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Marine bacteriophage reproduction under nutrient-limited growth of host bacteria. I. Investigations with six phage-host systems

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ABSTRACT: Bacteriophage reproduction was investigated with 6 phage-host systems (PHS) isolated from the North Sea near Helgoland, Germany, with the hosts adapted to growth at 6 or 0.6 mg organic nutrients l^{-1}. For 5 of the PHS, similarities in production were observed to depend upon the time of infection during a transition period which included the last hours of the logarithmic growth phase and the first 1 to 2 d of the stationary growth phase of the host bacteria. Over this period the extent and/or the rate of phage production d^{-1} decreased greatly. After longer incubation before addition of phage, the relative ability of host cells to propagate phage either ceased (3 PHS) or was regained (1 PHS) or remained the same as during the transition period in regard to final phage concentrations (1 PHS). The remaining PHS showed no phage reproduction at the low nutrient concentration. With phage-resistant mutant bacteria serving as competitors for nutrients, phage production was drastically reduced. The present findings are in agreement with observations concerning concentrations of infective virions in fresh seawater samples. They failed, however, to provide evidence for the hypothesis that release of mature phage in starving marine bacteria is delayed until sufficient nutrients become available.

KEY WORDS: Virus · Phage · Bacteria · Reproduction

INTRODUCTION

For the first time in nearly 40 years in the field of marine bacteriophage research, considerable interest in marine virology was aroused as a result of the studies by Berg et al. (1989) and Proctor & Fuhrman (1990). By means of electron microscopy, these authors provided evidence that viral particles in marine water samples occur at concentrations of up to 10^7 particles ml^{-1} (Berg et al. 1989) and play an important role in the mortality of marine bacteria (Proctor & Fuhrman 1990). Meanwhile other reports (Suttle et al. 1990, Heldal & Bratbak 1991, Paul et al. 1991, Steward et al. 1992b, Wommack et al. 1992, Cochlan et al. 1993, Weinbauer et al. 1993, Jiang & Paul 1994, for review see also Børsheim 1993) confirmed that viral particles in marine waters are generally found at concentrations ranging between about 10^5 and 10^7 particles ml^{-1}, depending on season and location. Such high numbers by far exceed any of the estimations based on observations of marine bacteriophage employing biological methods (Moebus 1987, 1992a, b).

The observations of high concentrations of viral particles in marine environments give prominence to the long ignored importance of viruses as an integral part of marine life. However, data based solely on electron microscopy do not provide information about hosts and/or infectivity of the particles observed. Steward et al. (1992a, b), using radioactive tracers to estimate viral production, obtained highly important information regarding groups of host organisms (bacterial vs non-bacterial) as well as reproductive activity in inshore and offshore environments.

Information concerning the maintenance of specific phage-host systems (PHS) in nature must still be obtained by classical methods, i.e. the observation of

bacterial growth and reproduction of infective virions. With regard to marine PHS, apparently no investigations have been reported in which media with nutrient concentrations characteristic for marine environments were employed. Information is also scant regarding limnetic PHS. Kokjohn et al. (1991) reported greatly reduced burst size when host *Pseudomonas aeruginosa* was starved for about 20 h in autoclaved river water.

Maintenance of PHS depends on the presence of host cells and phage particles which are suited to each other and on physico-chemical conditions suitable for either phage reproduction or the establishment of lysogeny. However, beside this truism, our knowledge regarding the important parameters (burst size, latent period, physiological status of host cells, and others) related to phage reproduction is still very limited as far as this process in nature is concerned.

This paper deals with results obtained with 6 PHS incubated in synthetic seawater under conditions simulating natural ones in regard to organic nutrient concentration and competition for nutrients.

MATERIAL AND METHODS

Media. Synthetic seawater (SSW), prepared with ion-exchanged, quartz-distilled rainwater according to the formula given by Burkholder (1963), contained (per litre) 23.48 g NaCl, 10.61 g $MgCl_2 \cdot 6H_2O$ (*), 3.92 g Na_2SO_4, 1.469 g $CaCl_2 \cdot 2H_2O$ (*), 0.66 g KCl, 0.19 g $NaHCO_3$, 0.096 KBr, 0.04 g $SrCl_2 \cdot 6H_2O$ (*), and 0.026 g H_3BO_3. Salts marked with (*) were dissolved separately.

For nutrient enrichments of SSW, a stock solution of 10 g Difco yeast extract l^{-1} and 50 g Difco peptone l^{-1} (YEP) was prepared with distilled water, autoclaved in 5 ml portions and stored in the refrigerator.

Seawater agar (SWA), soft seawater agar (sSWA), reduced seawater bouillon (SWB/5), and seawater mixture (SM) were of the same composition as given by Moebus (1992c).

Glassware. Before use, flasks, tubes and Erlenmeyer flasks used for the preparation of SSW or during experiments employing SSW were cleaned by an initial overnight treatment with HCl, rinsed with tapwater and placed in a hot bath containing the detergent Mucasol. After about 1 d the glassware was treated with several rinses of hot and cold tapwater, followed by at least 3 rinses with ion-exchanged rainwater and a final rinse with distilled rainwater. Cleaning with Mucasol was done after each use of the glassware, but treatment with HCl was repeated only once in a while. Sterilization was performed at 170°C for 4 h.

Table 1 Strains of host bacteria and bacteriophages used in this study. Plaque types: (1) 3–4.5 mm, translucent, sharp edge; (2) 1.0–1.2 mm, clear centre of up to 0.6 mm, increasingly turbid toward edge; (3) about 3 mm, clear, edge less sharp than with type 1; (4) about 1.5 mm, clear, sharp edge

Host	Isolated	Growth	Phage	Isolated	Taxonomic group	Plaque type
H2	1978	medium	H2/1	1978	Myoviridae	1
H3	1978	fast	H3/1	1978	Myoviridae	2
H11	1978	medium	H11/1	1978	Myoviridae	1
H40	1978	fast	H40/1	1978	Siphoviridae	3
H54	1974	slow	H54/1	1976	Siphoviridae	2
H85	1976	slow	H85/1	1976	Siphoviridae	4

Bacteria and bacteriophages. The strains used during this investigation are listed in Table 1. All were isolated from seawater collected near Helgoland, North Sea. The bacterial strains have been kept for years on SWA slants and were prepared for this investigation as described below. Preparation of phage lysates was as given by Moebus (1980). Only lysates containing at least 5×10^9 ml^{-1} of plaque-forming units (PFU) were used. Selection of phage-host systems was not according to the viral family of the phage as Table 1 might indicate (see 'Results').

