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Persisting Phage Infection in *Halobacterium salinarium* str. 1

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SUMMARY

Cultures of *Halobacterium salinarium* str. 1 are persistently infected with the virulent and extremely halophilic phage Hs1. The nature of phage infection depended on the salt concentration in the medium, changing from lytic to persistent as the salt concentration increased from 17·5 to 30% (w/v) NaCl. At salt concentrations below 25% (w/v) NaCl, phage infection resulted in a lytic development with phage production. The lytic development was characterized by a constant eclipse and latent period, irrespective of bacterial growth rate or salt concentration. At salt concentrations above 25% (w/v) NaCl phage infection resulted in the establishment of a carrier state in which lysis of the infected bacteria was delayed for several generations. In this carrier state the infected bacteria continued to multiply at the same rate as uninfected cells. Bacteria infected under conditions favouring lytic development could survive if transferred to a medium which favoured the formation of carrier cells. More than 77% of the bacteria infected with phage in a medium containing 20% (w/v) NaCl were able to form colonies if plated 90 min p.i. on agar plates containing 30% (w/v) NaCl. A majority of the colonies carried phage.

INTRODUCTION

Only a few bacteriophages for extremely halophilic bacteria have been isolated (Torsvik & Dundas 1974; Wais *et al.* 1975), and little is known about the relationship between *Halobacteria* and their phages (Wais *et al.* 1975; Torsvik & Dundas, 1978).

Bacteriophage Hs1 is host-specific and was isolated from a culture of *Halobacterium salinarium* str. 1 some 18 years after this bacterium was first isolated from salted codfish. The phage is extremely halophilic, being dependent on high concentrations of salt to retain structural integrity and activity. Cultures of *H. salinarium* str. 1 are persistently infected with Hs1. The co-existence of bacteria and phage has been observed in our laboratory for more than 5 years and the present evidence indicates that this bacterial strain has been infected since it was first isolated in 1958. Persistently infected cultures of *H. salinarium* str. 1 show two main characteristics: sporadic lysis of most bacteria accompanied by the massive release of phage particles, and the ability of infected bacteria to form colonies. Stable lysogenic bacterial clones have not been isolated (Torsvik, 1976; Torsvik & Dundas 1978).

The formation of phage-infected colonies from single infected bacteria suggested the existence of a carrier state in which the infected bacterium might continue to multiply for a limited period of time prior to lysis. We have investigated the salt dependence of phage production, phage adsorption and bacterial growth. In this paper we describe the effects of these parameters on the interrelations between phage and its bacterial host.

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METHODS

Organisms. *Halobacterium salinarium* str. 1.7, a phage-free bacterial clone isolated from an infected culture of *H. salinarium* str. 1, was used in all experiments. Bacteriophage Hs1 from an isolated plaque was cultivated in batch cultures with *H. salinarium* str. 1.7 until lysis occurred. The cultural lysates were treated with chloroform and bacterial debris removed by centrifugation. The resulting lysates containing phage were stored at 4 °C until used.

Growth media. All media contained 0·5% (w/v) of the following basal salts: KCl, NH_4Cl, $MgCl_2.6H_2O$ and $MgSO_4.7H_2O$, and as carbon sources 0·5% (w/v) Difco yeast extract and 0·25 % (w/v) Difco tryptone. NaCl (analytical grade) was added to the basal salt solution to obtain the desired salt concentration and the pH adjusted to 7·3 with 1 M-NaOH. Mineral salts and carbon sources were autoclaved separately. Solid media were prepared by adding 2% (w/v) Difco agar to the mineral solution before autoclaving. Top agar (TA) for the plaque test consisted of basal salts, 17·5% (w/v) NaCl, 0·01 M-tris-HCl and 0·8% (w/v) Difco agar, pH 7·3. Where concentrations of NaCl are referred to in the text the concentrations are given as percentage wt./vol. (w/v).

Growth conditions. Batch cultures were normally grown in 500 ml flasks containing 100 ml medium, incubated on a New Brunswick gyratory shaker at 250 rev/min at 37 °C. Bacterial growth was measured as the increase in light absorption at 600 nm (A_{600}). Phage adsorption and one-step growth experiments were carried out in 100 ml flasks containing 20 ml medium, incubated at 37 °C in a shaker water bath.

