was probably related to the lack of fermentable carbohydrate needed for anaerobic cell growth but perhaps also to massive Qβ infection of the culture. The dynamics of phage replication in the anaerobic LB broth was different from that observed for aerobic incubation: maximum Qβ concentrations were lower and were reached more quickly in the anaerobic culture. The similarity of Qβ replication in anaerobic cultures to one-step replication curves suggests that the replication cycle of Qβ was shorter than the doubling times of host cells under anaerobic conditions. The early end of Qβ replication and the decrease in CFUs in the infected cultures suggest that insusceptible cells were selected for, as was found in related experiments (data not shown).

Significance of temperature and growth phase to replication in the environment. On the basis of temperature alone, infection of F^+ host cells by FRNA coliphage is unlikely in groundwater in Wisconsin and areas with similar climates. Few cells would have F pili at 25°C and could therefore be infected. If infection occurs at 25°C, the extent of replication will be much lower relative to the optimum at 37°C, and infected cells may not be able to sustain phage replication. Only shallow aquifers would reach 25°C or higher by the end of summer. Temperatures in wastewater can exceed 25°C (29) but would fluctuate. The gastrointestinal lumen of endothermic animals, however, can maintain near-optimal temperatures; therefore, it is not surprising that FRNA coliphages can be maintained in animals established with F^+ bacteria (3). The effects of temperature suggest that the gastrointestinal lumen of warm-blooded animals is more likely to be a habitat of these phages than groundwater or wastewater. Little phage replication is expected in either environment.

The most likely growth phases of potential host cells while in wastewater or groundwater are difficult to model, but because neither environment is nutrient rich, the cells would unlikely be in the rapidly growing mid-logarithmic phase, which provides the optimum conditions for phage replication. We predict that host cells would be in stationary phase when in groundwater and would therefore be unable to sustain phage infection. We have observed some host cell growth in wastewater samples, but the absence of phage replication while host cells were able to grow indicates that logarithmic growth of host cells is necessary, but not sufficient, for extensive FRNA coliphage replication. Even if FRNA coliphages encounter host cells in wastewater warmer than 25°C, our experiments indicate that FRNA coliphage replication would be limited. Anaerobiosis typical of septic tank effluent would further constrain FRNA coliphage replication. We therefore conclude that FRNA coliphage fulfills the requirement that a model of human enteric viruses in wastewater or groundwater not replicate significantly in either environment.

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