Methods. Adaptation of host bacteria to low nutrient concentration was started only once, at the beginning of this investigation. Material from freshly grown colonies was suspended and diluted in SSW, then inoculated into SSW enriched with nutrients to 6 mg YEP l^{-1}. After a few subcultures, all 6 host strains grew well at this YEP concentration. Adaptation to lower nutrient levels proceeded from such subcultures.

Subcultures of 5 ml (in tubes 18 mm in diam.) were started with about 10^3 colony-forming units (CFU) ml^{-1}. Dilutions in between were carried out in SSW. Initially the cultures were incubated in a tube roller at 1 rpm, later they were incubated as still cultures. No difficulties were encountered in growing the 6 strains at 6 mg YEP l^{-1}, and most of the strains grew successfully as still cultures at 0.6 mg YEP l^{-1}. When roll culture was used, adaptation from 6 to 0.6 mg YEP l^{-1} had to be repeated several times, as it also did with the more exacting strains in still culture. In these cases, new attempts were started from either the last or second to last subculture stored in the refrigerator.

If higher nutrient concentrations were to be used, 3 to 5 subcultures were run for adaptation, starting with inocula from subcultures with the lowest YEP concentrations available.

Generally, experiments with added phage were performed employing 100 ml Erlenmeyer bottles initially containing 18 or 20 ml of medium. These samples were either prepared before the start of an experiment or withdrawn from 200 ml batch cultures after different durations of incubation. In early experiments, the cultures (samples or batches) were incubated in a shaking water bath at 160 strokes min^{-1}, but later still culture was preferably employed (for details see 'Results'). Concentrations of bacteria at time zero were about 10^3 ml^{-1}. Phages were added at an appropriate time to give an initial titre of about 10^3 PFU ml^{-1}. Dilutions of bacterial and phage suspensions were carried out in SSW

Concentrations of CFU were determined by platings on SWA, those of PFU by means of double layer plates (Adams 1959). Dilutions were prepared in SM if SSW did not have to be used.

All incubations of liquid cultures were at 20°C in the dark. Plate cultures of bacteria and phage were incubated at 25°C. Plaque counts usually were done after 1 d, colony counts after 2 d of incubation.

Phage sensitivity of subcultures to be used in experiments was tested by plating 0.1 ml of undiluted culture over a narrow area on SWA and spreading a drop of high-titre stock of the respective phage strain on top of the dried-on bacteria. Sensitivity tests with colonies grown from experimental cultures generally were done by suspending a colony in 0.5 ml of SM and cross-streaking the suspension over dried-on high-titre phage suspension applied in several lines per plate.

After prolonged incubation, phage-resistant mutants were selected either from SWA heavily sown with high-titre phage stock and a dense suspension of a colony of wild-type bacteria or from liquid cultures. The latter were set up with wild-type bacteria and the respective phage in SWB/5 and incubated for about 24 h. At that time, most of the sensitive wild-type bacteria were lysed and a population of resistant mutant cells was established. They were retrieved by plating on SWA followed by several successive streaks of colonies on SWA and concomitant tests for resistance.

To examine for the presence of infected cells that could not release progeny phage prior to the availability of fresh nutrients, samples (or parts of samples) were replenished with YEP to adjust concentrations to between 30 and 0.6 mg fresh nutrients l^{-1}. If whole samples were used, the procedure took into account the volumes withdrawn for titrations and was controlled by weight determinations and addition of sterile distilled water to compensate for losses due to evaporation.

The term sample will be used throughout this paper only in connection with the phage-seeded 18 or 20 ml aliquots. Cultures of 100 or 200 ml volume will be referred to as batches.

RESULTS

Preparatory experiments

Initially, chemically defined media, such as that used in starvation experiments and for bacterial cultivation, and 2 sources of organic nutrients were tested. Among the defined media, the SSW of Burkholder (1963) was found to be preferable since it supported good growth of the bacteria and because of its relatively simple composition. As a nutrient source, a preparation made from heavily enriched plankton, predominantly composed of diatoms, was compared with YEP solution. With both substrates the same growth patterns were observed, but YEP was preferred because of reproducibility in its preparation.

Ten strains of marine bacteria were tested for their ability to grow at 6 mg organic nutrients l^{-1}. They could be placed into 3 groups characterized by (1) fast growth resulting in CFU titres $>10^7$ ml^{-1} within 24 h, (2) growth to CFU titres $>10^6$ and $<10^7$ ml^{-1} within 24 h, and (3) slow growth with final CFU titres $>10^7$ ml^{-1} attained not before at least 2 d of incubation. Two strains from each group were selected at random for this investigation (Table 1).

During several months, subcultures were incubated at 1 rpm. Irregularities in growth were observed with all strains, though to differing degrees. Attempts to find out the reason(s) for weak or sometimes lack of growth generally failed. When the modus of incubation was changed to still culture, almost all of the difficulties met with when using roll culture were avoided. The remaining difficulty involved the adaptation of strains H2 and H11 to less than 6 mg organic nutrients l^{-1}. Strain H3 was the most reliable of all strains tested under any of the conditions used.

Phage reproduction experiments

The aim of these experiments was to determine the influence of conditions characterized by nutrient limitation and depletion on phage reproduction. Therefore, phage was added to separate samples shortly before bacterial populations were about to enter their stationary phase of growth and after varying periods of time of prolonged incubation.

In the first experiment, H3 incubated in tubes at 1 rpm showed increasingly reduced capability for phage reproduction during the first week of incubation after reaching the stationary phase. In a follow-up experiment 15 samples of 20 ml each were set up at time zero and incubated shaken. Phage was added after 1 to 13 d and incubation proceeded either as still culture or shaken until Day 21 of the experiment, which lasted for 36 d.

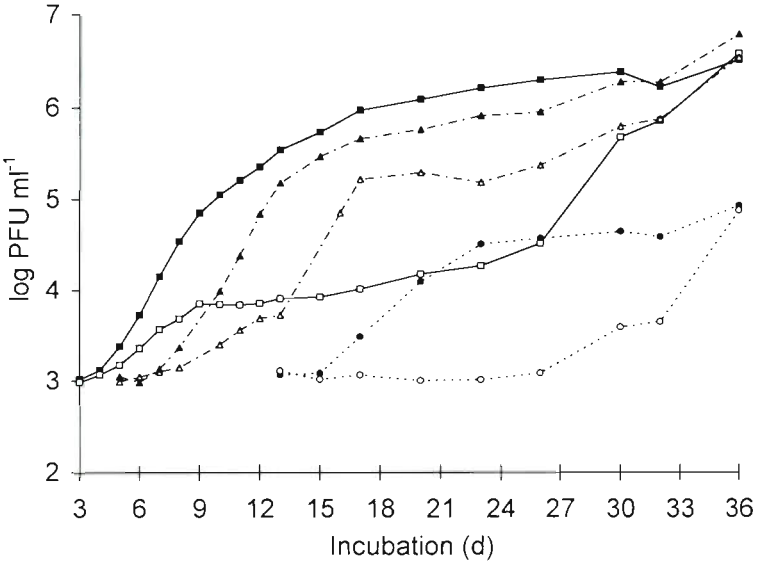

Fig. 1. Phage production dependence of mode on incubation. Phage-host system [H3:H3/1] at 0.6 mg organic nutrients l^{-1} in synthetic seawater. Pairs of 20 ml samples incubated in a shaking water bath until inoculated with phage H3/1 after 3 (■, □), 5 (▲, △), and 13 (●, ○) d. Afterwards 1 sample from each pair was incubated as still culture in an incubator (solid symbols), the other was put back into the shaking water bath (open symbols). On Day 20, shaken incubation ended for all samples