Viable counts. Viable counts of bacteria were made on agar plates containing 25% NaCl unless otherwise stated. Dilutions were made in complete medium with the same salt concentration as the growth medium. The inoculum was evenly distributed on the agar surface using an L-shaped glass rod. Plates were routinely incubated for 1 week at 37 °C before counting.

Plaque test. *H. salinarium* str. 1.7 was used as host. The host bacteria were incubated overnight in a medium with 17·5% NaCl and diluted to an A_{600} of 1·0 before use. Reagent tubes containing 3 ml melted TA were kept at 50 °C in a water bath. A 0·1 ml amount of phage suspension and 0·15 ml of host suspension were added and the mixture poured over a plate with 17·5% NaCl agar medium. Plates were incubated at 37 °C for 1 week before counting.

Adsorption of phage. The rate of phage adsorption was studied by assaying for unadsorbed phage as described by Adams (1959). Phage was added to a bacterial culture (10^8 to 10^9 bacteria/ml) which had been grown overnight in a medium with the same salt concentration as that used in the actual adsorption experiment. At the cell concentrations used the bacterial generation period exceeded 6 h regardless of the salt concentration in the medium. During the adsorption periods used (30 to 90 min, depending on the salt concentration) the bacterial concentration could thus be considered constant. Adsorption of phage was stopped by diluting the infected culture $1:10^5$ into fresh medium. Unadsorbed phage particles were determined after treatment of the diluted culture with chloroform.

One-step growth experiments. One-step growth experiments were performed with bacterial cultures grown overnight in media with the same salt concentration as that used in the actual experiment. Adsorption of phage was carried out as described above; the adsorption period was 30 min. In media with 20% or higher concentrations of NaCl, adsorption of phage was stopped by diluting the culture $1:10^5$ in fresh medium. Samples of 0·1 ml were subsequently diluted tenfold into fresh medium and assayed for total p.f.u. Phage particles were determined after treatment of the diluted culture with chloroform.

A $1:10^5$ dilution of cultures with 17·5 % NaCl resulted in rapid death of bacteria regard-

less of whether they were infected with phage or not. Phage adsorption at 17·5% NaCl was accordingly arrested by carefully washing the cells by centrifugation and diluting the washed culture 1:10 into fresh medium. After washing, viability was retained by more than 80% of the bacteria. The surviving bacteria grew without a lag phase at the normal rate for untreated bacteria in 17·5% NaCl medium.

Formation of phage-infected colonies. Cultures of *H. salinarium* str. 1.7 growing in 20% NaCl were infected with an excess of phage Hs1 and infection was stopped by diluting the culture into fresh medium. The average number of phage adsorbed to each bacterium was determined by assaying for unadsorbed phage in the diluted culture. Viable counts of bacteria were determined by plating on media with salt concentrations ranging from 17·5 to 30% NaCl. Colony numbers were determined after 18 days incubation at 37 °C. Presence of bacteriophages in the colonies formed on media with different salt concentrations was determined by inoculating each colony into liquid medium containing 17·5% NaCl, incubating for a week at 37 °C to ensure eventual phage production and assaying for phage after addition of chloroform to the cultures.

RESULTS

Isolation of phage-free bacteria

In initial experiments all bacterial clones isolated from stock cultures of *H. salinarium* str. 1 were found to carry phage. During these experiments agar plates were prepared with 23% crude solar salt. The agar surface was allowed to dry prior to inoculation and the growing colonies were thus exposed to varying salt concentrations well in excess of the initial 23%, even approaching saturation (Torsvik, 1976). Phage-free bacteria were later isolated from phage-infected colonies by pre-incubating the bacteria for 70 h in liquid media containing 25% NaCl and plating the growing bacteria on freshly prepared agar media with 25% NaCl. During these later experiments the growing bacteria were never exposed to salt concentrations in excess of 25% NaCl and 98% of the colonies were found to be free of phage. One phage-free bacterial clone, str. 1.7, was selected and used in all the following experiments.

Bacterial growth rates

Bacterial growth rates depended strongly on the salt concentration of the medium. In media with 17·5% NaCl generation periods of 11 to 13 h were generally recorded and in one experiment the generation period was 29 h. Increasing the salt concentration in the medium to 20% NaCl resulted in more rapid growth with generation periods of 8 to 10 h. Maximal growth rates were obtained in 25% NaCl with generation periods of 4 h. Such high growth rates were only observed at low cell densities in cultures containing 10^3 to 10^7 bacteria/ml, as used in experiments concerning the one-step growth of phage. At higher cell densities in late exponential growth phase, the generation period was 6 to 7 h even in media with 25% NaCl. In 30% NaCl the generation period was 5 h (dilute cultures) and 6 to 8 h (in late exponential growth phase).

