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Ecophysiology of Bacteriophage S5100 Infecting *Halobacterium cutirubrum*

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Increasing salinity reduces burst size and increases the latent period of infection of *Halobacterium cutirubrum* by lytic bacteriophage S5100. Cells become reversibly and persistently infected at saturation-level concentrations of NaCl. We propose that high salinity provides a natural refuge for sensitive host bacteria and that phage S5100 acts as a scavenger, proliferating when host viability is threatened by dilution of the environment.

The discovery of a high incidence of bacteriophage particles (3) and phage-infected bacterial cells (9) in aquatic ecosystems has focused attention on the possible roles and ecological significance of phages. The principles that govern natural phage populations and account for their coexistence with susceptible hosts remain largely unknown (6, 8). One factor that may contribute to the coexistence of hosts and virulent phages is the existence of a refuge in which the host is safe from phage attack (2, 8). The refuge may be thought of as either a physical portion of the niche of the host or a physiological or environmental condition that renders genetically susceptible cells resistant. In mathematical models of phage-host populations, the existence of a refuge reduces oscillations that may lead to extinction (8).

Another possible mechanism leading to stable coexistence of phage and host is the ability of the phage to act principally as a scavenger that somehow recognizes and attacks cells that are not likely to contribute genes to future populations because of environmental or physiological factors. Phage growth within and lysis of such cells would not result in selective pressure against the susceptible host genotypes (4).

The concepts of virulent phages acting as scavengers and of their host populations possessing refuges provided a plausible explanation for the variations observed in the population densities of phage infecting *Halobacterium cutirubrum* NRC 34001 in a natural brine pool of variable salinity (12). Phage population densities were observed to be low during periods of high salinity, which coincided with very dense host populations. When salinity was reduced dramatically, thereby causing severe damage to the salinity-dependent (7) host population, phage population density increased significantly, suggesting that high salinities (near saturation) provide a refuge for the host, whereas the phage may multiply significantly on a host population doomed to destruction by dilution of the environment. In this study, we sought to determine whether the ecophysiological properties of a phage isolate were consistent with the observed behavior of natural populations.

A phage enrichment culture was prepared with a 100-ml sample of brine from the Little Salt Pond in Yallahs, Jamaica (where population densities of halobacteria fluctuate dramatically because of changes in salinity), and a 900-ml log-phase culture of *H. cutirubrum* NRC 34001. A phage isolate (phage S5100) was obtained and was determined to belong to Bradley's morphological group A1 (10), as described previously (5). To determine the effect of salinity on bacterial and phage growth, 3.0 M NaCl (3.0 S), 3.5, 4.5, and 5.2 S broth media were prepared having the same composition as 3.5 S broth (5), except that the pH was adjusted to 6.5 and that 3.0, 4.0, 4.5, and 5.2 M NaCl was used, respectively. Plates of 3.0, 4.0, 4.5, and 5.2 S agar were prepared by adding 1.2 to 1.5% agar (Difco) to the respective broth media. Agar plates were routinely dried prior to use to remove excess moisture, a process that caused their salt concentrations at the time of use to be about 5% higher than the figures noted above.

The plating efficiencies of phage S5100 on 3.0, 3.5, 4.0, and 4.5 S agar were determined to be 0.4, 1.0, 0.7, and 0.001, respectively, indicating that multiplication of this phage is reduced at high salinities. The effect of salinity on phage adsorption was determined by assaying for unadsorbed phage (1). Phage was added to mid-log-phase *H. cutirubrum* cells that had been grown at the same salt concentration as was used in the adsorption experiment and that had been washed once by centrifugation and suspended in fresh prewarmed medium to give approximate final concentrations of 10^9 viable cells ml^{-1} and 10^8 PFU ml^{-1}. The resulting suspension was maintained at 38°C with gentle agitation. At intervals, the number of unadsorbed phage was determined by diluting with low-salt (LS) buffer (pH 7.4) containing 0.05 M $MgSO_4$–0.02 M Tris and then plating on 3.5 S agar. LS buffer lyses cells of *H. cutirubrum* but does not affect the viability of phage particles. Titers of unadsorbed phage plus infective centers were obtained by dilution with high-salt (HS) buffer containing 3.5 M NaCl, 0.1 M $MgSO_4$, and 0.02 M Tris (pH 7.4). HS buffer maintains the viability and cellular integrity of halobacteria. No significant difference in the rate of phage adsorption was detected over a salinity range of 3.0 to 5.2 M NaCl.