Beside corroboration of the aforementioned findings, the most important observation made was made with several pairs of samples, each one inoculated with phage at the same time: phage reproduction was faster in samples which, after phage addition, were incubated as still culture than it was in samples incubated shaken (Fig. 1).

This observation triggered the change to still culture as the method mainly used in subsequent experiments. Concomitantly, the change provided the opportunity to investigate greatly increased numbers of samples simultaneously. Two additional observations made with strain H3 prior to the change to still culture were of importance in regard to experiments with the other bacterial strains and shall be described in the following 2 sections.

Influence of competition for nutrients on phage reproduction

To further simulate natural conditions, phage reproduction was investigated under conditions of competition for nutrients. For this purpose, samples containing 1 part H3 wild-type bacteria ($H3_{WT}$) and 9 or 99 parts phage-resistant mutant bacteria were used. Two phage-resistant mutants of strain H3, designated H3-1 and H3-2, had been isolated and thoroughly tested beforehand. Mutant H3-1 was reliably distinguishable from $H3_{WT}$ by colony morphology and colour, and both mutants grew with almost the same generation time as the wild-type strain.

Batches of 100 ml, each inoculated with either $H3_{WT}$, the mutants or wild type-mutant mixtures, were incubated shaken in 300 ml Erlenmeyer flasks; 18 ml samples were withdrawn after 12, 24 and 72 h of incubation and inoculated with phage. Control samples without phage addition were set up after 12 h of shaking. Sample incubation was as still culture.

Results of the final PFU titrations, presented as percentages of the highest value found with the 12 h sample of $H3_{WT}$ pure culture, are compiled in Table 2. They demonstrate the impact of the time lag between the start of incubation and addition of phage as well as that of competition for nutrients.

Fig. 2 presents the findings obtained with the 3 samples collected from the batch set up with 10% $H3_{WT}$ and mutant H3-1. In the 12 h sample, no secondary growth of phage-resistant CFU was found. In contrast, in the 12 h sample with pure $H3_{WT}$, faster breakdown of the population was followed by strong secondary growth of 2 types of phage-resistant CFU. Phage reproduction ceased after 5 d of incubation: apparently $H3_{WT}$ was completely eliminated.

Die-off by lysis of $H3_{WT}$ in the 24 h samples with or without 90% resistant H3-1 occurred later and to a lesser extent than it did in the respective 12 h samples; these differences were even more marked between the 72 and 12 h samples.

Table 2. Phage-host system [H3:H3/1]. Influence of phage-resistant mutant cells as competitors for nutrients on phage reproduction by host H3. Relative concentrations of plaque forming units (PFU) in relation to composition of bacterial population. Findings presented in % of maximal PFU concentration found with pure culture of $H3_{WT}$ on Day 20, which for all samples was the highest PFU concentration established. Phage H3/1 was added to samples 12, 24 and 72 h after set up of experiment. Initial PFU concentration was about 10^3 ml^{-1} $H3_{WT}$: marine bacterial strain H3 (wild type); H3-1 and H3-2: mutants resistant to phage H3/1. 100% = 3.62×10^8 PFU ml^{-1}

Composition of CFU population	% max. PFU concentration 12 h	24 h	72 h
$H3_{WT}$	100.00	14.64	3.51
H3-1 + 10% $H3_{WT}$	14.78	6.27	2.96
H3-1 + 1% $H3_{WT}$	0.22	0.20	0.35
H3-2 + 10% $H3_{WT}$	5.97	2.49	0.54
H3-2 + 1% $H3_{WT}$	0.12	0.04	<0.01

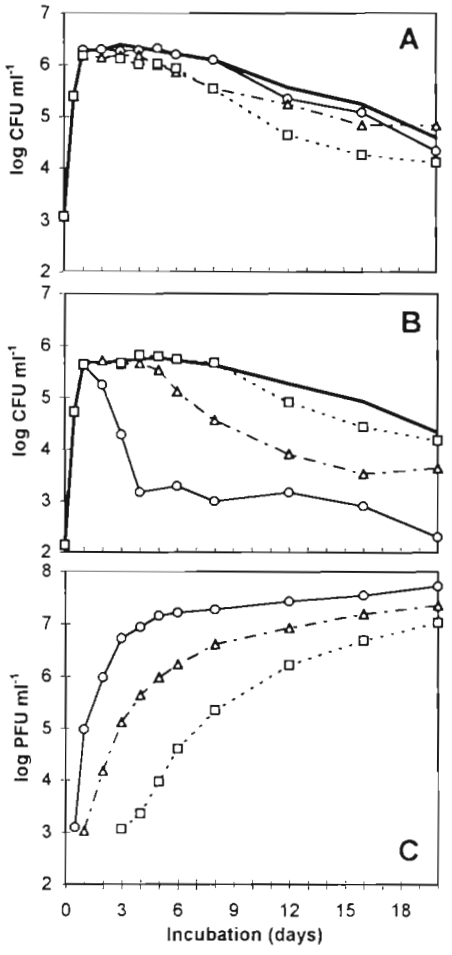

Fig. 2. Development of bacterial populations and reproduction of phage. (A) phage-resistant H3-1; (B) host $H3_{WT}$; (C) phage H3/1. Thick lines: CFU in batch culture. Phage-host system [H3:H3/1] at 0.6 mg organic nutrients l^{-1} in synthetic seawater. Batch culture (100 ml) set up with 9 parts phage-resistant mutant H3-1 and 1 part wild-type host $H3_{WT}$ Samples of 18 ml withdrawn after 12 (○), 24 (△), and 72 (□) h and seeded with phage H3/1. Incubations as still culture

In samples set up with the mixed population containing only 1% $H3_{WT}$, the concentrations of $H3_{WT}$ CFU in the control and the 3 phage-inoculated samples did not differ significantly at any time during the experiment. Similarly, no differences were found in the concentrations of H3/1 PFU in the 3 samples between 12 and 20 d of incubation.