Rate of phage adsorption

The rate of adsorption, and accordingly the adsorption rate constant *K*, was very dependent on the salt concentration (Fig. 1), decreasing with increasing salt concentration. Decreasing the salt concentration from 30 to 17·5% NaCl resulted in a 30-fold increase in the phage adsorption rate. At low salt concentration the adsorption rates approached the values found in other phage systems, for which adsorption rate constants in the range 10^{-8} to 10^{-9} ml/min are to be expected under optimal conditions (Adams, 1959).

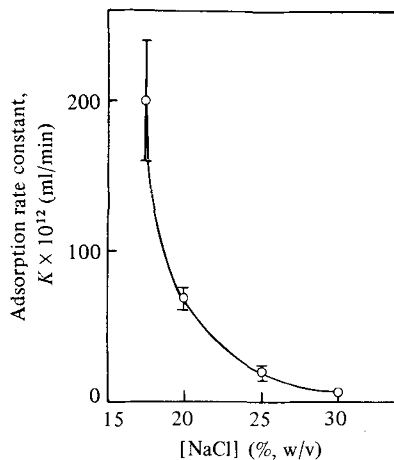

Fig. 1

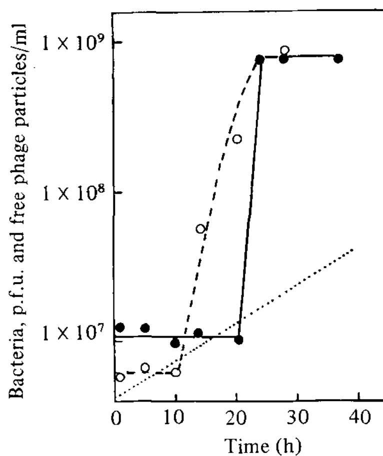

Fig. 2

Fig. 1. Rate of adsorption of bacteriophage Hs1 to *H. salinarium* str. 1: dependency of adsorption rate constant (K; ml/min) on salt concentration. K values at a specific salt concentration were calculated from the equation: $\ln (p/p_0) = -KBt$ in which p/p_0 is the fraction of unadsorbed phage, B the bacterial concentration and t the adsorption period. Plots of $\ln (p/p_0)$ as a function of time were prepared and the best fit line calculated using linear regression analysis. K values were calculated from the slopes of the regression lines. Average values for seven determinations at 25 % NaCl and two determinations at the other salt concentrations are shown.

Fig. 2. One-step growth of Hs1 on *H. salinarium* str. 1.7 in 17·5 % NaCl. 4×10^7 bacteria/ml were infected with 2×10^9 phage/ml and adsorbed for 30 min. Adsorption was stopped by washing the bacteria once by centrifugation and subsequent dilution 1:10 into fresh medium. ●——●, P.f.u.; ○ – – – ○, number of free phage particles after chloroform treatment; . . . , growth of uninfected bacterial control culture (cells/ml).

One-step growth of phage

Results from one experiment carried out in 17·5% NaCl are shown in Fig. 2. The results from this experiment and from additional experiments carried out at salt concentrations of 17·5, 20 and 25% NaCl are shown in Table 1. The eclipse period was not influenced by the salt concentration and lasted for an average of 12 h. The latent period was also independent of salt concentration and lasted an average of 17 h. The lytic period from the onset of phage liberation to the complete lysis of infected bacteria varied from one experiment to another, ranging from 4 h (at 17·5% NaCl) to 32 h (at 25% NaCl). The average burst size, irrespective of salt concentration or the duration of the lytic period, was 324 ± 134 phage per bacterium. In contrast the number of bacteria produced from a single ancestor during one lytic cycle increased by three orders of magnitude as the salt concentration increased from 17·5 to 25% NaCl.

