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Experimental Examination of Bacteriophage Latent-Period Evolution as a Response to Bacterial Availability

Stephen T. Abedon,[1]* Paul Hyman,[2] and Cameron Thomas[1]

Department of Microbiology, Ohio State University, Mansfield, Ohio,[1] and NanoFrames LLC, Boston, Massachusetts[2]

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For obligately lytic bacteriophage (phage) a trade-off exists between fecundity (burst size) and latent period (a component of generation time). This trade-off occurs because release of phage progeny from infected bacteria coincides with destruction of the machinery necessary to produce more phage progeny. Here we employ phage mutants to explore issues of phage latent-period evolution as a function of the density of phage-susceptible bacteria. Theory suggests that higher bacterial densities should select for shorter phage latent periods. Consistently, we have found that higher host densities ($\geq\sim10^7$ bacteria/ml) can enrich stocks of phage RB69 for variants that display shorter latent periods than the wild type. One such variant, dubbed sta5, displays a latent period that is ~70 to 80% of that of the wild type—which is nearly as short as the RB69 eclipse period—and which has a corresponding burst size that is ~30% of that of the wild type. We show that at higher host densities ($\geq\sim10^7$ bacteria/ml) the sta5 phage can outcompete the RB69 wild type, though only under conditions of direct (same-culture) competition. We interpret this advantage as corresponding to slightly faster sta5 population growth, resulting in multifold increases in mutant frequency during same-culture growth. The sta5 advantage is lost, however, given indirect (different-culture) competition between the wild type and mutant or given same-culture competition but at lower densities of phage-susceptible bacteria ($\leq\sim10^6$ bacteria/ml). From these observations we suggest that phage displaying very short latent periods may be viewed as specialists for propagation when bacteria within cultures are highly prevalent and transmission between cultures is easily accomplished.

Phage serve as important viral predators of bacteria (17, 38, 39), and the rate that bacteria are phage adsorbed is proportional to phage density (2, 24). Here we consider two factors affecting lytic-phage population growth to higher densities: fecundity and generation time. The fecundity of an individual phage is its per-bacterium burst size. Generation time is less straightforward, consisting of a diffusion-limited extracellular search for bacterial hosts followed by a latent period. The latent period encompasses the active phage infection of an individual bacterium. The extracellular search is the period of inert phage dissemination. A higher host densities—e.g., 10^7 versus 10^5 bacteria/ml—phage populations can grow more rapidly simply because phage can find bacteria faster (7).

As originally demonstrated in the classic work of Doermann (15), the truncation of an active phage infection, e.g., as occurs following artificial lysis, reduces the duration of intracellular phage progeny maturation. This truncation results in a decline in a phage's burst size. Various authors nonetheless have theorized that individual phages can achieve faster population growth through latent-period reduction despite the associated reduction in burst size (1, 8, 11, 27, 37). This advantage occurs only at higher bacterial densities. Only then are phage latent periods long relative to their diffusion-limited extracellular search, and thereby can shorter latent periods (SLPs) substantially reduce phage generation times.

At lower bacterial densities, because extracellular search times already are relatively long, it is not small latent-period reductions that are thought to bestow a significant advantage but larger burst sizes, i.e., as seen with longer latent periods (LLPs). In other words, at lower bacterial densities bacteria are more valuable to phage and therefore are most effectively exploited by producing more phage progeny per infected bacterium. These larger burst sizes are advantageous even though latent-period extension delays phage progeny acquisition of new bacteria. SLPs thus are thought to represent a specialization for the exploitation of bacteria growing at higher densities, while LLPs, as a distinct, alternative life history strategy, may be viewed instead as more specialized for the exploitation of bacteria growing at lower densities. Higher bacterial densities therefore should select for phage capable of displaying SLPs, while lower bacterial densities may select for phage capable of displaying LLPs.

In this study we emphasize the impact of phage generation time on the population growth of actively infecting, obligately lytic (also known as virulent) phage, particularly as latent periods vary between phage mutants and their wild-type (WT) parent. Lysogeny, pseudolysogeny, and chronic phage infections also may be addressed when considering the impact of phage generation time on phage population growth, but these phenomena are reflected on elsewhere (1, 7, 11, 20). Here we report on the isolation of a number of mutants of the T-even-like phage RB69 (9, 34) that display latent periods that are shorter than the latent period displayed by their WT RB69 parent. By using one of these mutants, we confirm that SLP phage can indeed outcompete LLP phage at higher but not at lower bacterial densities. We also confirm that this SLP advantage may be realized only given direct competition between two phage populations within the same culture (1). Indeed, when phage are grown individually we would deem the SLP

* Corresponding author. Mailing address: Department of Microbiology, Ohio State University, 1680 University Dr., Mansfield, OH 44906. Phone: (419) 755-4343. Fax: (419) 755-4327. E-mail: abedon.1@osu.edu.

phage "sick" since maximum phage population size (i.e., final titer) following broth culture growth typically has been less then one-quarter that of the parental LLP WT. From these observations we argue that SLPs may be advantageous when phage-susceptible bacteria are present at higher densities. This advantage may be lost, however, should final-titer differences between cultures play a larger role in phage competitiveness than within-culture rates of phage population growth.