As for the resistant mutant H3-1, its growth and survival probably were most severely affected by the concentration of $H3_{WT}$ present (see Fig. 2A, B). Deviations from findings obtained with the control sample were most pronounced with the 72 h samples, which contained the highest $H3_{WT}$ concentrations for the longest time. With only 1% $H3_{WT}$ present, corresponding observations were made.

Phage-resistant mutant H3-2 could not be distinguished from $H3_{WT}$ by colony morphology. Its growth in pure culture was as efficient as that of $H3_{WT}$ and mutant H3-1, and its survival rate ranged between those of $H3_{WT}$ and H3-1. As observed with H3-1, propagation of phage H3/1 by $H3_{WT}$ was greatly reduced in the samples with 90 or 99% H3-2 initially present (Table 2).

Information on competition experiments performed with other phage-host systems will be presented below.

Survival mechanisms of strain H3

In experiments with $H3_{WT}$, more or less complete phage-induced breakdown of the wild-type population, sometimes followed by secondary growth of resistant CFU, was observed with samples collected after 12 or 24 h of incubation before phage addition. Secondary growth mostly resulted in a seemingly pure culture giving rise to colonies resembling either of the known mutant strains H3-1 and H3-2, and sometimes it was a mixture of both mutant types.

Similar observations were never made with samples taken after longer incubation before phage inoculation, even though in many of these samples the same final phage concentrations were attained as in the samples referred to above. With such samples, no conspicuously rapid reduction in CFU numbers was observed; and, if mutant colonies of the H3-1 type occurred, they did so in very low numbers.

First clues as to what might be the reason(s) for these observations were found in the experiments presented above. Colonies grown in platings made from samples containing $H3_{WT}$ and high concentrations of phage H3/1 were tested for sensitivity to phage H3/1. These test results indicated that many of the colonies contained either 2 types of cells of different sensitivity or only 1 type which retained immunity after passing sensitivity. With some colonies, the findings pointed to the possibility that they contained large amounts of phage at the moment of isolation. In sum, these early observations strongly hinted at the ability of $H3_{WT}$ to develop pseudolysogeny with phage H3/1.

The collective term pseudolysogeny (Hayes 1964; for review see Baess 1971) refers to immunity against the infecting phage strain which, in contrast to lysogeny, is not inheritable. It develops in various ways during phage reproduction and is maintained as long as the mixed culture of host bacterium and phage retains its status, i.e. if host and phage are not completely separated by any means. After dilutions that do not lead to exclusion of phage, immunity by pseudolysogeny will be temporarily lost and restored during phage reproduction. For this reason the immune material of a pseudolysogenic colony, after being suspended in a

small volume of a medium and placed on SWA, will not produce a confluent bacterial lawn, as any 'normal' colony of equal size would do, but will give rise to more or less numerous single colonies. The cells of these single colonies are immune.

A more detailed study of $H3_{WT}$ including this topic is presented by Moebus (1996 in this issue).

Observations with different host bacteria under identical conditions

Experiments were set up with varying sets of PHS. The nutrient concentration employed in most cases was 0.6 mg YEP l^{-1}. Still culture was employed throughout. Samples of 18 ml were taken from 200 ml batches after varying periods of incubation and inoculated with the respective phage. In most cases the period between set up of the experiment and sample withdrawal and phage addition lasted for at least 2 wk.

The results obtained with the 6 PHS differed dramatically, especially with regard to phage production. Reproducibility of experimental findings was reasonably good with regard to phage production, but problematic with some hosts with respect to survival of CFU after addition of phage.

Phage-host system [H2:H2/1]. Bacterial strain H2 could be adjusted to 0.6 mg of YEP l^{-1}, however, it failed twice when experiments were set up with this nutrient concentration. Therefore, 6 mg YEP l^{-1} was used in experiments with H2. In a sample taken after 12 h of incubation and seeded with phage H2/1, the phage was effectively reproduced to 3.5×10^7 PFU ml^{-1} within $3\frac{1}{2}$ d and to a maximal 10^8 PFU ml^{-1} on Day 12. In the sample from the same batch collected and seeded after 1 d, phage production was reduced to a mere 4.4×10^4 PFU ml^{-1}, the maximal PFU concentration attained 3 d after seeding. With samples seeded after $3\frac{1}{2}$ and 8 d, slight phage inactivation instead of phage production was found to occur.

Regarding CFU, the results were typical for PHS comprising a strictly virulent phage. In the sample collected and seeded 12 h after set up of the batch, breakdown of the wild-type population started at little more than 12 h after phage addition, followed by secondary growth of resistant mutant CFU after more than 4 d. The only other sample undergoing CFU reduction (to $\frac{1}{10}$ of original) relative to the phage-free control was the 1 d sample.

In a mixed population of phage-resistant CFU initially containing 7% wild-type H2, but under otherwise identical conditions, a negligible increase in PFU concentration (by a factor of 2 to 3 only) was observed, even in the 12 h sample.

Phage-host system [H3:H3/1]. In agreement with earlier observations, reproduction of phage H3/1 at 0.6 mg YEP l^{-1} gradually decreased in pace and extent until Day 11 However, in a 16 d sample, following a lag of 2 d, a steep increase in phage concentration occurred: within 2 d it rose from about 3.7×10^3 to 7×10^6 PFU ml^{-1} Hence, after 24 d of batch culture 2 samples were collected and treated in parallel. In both samples phage H3/1 was effectively reproduced without any lag, with the PFU titre increasing from about 10^3 PFU ml^{-1} to 1.3×10^6 and 3.3×10^7 PFU ml^{-1}, respectively, within 2 d. The final phage concentrations of between 3 and 10×10^7 PFU ml^{-1} were in the same range as those in samples inoculated with phage H3/1 on Days 1 to 4 of the respective experiment.

Breakdown of H3 was observed with the 12 h and 1 d samples, followed by secondary growth. In samples collected later, direct correlation between sampling time and reduction of CFU titre was found; this correlation was relatively independent of the PFU titre. Lytically injured colonies were very rarely observed.