The roles of intracellular stages of phage development and of lysis of host bacteria in mediating the effect of high environmental salt concentrations were investigated by determining single-step growth kinetics of phage S5100 in 3.0, 3.5, and 4.5 S broth. A total of 10^9 viable cells ml^{-1} were first allowed to adsorb 1×10^8 to 2×10^8 PFU ml^{-1} for 1 h as described above; this was followed by a 10^{-3} dilution into fresh prewarmed medium. The resulting suspensions were diluted at intervals with HS buffer and LS buffer and were plated on 3.5 S agar to obtain the number of free phage plus infective centers and the total number of complete phage particles, respectively.

Figure 1a through c shows that the length of the phage eclipse period depends on the NaCl concentration. Eclipse periods of 3½, 5½, and 9½ h were obtained in 3.0, 3.5, and

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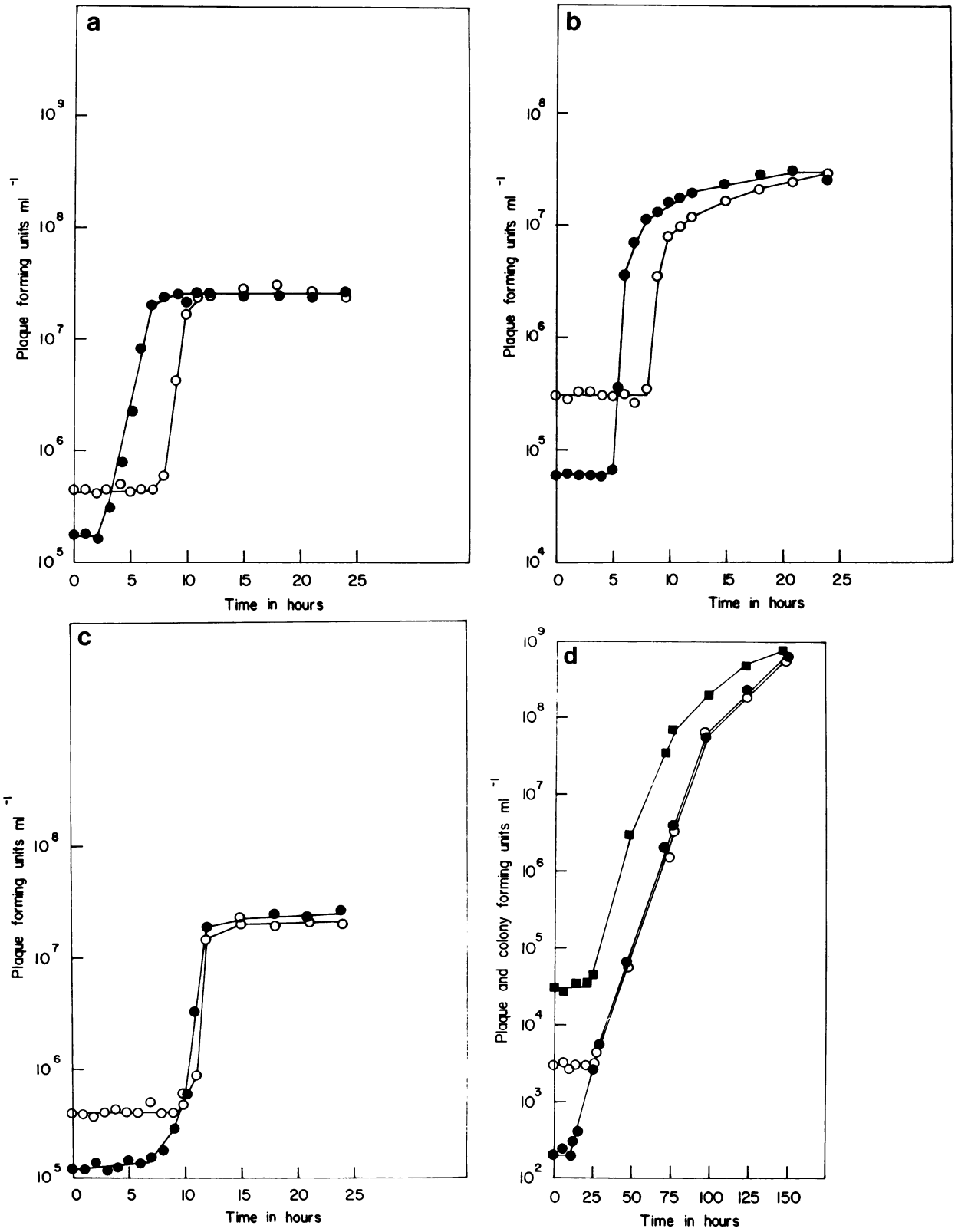