MATERIALS AND METHODS

Phage and bacterial strains. We obtained the following WT (and duplicate WT) phage and bacterial strains: phage T4D from Harris Bernstein; phage RB69 (as primarily employed in this study) along with phages T2H, T2L, T6, and RB69 mutant "#1" and *Escherichia coli* CR63 and a K12 WT strain from John Drake; phages T2, T4, T6, and RB69 from Sean Eddy (phage T2 presumably is equivalent to phage T2L; see reference 6); phage T4D from Edward Goldberg's collection; phage T4D++ from Elizabeth Kutter; and phage T6 from John Obringer.

Phage growth and whole-organismal characterization. All experiments were done by employing the *E. coli* CR63 strain except for one of a total of three serial-transfer protocols which employed a WT *E. coli* K12 host instead. Though all three protocols yielded similar enrichment for SLP phage, the SLP phage primarily characterized here (sta5) was obtained via the single serial-transfer protocol employing the *E. coli* K12 host. The media employed along with adsorption, single-step-growth, and "constant"-bacterial-density experiments are all as described in reference 8. For some latent-period determinations (lysis profiles), we observed culture turbidity that we determined by using a Klett-Summerson Colorimeter (Klett Manufacturing Co., Inc., New York, N.Y.) rather than via PFU (3, 5). For these lysis-profile experiments, bacteria were grown with aeration in larger vessels before their transfer, prior to phage infection, to test tubes of a diameter appropriate for use in a Klett-Summerson Colorimeter (part no. 2573-1413B; Bellco, Vineland, N.J.). Tubes were vigorously vortexed before each turbidity determination and otherwise were continuously shaken by a gyrotory water bath shaker. We distinguished SLP and LLP phages on the basis of plaque morphology, with differences accentuated given soft-agar overlay incubation at room temperature. Experiments otherwise were done at 37°C with phage addition to bacteria occurring at time zero.

RB69 *t*-gene sequencing and sequence comparison. DNA sequencing was done by the Tufts University Core facility (Department of Physiology, Tufts University School of Medicine, Boston, Mass.) from PCR products and employed various combinations of the following phage-RB69-based primers: rb69-38-t-5f (GGCG GTGGTGCTCCTGGCAGAGC), rb69-t-5f (GCTTTAGAACAACTACAAAT AGTCC), rb69-t-5r (GGTAACTTACCTTCATACGC), and rb69-asiA-t-3r (CT AATTACAAATTTAACTGCCG). The original sequencing of phage RB69 WT employed two T4 gene-*t*-based primers: t4-t-1f (ATGGCAGCACCTAGAATA TCA) and t4-t-1r (TTATTTAGCCCTTCCTAATAT). An RB69-specific primer, rb69-t-3r (CCAAATAAAATATCACTAGGCG), was also used to sequence RB69 genomic DNA upstream from the RB69 gene *t*. Our PCR protocol is that of Jozwik and Miller (23) with usage of 5 mM Mg^{2+}. The complete phage RB69 sequence can now be found at NC_004928 (RB69). Accession numbers and publication references of comparison gene *t*-region sequences are NC_000866 (T4) (29), X55191 (TuIb) (28), AF060870 (Ac3) (36), AF208841 (AR1) (43), X05312 (K3) (32, 33), M16812 (K3), X05676 (M1) (29), X05675 (Ox2) (29), X05312 (T2) (33), AF052605 (T6), AJ508254 (RB49), and NC_005135 (44RR2.8t).

RESULTS

Mutant isolation. Testing hypotheses on the selective benefits associated with different phage latent periods requires pairs of otherwise similar phage strains, one displaying an SLP and the other an LLP. A number of such "clock" mutant phages are available, ones whose latent periods differ from those of their parental WTs (e.g., see reference 31). Rare, though, are phage mutants whose latent periods are shorter than those of WT and which display burst sizes of greater than zero, a combination well suited to testing predictions that SLP phages can outcompete LLP phages particularly under conditions of higher host densities. To reduce experimental and conceptual complexity, it is also advantageous to employ sufficiently virulent phages such as the T-even coliphages, i.e., phages T2, T4, and T6, which are both obligately lytic and efficient lysers of broth cultures of bacteria (Fig. 1A).