Results from a representative experiment carried out in 30% NaCl are shown in Fig. 3. At such high salt concentrations the events taking place after phage infection were markedly different from those occurring at lower salt concentrations. The values for p.f.u. and the viable counts of bacteria increased in parallel, and the p.f.u. values remained roughly one order of magnitude higher than the number of free phage throughout the experiment. At the bacterial and phage concentrations used, adsorption of phage was completely arrested and secondary infection of bacteria could be ruled out. The rise in p.f.u. values therefore reflected the ability of phage-infected bacteria to divide. During a period exceeding 32 h, corresponding to six bacterial generations, the multiplication of infected bacteria took place at a rate equal to that of uninfected bacteria.

Table 1. *Production of phage and bacteria in media with different salt concentrations**

NaCl (%, w/v)	Eclipse period (h)	Latent period (h)	Lytic period (h)†	Burst size	Bacterial generation period (h)	Bacterial cells formed per lytic cycle‡
17·5	12	17	14	300	29§	2·1
17·5	10	21	4	150	12	4·2
20·0	12	15	15	310	8	13·5
20·0	10	17	14	470	9	10·9
25·0	13	15	23	300	4	724
25·0	14	18	18	210	4	512
25·0	—	18	32	530	4·5	2200

* Experiments were peformed at different times, with different salt concentrations.

† Lytic period is the length of time (h) from the first release of phage until there is no further increase in the number of phage particles.

‡ Number of bacterial cells formed from one ancestor during one lytic cycle.

§ The exceptionally long generation period of 29 h in one experiment at 17·5 % NaCl may reflect minor variations in the salt concentration which in this case was close to the lower limit for bacterial growth.

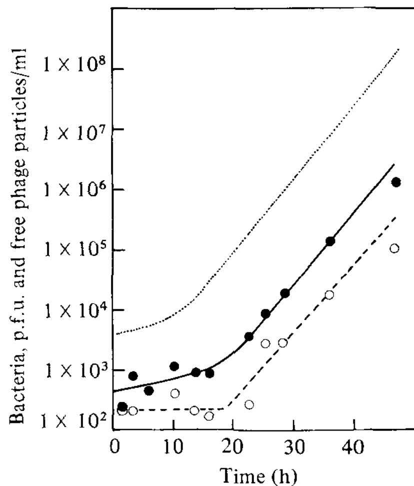

Fig. 3. Growth of *H. salinarium* str. 1.7 infected with phage Hs1 in 30 % NaCl. 8×10^8 bacteria/ml were infected with 6×10^8 phage/ml and adsorbed for 90 min. Adsorption was stopped by diluting the culture 1 : 10^5 into fresh medium. ● —— ●, P.f.u.; ○ - - - ○, number of free phage particles; . . . , growth of uninfected control culture.

The carrier state

Viable counts of bacteria obtained from phage-infected cultures varied with the salt concentration in the agar medium used for plating (Table 2). A bacterial culture was infected with an excess of phage in a medium containing 20% NaCl (favouring a lytic development of phage). After the adsorption period of 90 min an average of 1·8 phage particles had adsorbed to each bacterial cell, ensuring the infection of most bacteria in the culture. Immediately after the adsorption period the infected culture was assayed for surviving bacteria. Viable counts obtained using agar plates with 17·5 to 25% NaCl indicated that only 12 to 14% of the bacterial cells had survived the infection. Viable counts obtained using agar plates with 27·5 to 30% NaCl indicated that as many as 50 to 77% of the bacterial cells survived after the infection. While only few of the colonies

Table 2. *Colony formation by phage-infected bacteria**

NaCl (%, w/v)	Viable count/ml non-infected culture (× 10⁻⁶)	Viable count/ml infected culture (× 10⁻⁶)	Survival (%)	Number of phage-infected colonies (× 10⁻⁶)
17·5	540	78	14·4	—
20	565	67	11·9	9
22·5	623	72	11·6	—
25	643	80	12·4	12
27·5	525	265	50·5	89
30	450	347	77·1	260

* A culture of *H. salinarium* str. 1.7 containing $6{\cdot}4 \times 10^8$ bacteria/ml was infected with $1{\cdot}2 \times 10^9$ phages/ml in medium containing 20 % NaCl; 90 min after the addition of phage the culture was diluted to stop phage adsorption and the number of surviving bacteria in the culture was determined by plating on agar plates with salt concentrations ranging from 17·5 to 30 % NaCl. The colonies formed were counted after 18 days incubation at 37 °C. Colonies were tested for phage infection by subculturing in a medium with 17·5 % NaCl prior to assaying for plaque formation.

formed by surviving cells on agar plates with 17·5 to 25% NaCl carried phage, as many as 75% of the colonies formed on agar plates with 30% NaCl carried phage (Table 2). Increased salt concentration in the plating media thus enhanced the ability of phage-infected bacterial cells to multiply and form colonies.

