Vol. 148: 241–250, 1997 | MARINE ECOLOGY PROGRESS SERIES Mar Ecol Prog Ser | Published March 20

Investigations of the marine lysogenic bacterium H24. III. Growth of bacteria and production of phage under nutrient-limited conditions

Karlheinz Moebus

Biologische Anstalt Helgoland (Meeresstation), D-27498 Helgoland, Germany

ABSTRACT: The marine lysogenic bacterium H24, known for the genetic instability of the resident wild-type phage ϕH24 when propagated in nutrient-rich medium, was investigated by cultivation in synthetic seawater enriched to various levels of yeast extract plus peptone (YEP, in a relation of 1:5). The incidence of phage mutants was found to depend on a balanced equilibrium of nutrient concentration (tested between 0.6 and 600 mg l^{-1}) and the bacterial yield allowed by it on the one hand, and on the duration of incubation and the dilution between successive subcultures on the other. With too-low dilutions used in combination with high nutrient levels, non-virulent mutants, followed by virulent ones, increased in concentration until breakdown of the bacterial population occurred. Conversely, extinction of populations was caused by combinations of low nutrient levels with too-high dilution after too-short incubation periods. At low nutrient levels phage mutants did not influence the outcome of the experiments. However, at nutrient concentrations of 6 and 0.6 mg l^{-1} release of particles of wild-type phage ϕH24 was found to be initiated by nutrient addition inducing sufficiently rigorous bacterial multiplication. The latter findings are discussed as an ecologically promising explanation of how phage-host systems are maintained in nature. The number of free virions of ϕH24, which always remained low, rapidly decreased in the cultures but survived in cell-free filtrates stored in the refrigerator for the same period of time. The loss of virions in cultures is attributed to infection of $H24_{wt}$ cells which due to their immunity remained unharmed. Pseudolysogenization was indicated at nutrient levels of 600 and 60 mg YEP l^{-1}, but not at lower YEP concentrations.

KEY WORDS: Marine · Bacteria · Lysogeny · Pseudolysogeny · Phage · Mutation · Nutrients

INTRODUCTION

Since isolation of the first marine bacteriophage by Spencer (1955), marine viruses were all but ignored by the scientific community for decades. This is all the more surprising as the well-known rarity of phage sought after in seawater samples might have been expected to provoke interest in scientists in the problem of how phage-host systems in nature may be maintained. Unfortunately, this was not the case. Therefore, when Bergh et al. (1989) reported the observation of up to 10^7 virus particles ml^{-1} of seawater by electron microscopy, marine microbiologists were caught by surprise. Although considerable effort was made by a few scientists (for review see Moebus 1987, Børsheim 1993) during the 1970s and 1980s, our knowledge concerning the ecology of marine bacteriophages is still very limited.

The observation by electron microscopy of so high an abundance of virus particles in seawater, although highly important and welcome to those interested in marine phage, does not tell much more than numbers. It provides no information other than the circumstantial evidence that viruses in general and phages in particular must play a considerably more significant role in the marine environment than previously assumed.

Since the paper by Bergh et al. (1989), similar large numbers of virus particles observed in seawater, either by electron microscopy or by means of DNA-specific fluorescent dyes, have been reported in a steady flow of publications. The number of papers dealing with more direct approaches to the effects of phage on their

hosts, however, is still limited. Due to the lack of detailed knowledge about the 'life' of marine bacteriophage, the discrepancy between the generally very low numbers of host-specific infective particles observed with biological methods and the high numbers of viral particles detected by the aforementioned methods still defies meaningful explanation.

Lysogeny among marine bacteria is assumed with good reason to play a major role in regard to maintenance of specific phage strains and to the observed numbers of viral particles in marine waters. This idea is based on the known widespread lysogeny among non-marine bacteria and the lack of any information that would widely separate marine bacteria from non-marine ones in terms of their biology. Unfortunately, our knowledge concerning lysogeny in marine bacteria and temperate (i.e. lysogenizing) phage comprises a minute portion of that regarding marine phage in general. Only 4 papers have come to the attention of this author that reported facts related to lysogeny.