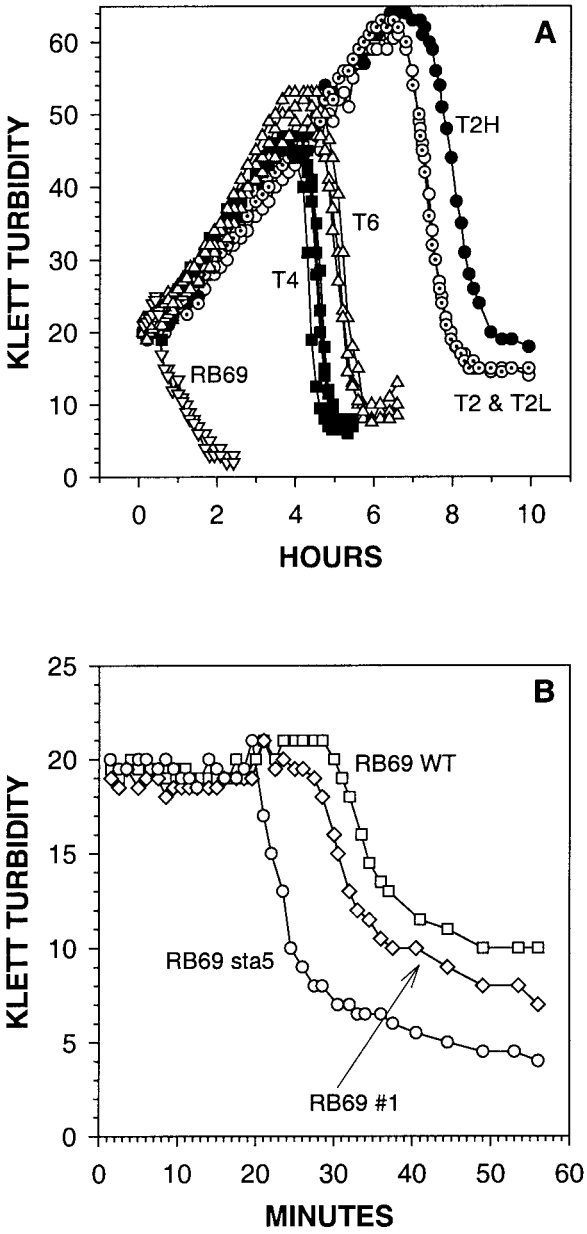

FIG. 1. Lysis profiles. Phage were adsorbed to *E. coli* CR63 growing at a density of approximately 10^8/ml. (A) Phages added with a multiplicity of 10 include T2 (○), T2H (●), T2L (⊙), T4 (■), T6 (△), and RB69 (▽), with each curve representing a phage stock obtained from a different source. Turbidity declines are indicative of phage-induced lysis (3, 41), and lysis somewhat later than 0.5 h is indicative of lysis inhibition. Infected-bacterium growth without division is thought to explain the rise in turbidity (3, 18, 19). (B) Phage RB69 strains, added to bacteria with a multiplicity of 5, are as indicated.

Acquisition of appropriate phages was inadvertently accomplished while attempting to repeat the Hershey method (21) of

selecting for T-even phages displaying lysis inhibition from stocks of lysis-inhibition defective phages. Lysis inhibition, a phenotype peculiar to T-even-like phages, is a delay in phage-induced lysis that is induced by the secondary phage adsorption (14) of an already T-even-phage infected bacterium (2, 4). We attempted to repeat this Hershey method experimental evolution because our WT stocks of RB69, a T-even-like phage, did not display lysis inhibition (Fig. 1, along with additional experiments not presented; see also reference 33a; for extensive discussion of the biology of lysis inhibition see references 2–5, 16, and 30). The Hershey method simply involves preparing broth-grown phage stocks. Growth is initiated via addition of low-multiplicity phage to growing bacterial cultures, which, after phage growth, is followed by culture-wide phage-induced lysis. Subsequent stocks are initiated by addition of a volume of the previous phage stock without plaque-purifying phage between transfers.

WT T-even phages generally display small, rough, cloudy-bordered plaques (16, 21). Though no such plaques were obviously present during the plating of serially transferred RB69 stocks, a number of plaques appeared to be larger than those associated with the parental RB69 WT. Previous experience with an RB69 mutant dubbed mutant #1 suggested, as consistent with plaque development theory (25, 40), that a phage displaying larger plaques than WT could also display an SLP (Fig. 1B). Consistently, upon testing we found that many of these RB69 large-plaque variants displayed latent periods shorter than those of the parental RB69 WT (data not presented). We further characterized one mutant, dubbed "serially transferred ancestry" mutant number 5 (sta5), which displayed the shortest latent period: ~70% of WT as determined by lysis profile and ~80% of that of the WT as determined by single-step growth, in each case with latent period defined as the start of population-wide lysis. RB69 sta5 also displayed the smallest burst size (~30% of WT).

Burst size and adsorption rate characterization. An SLP advantage in broth culture could result if mutants also adsorb bacteria faster or also display larger burst sizes. Plaque formation theory, however, suggests only that larger burst sizes should result in larger plaques. On the one hand, greater burst sizes can result in the formation of larger plaques by allowing at least marginally greater phage acquisition of uninfected bacteria (found on the periphery of growing plaques), though this effect should result in obviously larger plaques only if actual burst sizes are quite small and also so long as burst size increases do not come at a significant latent-period cost. On the other hand, faster adsorption (as distinct from faster phage diffusion) can interfere with plaque development by more readily associating phage with relatively immobile bacteria. The more time that phage spend infecting bacteria, rather than diffusing to the periphery of plaques, the smaller the resulting plaque. Similarly, SLPs should reduce the length of this infection delay, resulting in the production of larger plaques, with plaque size ultimately a function of the total time within plaques that free phage are allowed to diffuse (25).