DISCUSSION

Initially the presence of a temperate phage was proposed to account for the occurrence of phage-infected colonies of *H. salinarium* str. 1 (Torsvik, 1976; Dundas, 1977) but attempts to demonstrate the presence of a temperate phage in infected cultures of *H. salinarium* str. 1 were unsuccessful (Torsvik & Dundas, 1978). We therefore concluded that the persistent phage infection was established by interactions between a virulent phage and its host.

The results presented above show that there was a change from lytic to persistent infection as the salt concentration increased from 17·5 to 30% NaCl. The effect of lytic growth of phage on the bacterial population could be described by three parameters: (i) the rate of phage adsorption, (ii) the rate of phage production and (iii) the rate of bacterial growth. The salt concentration influenced these parameters in different ways as described below. The rate of phage adsorption decreased rapidly with increasing salt concentration (Fig. 1). The rate of phage production per cell per unit time was quite independent of salt concentration below 25% NaCl. The eclipse period and the latent period remained constant independent of salt concentration. The burst size varied by a factor of three, but increased burst sizes were largely counterbalanced by increased lytic periods, resulting in fairly constant rates of phage production. The bacterial growth rate on the other hand increased rapidly with increasing salt concentration, reaching a maximum at 25% NaCl. At this salt concentration the production of bacterial cells was faster than the production of phage by infected bacteria (Table 1). Phage production was accordingly favoured at low salt concentration while bacterial production was favoured at 25% NaCl and above.

At salt concentrations above 25% NaCl a high proportion of the bacteria entered a carrier state. The increase in p.f.u. in media with 30% NaCl (Fig. 3) showed that infected bacterial cells were able to multiply at the same rate as non-infected cells. From the number of free phage particles the probability of spontaneous lysis could be calculated to be 1×10^{-4} per bacterium per generation assuming a burst size of 150 phage particles per bacterium.

This value is comparable to those found for lysogenic bacteria (Adams, 1959) and illustrates the stability of the *Halobacterium*/Hs1 carrier state.

Infection of bacteria in the presence of 20% NaCl resulted in the killing of 88% of the cells (Table 2). Plating the infected bacteria on agar with more than 25% NaCl increased the chances of survival; 77% of the cells survived when plated on agar with 30% NaCl. Most of the cells survived by establishing a carrier state, as shown by the presence of phage in 75% of the colonies. The remaining 25% of the colonies could be due to uninfected cells (about 16%) and to cured cells (about 9%, calculated from the data given in Table 2). On agar plates containing 25% NaCl or less, 10% of the surviving bacteria carried phage. This indicated that a carrier state was established to a certain extent also at these salt concentrations. Carrier states in which the infected bacteria continues to divide for a limited period of time have been described in other phage/bacterium systems (Fraser, 1957; Zinder, 1958; Bott & Strauss, 1965; Kawakami & Landman, 1968). In several carrier systems the persistence of phage infection is also ensured by the very slow adsorption rate of the phage (Li *et al.* 1961; Jones *et al.* 1962; Barksdale & Arden, 1974). In the *Halobacterium*/Hs1 system the slow phage adsorption, the long latent period of the phage and the delayed lysis of the bacteria may lead to an equilibrium between phage production and bacterial growth. This mechanism would ensure the persistence of phage infection also at intermediate salt concentrations.

The change from lytic to persistent infection may be of ecological importance for bacteria and phage living in a solar saltern, the natural habitat of *H. salinarium* str. 1. During the salt production process the salt concentration in the terminal solar pans may approach the lower limit for the existence of *Halobacterium*. This is also the salt concentration which favours the production of phage particles, thus maximizing the chances of phage survival. At saturating concentrations of salt the bacteria reach their optimal conditions for growth. At this salt concentration the carrier state is established, which simultaneously protects the bacteria from extensive phage-induced lysis and provides for the perpetuation of the phage. The *Halobacterium*/Hs1 phage system is therefore well adapted to the variations in salinity occurring in the salterns.

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