FIG. 1. (a through c) Single-step growth of phage S5100 on *H. cutirubrum* at 3.0, 3.5, and 4.5 M NaCl, respectively; (d) persistent infection of *H. cutirubrum* by phage S5100 at 5.2 M NaCl. Symbols: ○, extracellular phage and infective centers; ●, extracellular and mature intracellular phage particles; ■, CFU on 4.5 S agar.

4.5 S media, respectively. The release of phage progeny commenced after latent periods of 9 h at 3.0 and 3.5 M NaCl, compared with 11 h at 4.5 M NaCl. The time span between the first appearance of intracellular phage particles and the complete release of progeny phage is significantly shorter at 4.5 than at 3.5 and 3.0 M NaCl, as is illustrated by values of 2½, 5½, and 7½ h, respectively. The sudden appearance of mature phage progeny prior to lysis in 4.5 M NaCl suggests the presence of a novel type of control function, perhaps acting at the level of phage assembly. Figure 1a through c also shows that the apparent average burst size decreases from 125 at 3.0 M to between 60 and 65 phage per infected bacterium at 3.5 and 4.5 M NaCl.

Figure 1d shows the results of a similar S5100 growth experiment in which infected cells were diluted by a factor of 10^{-5} in prewarmed 5.2 S medium (i.e., medium saturated with NaCl). After an eclipse period of approximately 12½ h, the maturation of phage particles began to produce an average of one complete particle per infected cell, a fact indicated by almost equal values being obtained for PFU diluted in HS buffer and for LS-buffer-resistant PFU. After the latent period and throughout the remainder of the experiment, the values for PFU diluted in HS buffer and viable counts of bacteria in the infection mixture increased in parallel, suggesting that the rise in the value of HS-buffer-diluted PFU is probably due to the ability of phage-infected bacteria to divide. The bacteria thus appear to be persistently infected in much the same way that cells of *Halobacterium salinarium* 1 are infected by phage Hs1 (11).

Phage multiplication did not retard the growth of cells, since similar generation times and growth lags were obtained with an uninfected cell suspension. Similarly, when host cells were infected with phage S5100 in 5.2 S broth and plated on 4.5 S agar, the majority survived and formed colonies that also contained phage. When the infected cells were plated on 3.0 or 3.5 S agar, there were few surviving colonies and they were not associated with phage (data not shown).

The state of persistent infection by phage S5100 induced by growth in 5.2 S broth is readily reversible when the salt concentration is reduced. Figure 2 shows the results of diluting a persistently infected culture of *H. cutirubrum* by a factor of 10^{-1} in 3.5 and 5.2 S prewarmed media. In 3.5 S broth, maturation of new phage particles begins immediately, although initially at a slower pace than in a normal infection. Normal rapid maturation of phage particles begins 4 h after dilution, or 1½ h sooner than it does when cells are initially infected in 3.5 S medium (Fig. 1b). Lysis begins after a latent period of 5 h in the diluted culture, 4 h before extracellular phage is detected emerging from cells infected in 3.5 S broth. Figure 2 also shows that persistently infected cells diluted 1:10 into fresh 5.2 S broth fail to produce any phage for 8 to 12 h after dilution. This result is likely due in part to the infected cells' experiencing a growth lag that always accompanies dilution of *H. cutirubrum* into fresh medium (data not shown).