Consequent to the above arguments, SLPs alone should result in the formation of larger plaques, particularly if burst sizes are not too greatly reduced by latent-period changes. Higher rates of phage attachment to bacteria, however, should result in smaller rather than larger phage plaques, while formation of larger plaques should result from reduced rates of attachment. To ascertain whether observed larger plaque sizes or more rapid rates of broth growth (below) were a function of SLPs rather than burst size or adsorption rate anomalies, we determined these parameters for the RB69 sta5 mutant. No apparent difference was observed between the broth adsorption rates of RB69 sta5 and RB69 WT (Fig. 2A), nor were adsorption rate differences observed between mutant no. 1 and RB69 WT (data not presented). In Fig. 2B we compare single-step growth curves. Again, no apparent difference between the two phages—RB69 sta5 versus RB69 WT—is observed other

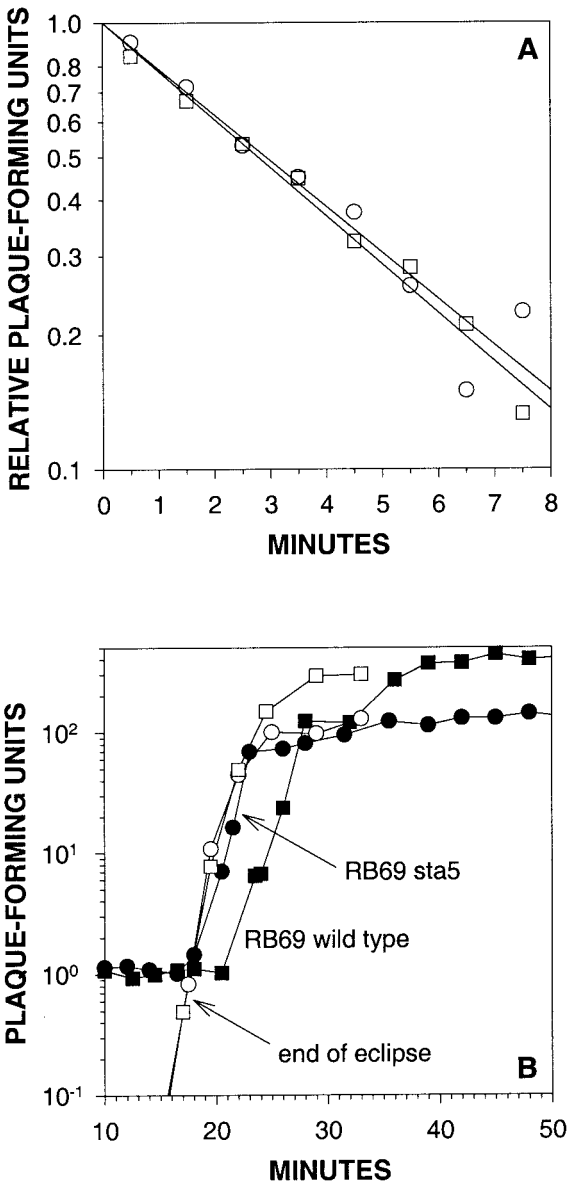

FIG. 2. RB69 sta5 phenotypic comparison. RB69 WT is shown as squares, while RB69 sta5 is shown as circles. (A) Phage adsorption to *E. coli* determined in broth via the chloroform-lysis method. (B) Single-step growth curve (solid symbols) with bacterial lysis induced by phage (lysis from within [41]) versus equivalent chloroform-lysis experiment run in parallel (open symbols).