Phage-host system [H11:H11/1]. As with H2, strain H11 at the lowest nutrient concentration grew unpredictably. Only 1 experiment with 0.6 mg YEP l^{-1} could be performed. Results obtained with H11 are demon-

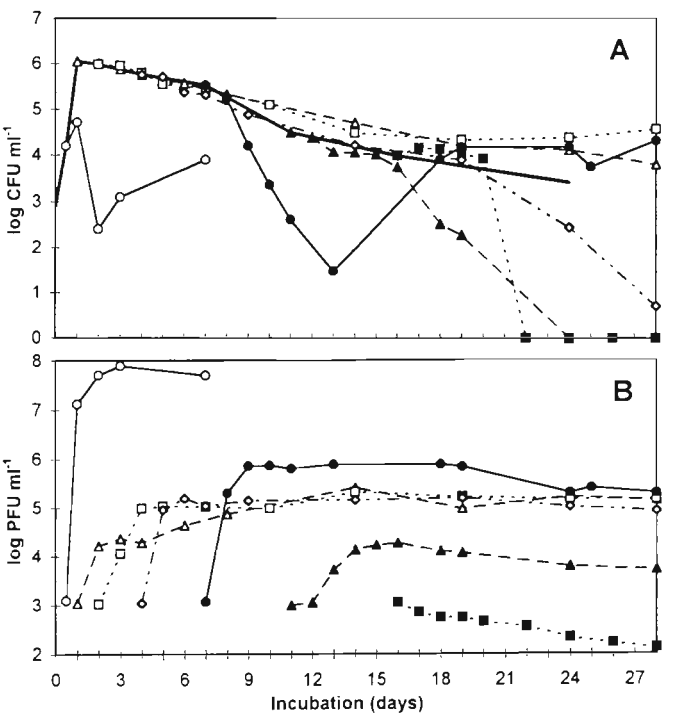

Fig. 3. Dependence of phage reproduction and development of H11 populations on time of seeding after set up of batch culture. (A) Bacterial populations, thick line refers to batch culture; (B) phage populations. Phage-host system [H11:H11/1] at 0.6 mg organic nutrients l^{-1} in synthetic seawater Incubations as still culture

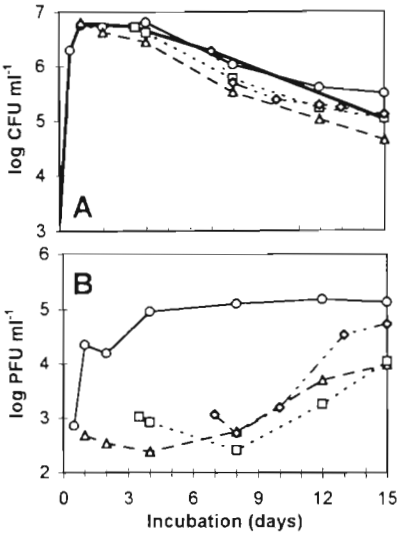

Fig. 4. As for Fig. 3, except phage-host system [H11:H11/1] at 6 mg organic nutrients l^{-1} in synthetic seawater

strated in Figs. 3 & 4. Phage H11/1 is strictly virulent and causes large clear plaques.

In the experiment performed at 0.6 mg YEP l^{-1} (Fig. 3), an abrupt decrease in the ability of H11 to produce H11/1, relative to that observed in the sample collected and seeded after 12 h, was observed in the sample collected and seeded after 24 h; the extent of this decrease was greater than that found with H3. However, in samples withdrawn over the subsequent days, phage production relative to that in the 24 h sample showed a gradual and continuing increase. Note that in these samples, including that withdrawn after 24 h, the final phage concentrations were identical.

In both of the most productive samples, i.e. those withdrawn and seeded after 12 h and 7 d, breakdown of the wild-type host's population took place and was followed by the build-up of a resistant one. The breakdown in these samples started 12 h and 2 d, respectively, after phage addition. In contrast, for the samples collected after 1, 2, and 4 d, breakdown of the H11 population occurred only in the 4 d sample and only after more than 15 d following inoculation of phage.

As long as no resistant population was dominant, lytic colonies were found for all samples except the one seeded after 16 d. Of course, their portions depended on the PFU concentration as well as on the dilution factor used before plating and generally increased with the duration of the experiment.

With H11 investigated in SSW enriched to 6 mg YEP l^{-1} (Fig. 4), the results differed in all aspects from the aforementioned ones. Possible reasons will be addressed in the 'Discussion'.

In a mixed culture prepared from phage-resistant CFU and 7% wild-type CFU, but under otherwise identical conditions (6 mg YEP l^{-1}), no phage production at all was found.

Phage-host system [H40:H40/1]. Phage H40/1 in SSW with 0.6 mg YEP l^{-1} was propagated in all samples to an almost identical extent, irrespective of the period of time that H40 was incubated before addition of phage (tested between 12 h and 24 d, Fig. 5). Only the course of phage titre increases during the first 2 d of the experiments resembled that observed with H3, insofar as the rate of phage production per day was reduced. Reproducibility of these observations was perfect.

With host H40 and in regard to CFU, extraordinary, but unreproducible, results were obtained. In an experiment performed in December 1994, the concentration of viable cells was generally reduced relative to increasing PFU concentration. However, the rate of reduction in CFU concentration (with 1 exception) was lower the older the cells were before phage addition. On the one hand, with younger cells (samples withdrawn 12 and 24 h after set up of the experiment), minimal CFU concentrations were observed on Day 19. On the other hand, with older cells (i.e. samples collected after 11 and 16 d), minimum CFU concentrations were found over short periods (only 5 and 6 d, respectively) after phage addition. Furthermore, in all samples secondary growth resulted in similar final CFU titres.

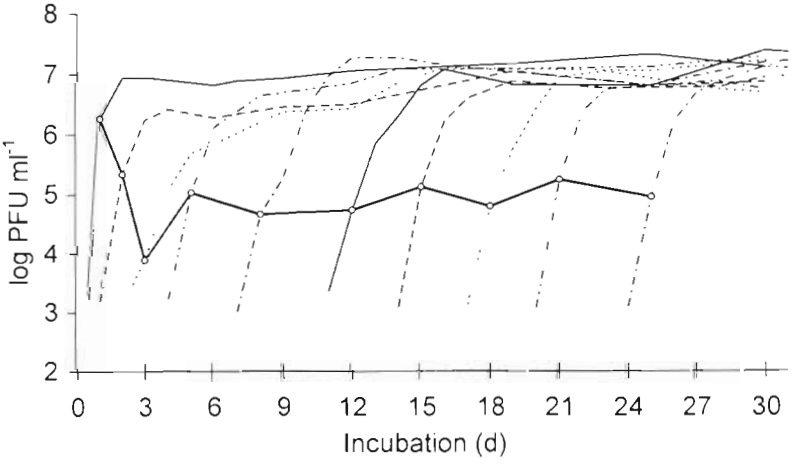

Fig. 5. Phage reproduction in 10 samples collected and seeded between 12 h and 24 d of incubation of 200 ml batch culture. (o—o) Dependence of PFU concentration on cell age as found 1 d after inoculation with phage. Phage-host system [H40:H40/1] at 0.6 mg organic nutrients l^{-1} in synthetic seawater. Still culture