The results presented here suggest that high levels of salinity provide a refuge for hosts sensitive to phage S5100 by causing a reduction in the burst size and a progressive delay in the appearance of mature particles in infected cells. At saturation levels of NaCl (Fig. 1d), phage maturation is limited to an average of only one phage per cell; the cells continue to divide in a state of persistent infection, and the majority are not severely affected by the infecting phage. Persistent infection by phage S5100 is readily and rapidly reversed by reducing the salinity (Fig. 2), with a small but

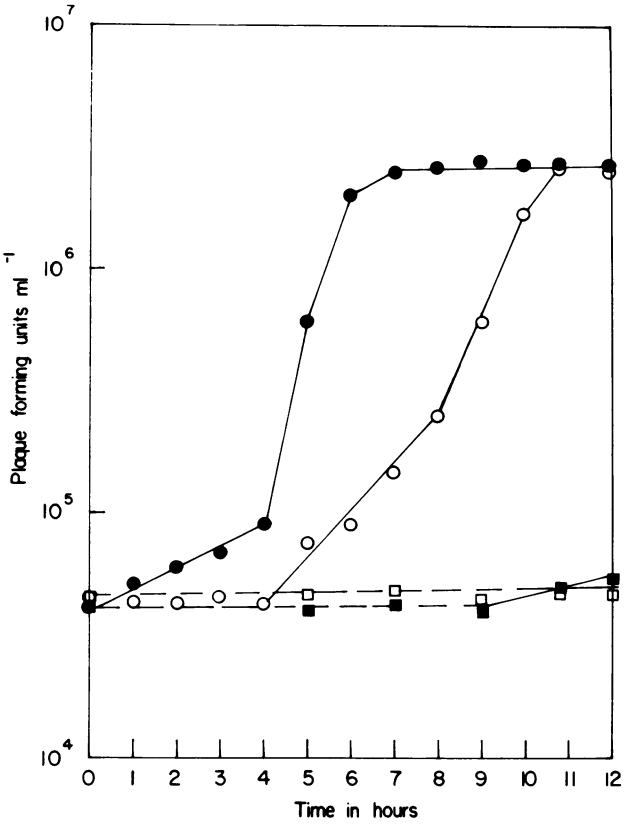

FIG. 2. Phage production in persistently infected cells of *H. cutirubrum* shifted from 5.2 to 3.5 M NaCl. Symbols: ○, extracellular phage and infective centers; ●, extracellular and mature intracellular phage in culture shifted to 3.5 S medium at time zero; □, extracellular phage and infective centers; ■, extracellular and mature phage in culture diluted in 5.2 S medium at time zero.

significant amount of phage maturation occurring immediately after the reduction in salinity. This suggests an ecological strategy whereby the phage adapts to proliferate on the host population immediately following the dilution of the environment to below saturation levels of NaCl. In the natural environment from which phage S5100 was isolated, this would allow it to act as a scavenger and to proliferate on an otherwise doomed host population, since very dense populations of host organisms are often destroyed by dilution of the environment following periods of heavy rain. Under natural conditions, dilution leading to host destruction takes place gradually enough to allow for phage maturation (and perhaps even growth), since the fresh water diluting the brine is of a much lower density than the brine and mixes in only gradually. The effects of salinity on phage growth reported here are not likely to be due to indirect effects on cell growth rate, since optimum growth occurs at a concentration of NaCl near 4.0 M, and the growth rate at 3.0 M NaCl is close to that obtained at 5.2 M NaCl. (Relative growth rates determined by measuring the rate of increase in optical density at 650 nm in 3.0, 3.5, 4.0, 4.5, and 5.2 S broth were 0.7, 0.8, 1.0, 0.8, and 0.6, respectively.) An NaCl concentration of 3.0 M is very close to the minimum required by *H. cutirubrum* for growth.

Torsvik and Dundas reported that halophage Hs1 enters a state of persistent infection at high salinity (11). Phage Hs1 differs from phage S5100 in that adsorption was strongly

inhibited at high salinity and no effect of salinity on the eclipse period was noted. The data for phage Hs1 are not easily interpreted in terms of the behavior of naturally occurring phage populations, since this phage occurred spontaneously in a culture of *H. salinarium* 1 that had been previously maintained in the laboratory for 18 years. Under these circumstances, some form of stable interaction between phage and host would be required for the phage to have survived up to the time of its discovery. Also, there may have been substantial opportunity for coevolution of phage and host to take place in the laboratory, given the long time that the host and phage were maintained in culture and the possibility of Hs1 undergoing the very high frequency of genetic rearrangements observed in another halophage, φH, which occurred spontaneously in a culture of *Halobacterium halobium* (13).

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