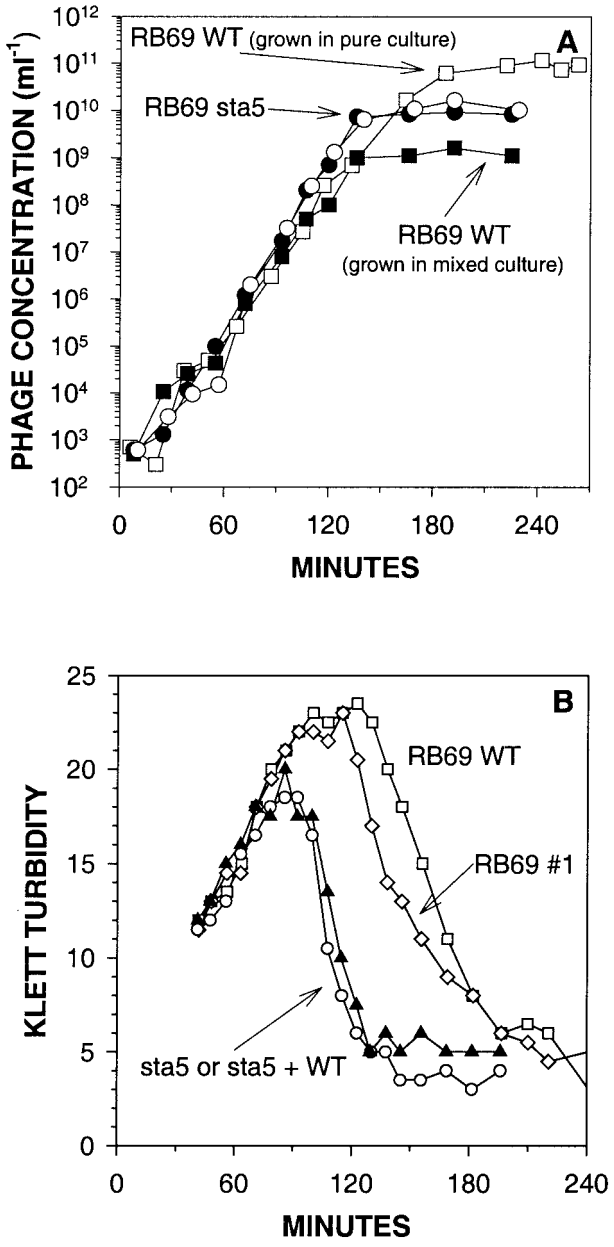

FIG. 3. Mixed- and pure-culture competition. Shown are RB69 WT (□), sta5 (○), and RB69 mutant #1 (◇) growing from initially low densities (initial bacterial density of ~10^7 bacteria/ml and initial phage multiplicity of ~0.0001; e.g., as for phage stock preparation in broth). Pure cultures are shown as open symbols, and 1:1 sta5-WT mixed cultures are shown as solid symbols. Panels A and B represent experiments run on different days. Note that the sta5 mutant and WT phages produce plaques that are easily distinguished upon visual inspection (A).

than that the sta5 mutant displays an SLP and a commensurately smaller burst size.

Mixed-culture advantage. Unlike the experiments presented in Fig. 1 and 2, where infections were initiated by using an excess of phage, for Fig. 3 phage were added to bacterial cultures at low multiplicities. Consequently phage population growth could be followed within a bacterial-resource-copious environment. In pure-culture experiments—that is, one kind of phage-susceptible bacterium mixed with one kind of phage—phage RB69 WT produced a final titer that was ~8-fold greater than that which the sta5 mutant produced (Fig. 3A, open symbols). This larger titer presumably is a consequence of RB69 WT's larger burst size but also could be due to the WT phage displaying slower population growth, which would allow bacterial populations to grow to higher peak densities. That is, final phage titers should be equal to the product of total cells infected and the per-infection burst size such that greater phage-susceptible bacterial densities ultimately should result in higher phage titers (at least so long as bacterial densities are not so high that they negatively impact on phage burst sizes). As shown in Fig. 3B, greater bacterial growth, as determined by culture turbidity, does appear to occur given growth of WT versus sta5 mutant phage growth. Via bacterial viable counts, as determined in parallel to the experiments shown in Fig. 3A, we additionally observed more bacterial growth in the presence of WT phages than in the presence of sta5 mutant phages (data not presented).

WT RB69, through some combination of larger burst size and by allowing greater bacterial growth, clearly displays a pure-culture final-titer advantage over the sta5 mutant. Final titers themselves, however, are not a measure of rates of phage population growth. Instead, phage population growth occurs as a function of phage generation time as well as phage fecundity (i.e., burst size), with shorter generation times, like larger burst sizes, capable of supporting faster phage population growth (e.g., see reference 8). Since phage RB69 WT has the larger burst size (Fig. 2B), we therefore would predict that any growth rate advantage seen by the sta5 mutant versus WT would stem from the sta5 mutant's display of an SLP (one component of the phage generation time). From Fig. 3A, however, it is clear that differences between growth rates of RB69 WT and the sta5 mutant—functions of the upward slope of phage population increase—are small. With direct competition for the same bacterial resource, however, we expect one phage, the one displaying faster population growth, to reduce the frequency of the other simply by infecting more bacteria sooner. A resulting decline in the titer of WT relative to that seen with pure-culture growth would be consistent with a sta5 population growth rate advantage over WT.

A representative experiment addressing the above prediction is presented also in Fig. 3A (closed symbols). With mixed-culture growth—that is, mixtures of phage-susceptible bacteria, the sta5 mutant, and WT phage RB69—the sta5 mutant's final titer is unchanged from its pure-culture final titer (closed versus open circles in Fig. 3A). In the same mixed culture, however, the WT phage's final titer is considerably less than that of the sta5 mutant (closed squares versus closed circles). In Fig. 3B a similar experiment is presented, though with lysis timing determined by measuring culture turbidity, a function of unlysed bacterial densities. The figure clearly shows that culture lysis induced by sta5 (and mutant #1) occurs sooner than that induced by RB69 WT and that the sta5 mutant's phenotype is dominant over WT in terms of the culture-wide timing of lysis. Our interpretation of these results is that the two mutants display a small growth rate advantage over RB69 WT, at least when bacterial densities are relatively high, and that this growth rate advantage results in a faster acquisition of