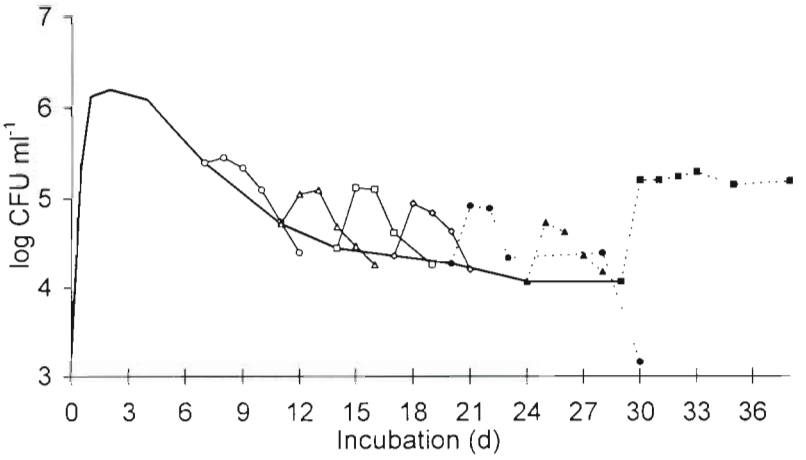

Fig. 6. Changes in CFU concentrations as observed with the batch culture (thick line) and with Samples 5 through 10 (○, △, □, ◇, ●, ▲, respectively) after transfer to fresh receptacles and inoculation with phage. Results of only the first 4 to 5 CFU titrations are presented. The additional sample (Sample 11, ■) was transferred but not seeded with phage. Phage-host system [H40:H40/1] at 0.6 mg organic nutrients l^{-1} in synthetic seawater. Still culture

During an experiment performed 3 mo later with 10 samples taken and seeded between 1 and 24 d after incubation began, the reduction in CFU concentration ran perfectly parallel to that in the phage-free batch culture until Day 25 of the experiment. Only during the final 5 d of the experiment did most of the 10 samples exhibit either an increase or a decrease in CFU titre.

Beside this deviation from observations made during the aforementioned experiment, one other deviation was observed and is shown in Fig 6. With Sample 5 (collected on Day 7 of the experiment) and all subsequent samples, a significant, if only passing, increase in CFU titre was found. When the final sample (Sample 11) was not seeded with phage, the result was an increased CFU number which remained unchanged for the next 8 d. This indicates that the increase in CFU titre was dependent only on the transfer into a fresh receptacle and that nutrients unintentionally introduced along with the phage were not involved.

Lytically damaged colonies were found with all samples; however, their relative numbers and those of typical H40 colonies greatly decreased with the increase in the age of cells before phage addition. With samples withdrawn and seeded early in the experiment, a slow, gradual change in the appearance of colonies was observed. In platings performed within a few days after addition of phage, typical H40 colonies predominated, many of them showing symptoms of phage attack occurring during incubation of the plates. In platings performed later on, the portion of typical H40 colonies decreased while that of colonies presumedly representing various types of phage-resistant mutants increased. In the samples collected and seeded after 7 or more days, such colonies appeared within increasingly shorter periods of time.

The investigation of presumedly phage-resistant cell lines met with difficulties caused by the paste-like consistency of H40 colonies, the material of which could not be evenly suspended. For this reason, and due to the lack of a serum to inactivate phage H40/1, it cannot be said with certainty whether strain H40 develops pseudolysogeny. However, there can be no doubt that the majority of the cell lines investigated were not phage resistant. Among them some were found to be even better suited to propagation of phage H40/1 than the original host H40.

Phage-host system [H54:H54/1]. No reproduction at all of phage H54/1 at 0.6 mg YEP l^{-1} was found with any sample investigated. In samples collected early in the experiment, inoculation of phage was sometimes followed by a reduction in CFU titre of up to 2 orders of magnitude as compared to the phage-free batch cultures. However, toward the end of the experiment the CFU concentrations in such samples started to increase until they were in the range observed with the batch culture and samples taken after 7 or more days.

The complete lack of phage reproduction at the lowest YEP concentration employed was unique among the PHS investigated. In a final experiment, nutrient concentrations of 6, 60, and 600 mg YEP l^{-1} in combination with initial PFU concentrations of about 10^3 and 10^5 PFU ml^{-1} were used. Samples were collected from the batch cultures after 0, 12 and 36 h as well as after $3\frac{1}{2}$ and 7 d.

In SSW enriched to 6 mg YEP l^{-1}, no phage reproduction at all was observed, irrespective of the initial PFU concentration. Also, with the exception of the first 4 d, no significant differences were found in the development of CFU populations. In the 0 and 12 h samples the CFU concentration decreased to 2 orders of magnitude lower than that in the batch culture; however, on Day 4 of the experiment, no significant differences in CFU concentration were found between any of the samples and the phage-free batch culture. No impact of differences in phage input was observed.

At 60 mg YEP l^{-1} very similar observations were made, with the exception of higher CFU concentrations and a more pronounced decrease of the CFU titre with the duration of the experiment. This, however, was independent of the initial PFU concentration and

Table 3. Phage-host system [H54:H54/1]. Maximal concentrations of CFU and PFU observed in samples set up with 600 mg YEP l^{-1}. Initial H54/1 concentrations were 1.6×10^3 and 1.6×10^5 PFU ml^{-1}. Cell age refers to the period of incubation before withdrawal of sample from batch culture and addition of phage. Time represents the point after addition of phage at which the maximum concentration was observed. The experiment lasted 15 d

Initial PFU ml^{-1} (×1.6):	10^3		10^5		10^3	10^5	
Cell age	PFU_{max}	Time	PFU_{max}	Time	CFU_{max}	CFU_{max}	Time
zero	2.6×10^8	1½ d	5.0×10^7	4 d	5.7×10^7	5.8×10^7	5 d
½ d	2.5×10^9	1½ d	1.3×10^9	1½ d	8.1×10^7	7.8×10^7	4½ d
1½ d	2.7×10^3	2 d	2.8×10^5	2 d	3.0×10^8	2.0×10^8	2½ d
3½ d	–		–		9.3×10^7	9.5×10^7	4½ d
7 d	–		–		2.0×10^8	1.7×10^8	1 d

hardly differed between the samples. Regarding phage production, there is a faint possibility that in the 0 and 12 h samples a doubling of PFU occurred within 36 and 24 h, respectively, after addition of phage.