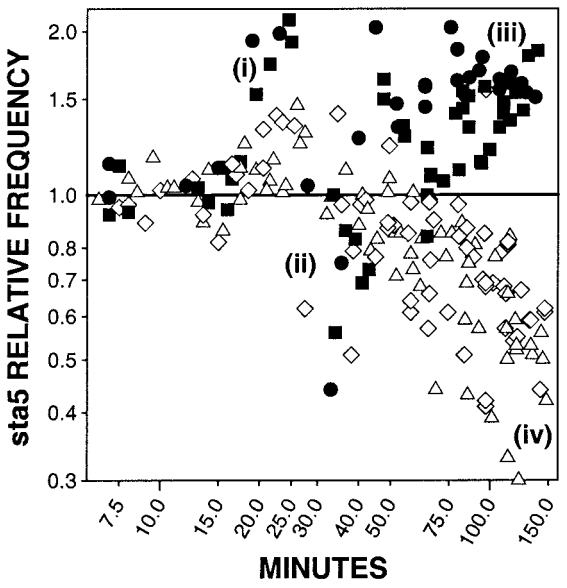

FIG. 4. Constant bacterial density competition. Log-phase *E. coli* was diluted to various densities in phage-containing fresh broth (to an initial phage density of $\sim 2 \times 10^3$ PFU per ml) with cultures, then split 1:1 to fresh broth every 30 min. Relative phage densities, RB69 sta5 versus WT, were determined as for Fig. 3A and are presented as the fraction of sta5 mutant (large plaques/total plaques) relative to the sta5 fraction at time zero (i.e., the sta5 "relative frequency" or rel-freq$_{sta5}$). Cultures are distinguished according to prelysis estimated arithmetic means of bacterial density: $\geq 10^{7.6}$ (●), $\geq 10^{6.6}$ (■), $\geq 10^{5.6}$ (◇), and $\geq 10^{4.6}$ (△). Roman numerals refer to (i) expected timing of post-sta5 lysis and pre-WT lysis, (ii) expected timing of post-WT lysis, (iii) approximate timing of lysis of all bacteria in higher bacterial-density cultures, and (iv) an ongoing cohabitation of cultures by phage and bacteria at lower initial bacterial densities.

bacteria. Consequently, while the sta5 final titer is little affected by the presence of phage RB69 WT, the WT final titer is greatly affected by the presence of phage RB69 sta5 (Fig. 3A).

Low-host-density disadvantage. While a high-host-density SLP advantage is strongly suggested in Fig. 3 ($\geq \sim 10^7$ bacteria/ml), this result does not serve as proof that the sta5 mutant's SLP is responsible for this advantage. Minimally, it is still necessary to show that the sta5 advantage is lost or, indeed, is reversed, given mixed-culture phage growth when host densities are lower. This consideration is addressed in Fig. 4. Shown are the results of three representative experiments where the sta5 mutant was competed against RB69 WT at various host densities. Curves are graphed as the ratio of sta5 phage (large plaques) to total phage, divided by this ratio at the time (zero) of initial phage mixing with bacteria. Thus, a sta5 relative frequency (rel-freq$_{sta5}$) is defined for different intervals as determined by the point of sampling such that a rel-freq$_{sta5}$ of >1.0 indicates an increase in the sta5 phage fraction (SLP advantage) while a rel-freq$_{sta5}$ of <1.0 indicates an increase in the WT phage fraction (LLP advantage). We identify four trends in this data as presented in Fig. 4:

(i) Around 20 min rel-freq$_{sta5}$ is greater than 1.0, corresponding to the initial lysis of sta5-infected bacteria. This increase is greater, given higher host densities, because a greater fraction of phage are infecting bacteria due to faster host adsorption.

(ii) Around 30 min rel-freq$_{sta5}$ falls below 1.0. This results from the lysis of WT-infected bacteria, which produce three or more times as many phage per bacterium as do sta5-infected bacteria (Fig. 2B). This WT advantage does not last for long, however, since by $\sim$40 min, at higher cell densities, a second round of sta5-induced lysis should begin.

(iii) In cultures containing higher bacterial densities (closed symbols), we ultimately see ratios of sta5 to WT in the range of approximately 3:1, up from a starting ratio of $\sim$1:1 [i.e., rel-freq$_{ta5}$ = 1.5 = 0.75/0.5 where 3:1 $\equiv$ 0.75, i.e., 3/(3 + 1), and 1:1 $\equiv$ 0.5, i.e., 1/(1 + 1)]. Though in theory we would expect the sta5 fraction of these cultures to continue to increase with time (8), in practice at higher host densities bacterial cultures are completely lysed by phage (e.g., Fig. 2B), which here results in a plateauing of rel-freq$_{sta5}$ by about 100 min.

(iv) Owing to an ongoing cohabitation of cultures by phage and not-infected bacteria, a plateauing of rel-freq$_{sta5}$ is not observed with lower bacterial densities (open symbols). Instead, rel-freq$_{sta5}$ declines from about 30 min onward, with a rel-freq$_{sta5}$ of 0.5 in Fig. 4 corresponding to a 1:3 ratio of large plaques (sta5) to small (WT).

These experiments successfully capture the SLP sta5 advantage at higher host densities but, more importantly, also show that this advantage is reversed when densities of bacterial resource are relatively low.

RB69 gene *t*. Bacteriophage holins, encoded by the phage gene *t*, are proteins that control the timing of phage-induced lysis and form holes in the bacterial plasma membrane that allow phage endolysins to reach the bacterial-host cell wall (42). A number of mutations in the T4 gene *t* confer LLPs (31). As a preliminary genetic characterization of RB69 sta5, we sequenced its gene *t* and compared it to that from RB69 WT as well as to a variety of additional T-even-like phages (Fig. 5 and 6). We note the following:

(i) There are two start codons near the beginning of RB69 gene *t*, separated by three sense codons (Fig. 5). This situation is similar to phage λ's holin-gene, *S*, which also displays two start codons at its beginning, resulting in the production of two different proteins, one (105 amino acids long) a lysis effector and the other (107 amino acids long) an inhibitor of the shorter protein (10). For phage RB69, however, only the second ATG is downstream of a consensus Shine-Dalgarno ribosome binding site (GGAGG [Fig. 5]). For sequence numbering of gene *t* (below) we therefore start with this second ATG. In addition to the two gene *t* start codons, we also note that the intergenic region between genes 38 and *t* of RB69 is larger than that of other T-even-like phages sequenced in this region (45 nucleotides versus 20, 30, 31, or 33), except for that of the newly sequenced phage 44RR2.8t (71 nucleotides—though there the upstream gene is not 38). The RB69 intergenic region also contains more stop codons, with three in frame with the upstream gene 38 plus two out of frame for RB69. This compares to a range of zero to one in frame and zero to three out of frame for the other phages sequenced (Fig. 6).

(ii) Gene-*t* translations from phage AR1 (43), Ox2 (29), TuIb (28), K3 (32, 33), and T4 (29) all possess a predicted isoleucine at amino acid position 39. On the other hand, the gene-*t* translations of the apparently lysis inhibition-defective

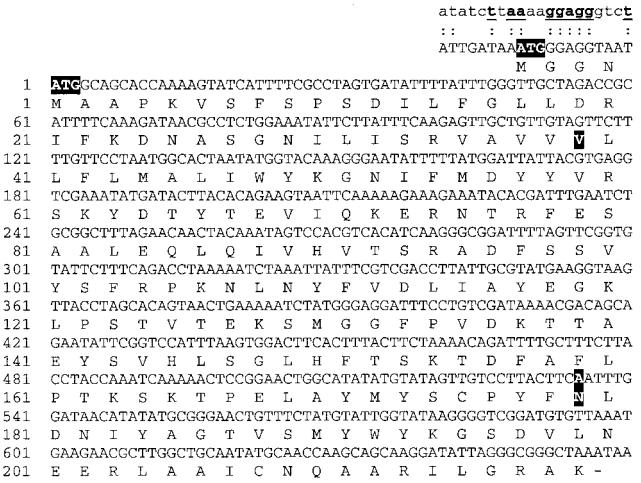

FIG. 5. Phage RB69 gene *t*. At top is a comparison of T4's intergenic region (lowercase) with RB69's (uppercase). Boldfaced and underlined sequences are consistent with other T-even-like phages for which sequence is known (Fig. 6). Highlights of subsequent RB69 sequence (in order) are (i) start codons (note that the first 11 nucleotides of the reading frame starting with the second ATG are identical for all phages listed in Fig. 6, except AC3 and T2, for which this sequence is not known, and RB49 and 44RR2.8t, for which overall sequence is highly divergent), (ii) amino acid position 39 (Val in RB69, Iso in others), (iii) nucleotide position 535 (A in RB69 WT and G in sta5), and (iv) amino acid position 179 (Asn in WT and Asp in sta5). Translated, RB69's gene *t* shares 71.7% identity with phage T4's versus 72.1% identity at the nucleic acid level.

RB69 WT and RB69 sta5 (Fig. 1) and the protein T (also a translation) of the lysis inhibition-defective T4 *rV* mutant dubbed "r2" (16) all display a predicted valine (Fig. 5). This correspondence at this amino acid position between RB69 and the phage T4 *rV* mutant is suggestive that our failure to observe phage RB69 displaying lysis inhibition (Fig. 1) could be associated with the presence of this T4 *rV* phenotype-associated amino acid.

(iii) For phage RB69, as well as the equivalent aligned position for WT phages 44RR2.8t, AR1, K3, RB49, and T4, an asparagine is found at position 179 of gene-*t* translations. For RB69 sta5, however, an aspartate is found instead at this position (Fig. 5). Since gene-*t* missense mutations have previously been associated with lysis timing defects (31), we speculate that this missense difference between RB69 sta5 and RB69 WT (A535G) could underlie observed lysis timing differences between these phages (Fig. 1). Note, however, that the identification of this genetic difference between these phages does not preclude the existence of additional as-yet-unidentified genetic differences, some of which could also underlie lysis timing differences. Of interest, a predicted tyrosine substitution at the same position, 179, in T4's protein T appears to result in an LLP (31).

DISCUSSION

Theory suggests that phage displaying SLPs can possess both larger plaques (25, 40) and—in broth at higher host densities—a population growth rate advantage over phage displaying LLPs (1, 8, 11, 27, 37). Here we describe a phage RB69 SLP, large-plaque variant, dubbed sta5, that we isolated following repeated serial transfers of phage RB69 WT to bacterium-containing broth cultures. Compared to RB69 WT we find that the sta5 mutant displays an SLP (~70 to 80% of WT), a commensurately smaller burst size (~30% of WT), and essentially identical eclipse period and adsorption kinetics (Fig. 2). The phage T4 holin (*t*) gene is thought to control the timing of phage-induced bacterial lysis (31), and upon sequencing we have found a single missense difference between the gene *t* of phage RB69 WT and that of the sta5 mutant (Fig. 5 and 6).