Only with 600 mg YEP l^{-1} did the findings in regard to phage production resemble those obtained with the other phage-host systems at much lower YEP concentrations. Table 3 presents the maximal concentrations of PFU and CFU and the time of observation. In samples collected and seeded after 3½ and 7 d no phage production took place.

The development of CFU populations in all but the 0 h samples was independent of initial phage concentration. In the 0 h samples 12 h after addition of phage, the CFU titre in the sample seeded to 1.6×10^5 PFU ml^{-1} was found to be only 19% of that in the sample seeded to 1.6×10^3 PFU ml^{-1}. However, independent of the initial phage concentration, a ca 90% reduction of CFU occurred in both samples between 12 and 36 h, followed by increases to the maxima presented in Table 3. In the two 12 h samples the initial increase in CFU numbers was seemingly halted by phage addition for about 1 d, with almost no difference in the CFU concentrations determined at that time. From Day 5 until the end of the experiment, only minor differences between CFU populations in all samples in this experiment were found.

It should be noted that at this high nutrient concentration in the samples taken 1½, 3½ and 7 d following phage addition, higher CFU counts were found on some days than were found in the phage-free controls. Lytic colonies were not observed.

Phage-host system [H85:H85/1]. The extent of reproduction of phage H85/1 at 0.6 mg YEP l^{-1} decreased from 1000-fold (12 h sample) to less than 100-fold (1 and 2 d samples) to zero (4 d to 16 d samples).

The CFU titre in samples with phage reproduction was generally slightly below that of the phage-free batch culture, while in samples without phage propagation it hardly differed from that of the batch culture. No lytic colonies were observed.

DISCUSSION

This investigation was performed to answer the question of how marine phage-host systems are maintained in nature. It was based on the following reasoning: (1) Cells of *Escherichia coli* which become infected by phage in their stationary phase of growth will produce progeny phage without releasing them at once (Propst-Ricciuti 1972, 1976). (2) As demonstrated by Proctor & Fuhrman (1990), mature phage particles will be found in appreciable portions of naturally grown marine bacteria when dissected and placed under the electron microscope. (3) If marine bacteria in their prevalent state of starvation become infected by phage and reproduce it, they may not immediately release the new generation of phage. Instead, they might keep the phage inside the cell until better nutritional conditions are met. In this ecologically reasonable way, infected marine bacteria could serve as a rapidly reacting source of phage.

As it turned out, this matter is much more complicated. First of all, with the 6 PHS investigated no generally valid scheme was found to describe the reaction of the host bacteria to phage infection. Similarities in reaction to phage infection were observed only during a short transition phase which includes the last hours of logarithmic growth and the first few days of the stationary phase. These similarities, however, exist only in regard to the tendency, but not to the degree, of changes in phage propagation. During this period the rate of phage reproduction per day, as observed in the first 1 to 3 d after phage addition, decreased sharply, even with host H40.

After longer periods of nutritional deficiency, the host bacteria employed reacted quite differently to phage addition. The one extreme, host H40, produced about the same maximal numbers of progeny phage in samples seeded with phage between 12 h and 24 d of incubation and appeared to be unaffected by nutrient deprivation. The other extreme, host H54, produced no phage at all at 0.6 and 6 mg YEP l^{-1}, hardly any at 60 mg

YEP l^{-1}, but did so effectively at 600 mg YEP l^{-1}, albeit only when phage was added before the end of logarithmic growth. Host H85 reproduced its phage only during the transient phase and with decreasing efficiency.

Host bacterium H3 resembled neither of the aforementioned strains in its reaction to phage. In samples withdrawn and seeded while H3 cells were passing through the transient state, as well as those seeded over the subsequent 8 to 12 d, phage reproduction relative to that of each previous sample was greatly and progressively reduced. In later samples, however, phage H3/1 reproduction resumed at an even higher rate, but to about the same extent, as observed immediately after the end of the logarithmic growth phase. More information on the phage-host system [H3:H3/1] is presented by Moebus (1996).

The remaining hosts, H2 and H11, at 0.6 mg YEP l^{-1} posed considerable difficulties due to unpredictable reactions to the transfer into fresh medium for subcultures. These problems could not be overcome by extending the time or increasing the number of generations for adaptation. Instead, it was observed more than once that both strains, although seemingly adapted after several subcultures, suddenly ceased to grow: the other 4 strains did not change their growth patterns in the same medium.

With a number of hosts, considerable losses in CFU, sometimes only passing, were observed without concomitant phage production. Excluding secondary growth of resistant populations, the respective observations made here support the assumption that different mechanisms were involved. With H11 at 0.6 and 6.0 mg YEP l^{-1} a relatively rapid decrease in CFU numbers of batches was observed during prolonged incubation, presumedly due to lack of nutrients. This process may have been intensified by the effect of sample transfer to fresh receptacles, such as is demonstrated in Fig. 6 (cf. also Moebus 1996). Abortive infection, i.e. killing of infected cells without phage reproduction, also cannot be ruled out.

With H40 and H54 at 0.6 mg YEP l^{-1}, the CFU concentration in many samples decreased more or less sharply, but toward the end of experiments increased again to values found with phage-free batches. Although effective phage reproduction was found only with PHS [H40:H40/1], release of nutritive matter from lysing cells may have enabled limited cell growth and division in the case of H54

Several attempts were made to demonstrate that at least part of the losses in CFU was due to cells which produced progeny phage after infection, but could not release them before nutrients became available. Such cells would not form colonies but would release phage on SWA. All tests performed in liquid culture failed. With none of the 5 hosts employed (H2 excluded) did the findings obtained point to anything other than direct correlation between cell growth and division and concomitant phage reproduction.

With most host strains during adaptation in subcultures run with 0.6 mg YEP l^{-1}, it was observed that the CFU concentration gradually increased and finally was relatively higher than would be expected after a reduction of the nutrient concentration to one tenth. This state is assumed to be due to reactivation of high affinity transport systems, is thought to represent complete adaptation to this nutrient concentration, and is usually reached after 50 to 100 cell generations.

Within the time needed for this investigation, changes in the populations of strains H3, H40 and H54 were observed. After more than 1 yr of repeated subcultivation of host H3 at 0.6 mg YEP l^{-1}, extensive flock formation occurred, but during further subcultivations almost completely disappeared. When grown in SWB/5, host H40 usually produces colonies of 2 sizes on SWA, the smaller colonies occurring in low fractions and being less opaque. With 0.6 mg YEP l^{-1} the smaller type almost completely outgrew the larger CFU, while with 6 mg YEP l^{-1} the larger type maintained its majority. Finally, for some time H54 tended to produce high portions of colonies which were smaller than is normally typical.