In cultures initiated with similar densities of both RB69 sta5 and WT (i.e., same-culture competition) the sta5 mutant ap-

FIG. 6. Comparison of 38-*t* intergenic region (shown unshaded). Presumptive gene *t* start codons (second start codon for phage RB69) and first gene-38 stop codons are shaded black. Double-underlined stop codons are in frame with gene 38, while other stop codons, not in frame, are singly underlined. The first RB69 start codon is shown unshaded but is boldfaced and underlined. Consensus sequence for phages T4, TuIb, and AC3 through T6 is shown as uppercase text. Sequences are arranged first by the similarity (at the DNA level) of their gene 38 (with T4 and TuIb's gene 38 both very different from those of phages RB69 through T6), with phage RB69 shown next, and with AC3 through T6 arranged alphabetically (and forming a second group based on gene 38 similarity). Also shown is the pseudo-T-even phage RB49 (13) and phage 44RR2.8t, which apparently do not possess a gene 38 in the same position as these other phages. Note that the RB69 gene 38 DNA sequence is not an outlier from the gene 38 sequence of phages AC3, AR1, K3, M1, Ox2, T2, and T6. Sequence references are shown parenthetically and in Materials and Methods.

pears to display a significant growth advantage over WT (Fig. 3). This sta5 advantage is present given relatively high bacterial densities ($\geq\sim10^7$; e.g., as may be observed for *E. coli* within mammalian colons prior to the formation of feces [1]) but is lost if bacterial densities, under the conditions employed here, are reduced to less than $\sim10^6$ bacteria/ml (Fig. 4). These results are qualitatively consistent with hypotheses that phage with SLPs, despite displaying smaller burst sizes, can exhibit a within-culture, broth growth advantage so long as bacterial densities are sufficiently high (1, 8, 11, 27, 37). It is difficult to extend this consistency to a more quantitative corroboration between theory and experimentation, however, since differences in population growth rates between phage RB69 WT and sta5, as we have observed (Fig. 3), are smaller than differences between actual and predicted growth rates as presented by Abedon et al. (8).

Though providing a selective benefit at higher bacterial densities (Fig. 3 and 4), shorter generation times still come at a fecundity cost (Fig. 2B). Indeed, during stock preparation the sta5 mutant is quite "sick," with WT stocks typically displaying titers that are fivefold or greater than sta5 stock titers. This fecundity cost should be felt not only when bacterial densities are low (Fig. 4) but also when free-phage decay rates are high (22). For example, a 0.01 survival rate (0.99 prereproduction rate of decay) would reduce a burst size of 100 to just 1, which represents a population growth rate of zero. The same decay rate would reduce a burst size of 300 to 3, implying instead a threefold population increase per phage generation. Of perhaps greater relevance, high phage decay rates as well as significant phage dilution could reduce the likelihood of greater-than-one phage multiplicities of transmission between bacterial cultures. LLP phage, upon repeated low-multiplicity dispersal to unexploited (i.e., phage-free) bacterial cultures, therefore—given the greater LLP-phage productivity when grown absent within-culture competition—could come to dominate extended phage populations, even if bacterial densities are habitually high within individual cultures (Fig. 3A). Similarly, the marginal value theorem from optimal foraging theory (12, 35), as has been applied to phages elsewhere to derive within-culture optimal latent periods (37), suggests that greater distances or costs between exploitable environments should select for more complete, e.g., LLP-like (Fig. 3A) exploitation of resources within individual environments.

Selection for SLP phage can also be viewed from the perspective of later-offspring discounting (22): offspring produced sooner can be more valuable but only if they themselves can quickly contribute to phage population growth. A quick contribution of phage offspring to population growth between environments, however, would be the case only if environments are sufficiently close together. As exploitable resource-containing environments become ever closer, then a well-mixed total environment is increasingly approximated, which is just the situation in which we would expect SLP phage to outcompete LLP phage (Fig. 3 and 4 and reference 8). In other words, if phage habitually initiate growth as significant-sized populations within bacterium-containing environments and if within-culture bacterial densities also are sufficiently high, then we may expect SLP phage to maintain a mixed-culture advantage both within and between cultures. Just such conditions were approximated during our original Hershey-type (21) serial transfers that enriched our RB69 stock for SLP phage.

The possibility of selection between cultures for LLP phage helps explain why, by and large, lytic phage do not display latent periods that are nearly as short as their eclipse periods, i.e., as we observe here with phage RB69 sta5 (Fig. 2B). That is, any selective advantage displayed by the very short sta5 latent period probably should be interpreted as a consequence during serial passage of a relaxed selection for more effective phage transmission between cultures (26). Thus, on the one hand SLP phage appear to be specialists for within-culture competition and then only when bacterial densities are sufficiently high. On the other hand, we suggest that LLP phage may be the more effective strategists, regardless of within-culture bacterial density, in terms of low-multiplicity transmission between cultures. We expect, therefore, that actual phage latent periods will represent an adaptive compromise between conflicting selection for SLPs when bacteria within cultures are increasingly available and for LLPs if new cultures are necessary for continued phage propagation and challenging to acquire.

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