Beside changes in colony morphology, certainly passing or persistent but less obvious variations of other traits occurred. The question arises to which degree phage production may have been influenced by such changes, whether visible or hidden, during the present investigation. The answer can only be speculative.

Unnoticed changes may have been responsible for the remarkably different findings for host H11 shown in Figs. 3 & 4. Regarding H3, it was observed that during this investigation increasingly longer incubation prior to phage addition was needed to obtain corresponding findings. Detailed information is presented in the accompanying paper (Moebus 1996).

With H40, due to changes in colony morphology after phage addition (as described in 'Results'), comparative observations were severely hampered. It was impossible to decide whether the morphologically non-typical H40 colonies developed only from CFU representing the smaller colony type, as could be deduced from observations made with samples collected early in the experiment, or from both CFU types. In the first case, one would have to conclude that CFU forming the larger H40 colonies were preferentially infected by phage in samples taken after prolonged incubation of the batches.

An investigation of the altered H40 colony types indicated that 11 isolates probably represented resistant lines, and 19 isolates presumedly pseudolysogenic

ones. As mentioned earlier, only coarse suspensions can be produced with material of H40 colonies. For this reason there was a high risk for transferring phage and cells together, so that immunity by pseudolysogeny could have been maintained in spite of 3 consecutive streaks on SWA, incubation in SWB/5 and subcultivation at 6 mg YEP l^{-1}. Some of these lines were found to reproduce phage H40/1 more effectively than the original host H40.

With H54 at 600 mg YEP l^{-1}, about 8×10^7 CFU ml^{-1} survived for $13\frac{1}{2}$ d in the presence of 2.5×10^9 infective phage particles ml^{-1}. This CFU concentration was attained by growth starting from 10^6 CFU ml^{-1} without breakdown of the population. Therefore, it is assumed that H54, too, is able to develop pseudolysogeny. However, no investigation was performed due to difficulties in producing adequate suspensions of H54 colonies.

The observation of unequivocal increases in CFU titre following transfer of H40 samples to fresh receptacles was unique. However, in the following paper (Moebus 1996) results obtained with H3 are presented which point out that such 'wall effects' must be taken into consideration during similar investigations.

Unfortunately, the observations made with host H2 and H11 are unsatisfactory. Even at 6 mg YEP l^{-1}, efficient phage reproduction to a final concentration of 10^8 PFU ml^{-1} was found only for H2 when samples were inoculated with phage 12 h after the start of the experiment. With H11 under identical conditions only 10^5 PFU ml^{-1} were produced.

Both of these hosts and their respective phages have also been used by other authors. An investigation by Proctor et al. (1993) was aimed at calibrating estimates of phage-induced mortality of marine bacteria. Although experiments were performed at considerably higher nutrient concentrations, differences between hosts H2 and H11 with regard to phage reproduction correspond with those found in the present investigation. Host H2 was found to be superior. With host H11, strikingly diminished efficiency of phage reproduction was found when the nutrient concentration was reduced (by a factor of 10) to 50 mg peptone l^{-1} plus 50 mg casamino acids l^{-1}, which still is about 10 or 100 times the nutrient level used in the present investigation.

Of course, the efficiency of phage infection in relation to propagation of a phage-host system greatly depends on the amount of available nutrients. As shown by Proctor et al. (1993), the number of progeny phage per cell (burst size) decreases with reduced nutrient concentration. Concomitantly, the duration of the latent period, i.e. the time between infection and release of first progeny virions, was found to increase.

Cells, however, must first become infected. In this respect, due to the appreciable portion of cells insensitive to a distinct type of phage, conditions in nature are much more restrictive than in pure laboratory cultures. This holds true even if the nutrient concentration is as low as that used in the present investigation. With 0.6 mg YEP l^{-1}, the employed hosts attained maxima of 1 to 5×10^6 CFU ml^{-1}. In most marine environments such a high concentration of 1 bacterial strain among many others cannot be expected to occur. Therefore, the portion of cells sensitive to the phage in question is another important factor to be considered. The same applies to the density of the population of the phage in question.

In this investigation an initial concentration of about 10^3 PFU ml^{-1} was employed. (Results obtained with lower initial PFU titres are presented in Moebus 1996.) According to the information available regarding the numbers of PFU found in freshly collected seawater samples, this is an unusually high concentration of 1 type of phage. With only sensitive cells present under the most favourable of conditions, i.e. when phage was added to samples collected prior to the end of logarithmic growth, rapid phage propagation was ensured. With only 5 to 10% sensitive cells present in experimental populations under otherwise identical conditions, phage reproduction was reduced: with H3 (at 0.6 mg YEP l^{-1}) to about 10%, with H2 and H11 (at 6 mg YEP l^{-1}) to zero. Such findings correspond well with observations relating to natural seawater samples.

With PHS [H3:H3/1] the PFU concentration continued to increase as long as sensitive host cells were present. It also occurred in populations containing only 1% sensitive cells. This observation also sets host H3 apart from the other strains employed.

Based on experience gained by handling about 500 marine PHS for at least the length of time needed to decide whether to keep a PHS for further tests, this author regards PHS [H3:H3/1] to be far more representative of marine PHS than [H2:H2/1] and [H11:H11/1]. Both of the latter PHS comprise phages which cause clear plaques of 3 to 4 mm in diameter, representing rarely encountered types of marine bacteriophages. (Phage types forming clear plaques often are obtained only during purification procedures, starting from plaques showing one to several concentric turbid zones and a small clear centre. This does not apply to phages H2/1 and H11/1.)

The vast majority of PHS isolated from seawater collected in the North Sea near Helgoland and in the Atlantic between the European continental shelf and the Sargasso Sea were characterized by small (diam. up to 1.5 mm) plaques, their turbidity decreasing toward a small fairly clear centre. Because of difficulties often met during preparation of high titre stocks, and because of the general difficulties in working with phage causing this type of plaque, most of these PHS

were excluded from further investigation. The remaining ones included PHS such as [H3:H3/1] and [H54:H54/1] and, with some restriction, [H40:H40/1].

Recent investigations by Moebus (1992c) and Suttle & Chen (1993) found that marine bacteriophages in raw seawater do not survive for extended periods of time. The present investigation was undertaken to examine whether infected host bacteria in a state of starvation-survival could function as a source of progeny phage by making them available as soon as more favourable nutrient conditions are met. No indication was found that such a mechanism plays any significant role. Lysogeny is assumed with good reason to be an inexhaustible source of phage. It may well play the prominent role in PHS maintenance. This also remains valid in view of the failed attempts of Wilcox & Fuhrman (1994) to prove lysogeny as a source of the high concentrations of viral particles ml^{-1} in seawater as seen in the electron microscope.

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