Hastings et al. (1961) isolated a phage from lysogenic luminescent bacteria, and Rambler & Margulis (1979) induced phage production with UV light in a marine vibrio. Jiang & Paul (1994) found 22 of 51 marine bacterial isolates from various locations to be inducible with mitomycin C but were unable to differentiate between lysogenic and bacteriocinogenic strains. Wilcox & Fuhrman (1994) reported experiments designed to enable differentiation between virulent and visible-light induced phage production. No indication of the latter was found.

In the companion papers, Moebus (1997a, b) reported on observations made with the lysogenic bacterium H24, isolated from a seawater sample taken near Helgoland (North Sea). This strain, after being kept for a number of years on agar slants, unveiled itself as a lysogenic bacterium by spontaneous plaque formation. When the cured derivative H24(L10) became available, the observed plaques were found to be produced by non-virulent (non-vir) or virulent (vir) mutants of the wild-type phage ϕH24 residing in H24. Investigations employing SWB/5 to grow $H24_{wt}$ showed that in general several consecutive subcultures in nutrient-rich medium are necessary to produce bacterial populations containing appreciable portions of 'cellular PFU', i.e. cells that only after being fixed in the SSWA overlay of a double layer plate start to release mutant particles of phage ϕH24. Free virions are present at this stage of development; however, their numbers are relatively low and, more importantly, the majority belong to the non-vir type of mutant phage. By definition this mutant type cannot overcome the immunity threshold of $H24_{wt}$ and does little if any harm to its cells in terms of bacterial growth. With further increase of 'cellular PFU' the chance of occurrence of virulent 'cellular PFU' and, subsequently, the release of free vir mutant particles increases too. These eventually would lead to breakdown of the bacterial population if pseudolysogeny did not develop in the meantime.

Wild-type phage ϕH24 was found to represent the vast majority of virions in tiny turbid plaques occurring in plates prepared with suspensions of $H24_{wt}$ diluted in excess of 10^2 and with H24(L10) used as indicator (Moebus 1997a). A similar situation was encountered in regard to the predominant vir mutant of ϕH24, mutant ϕH24-1. Free virions of this mutant with $H24_{wt}$ as indicator strain caused clear plaques with a diameter of about 1 mm. More often PFU_{vir} were indicated by turbid plaques of about 0.5 mm in diameter which generally occurred in advance of the larger clear ones. Investigations of such plaques indicated that they too originate from $H24_{wt}$ cells which release vir mutant virions during incubation in SSWA overlays (Moebus 1997a).

The wild-type bacterium, henceforth named $H24_{wt}$, was investigated under various nutritive conditions with regard to its growth, to the production of mutant phage, and to the spontaneous release of wild-type phage, ϕH24. Results of these investigations are dealt with in this paper.

MATERIALS and METHODS

Media. Seawater agar (SWA), soft seawater agar (SSWA), reduced seawater bouillon (SWB/5), seawater mixture (SM) and beef extract (BE) were of the same composition as given by Moebus (1997a). The synthetic seawater (SSW) used in experiments was prepared according to the formula of Burkholder (1963) as described by Moebus (1996a). For nutrient enrichment of SSW, a stock solution containing 10 g l^{-1} Difco yeast extract and 50 g l^{-1} Difco peptone in distilled water (YEP) was used after appropriate dilution.

Glassware. Only carefully cleaned glassware was used as detailed by Moebus (1996a).

Bacteria and bacteriophage. Wild-type strain $H24_{wt}$ and its cured (non-lysogenic) derivative H24(L10) were kept on SWA slants in the refrigerator. At the beginning of the investigation several consecutive streaks on SWA were incubated for 1 to 2 d at 25 or 20°C. Finally, colonies of about 2 mm in diameter were transferred into SSW and the suspensions after dilution in SSW inoculated into SSW samples enriched with YEP to various nutrient levels.

Methods. If not otherwise stated, the methods given by Moebus (1997a) were employed.

Adaptation of bacteria to the desired nutrient concentration was accomplished by successive subcultivation. In early experiments, samples of 5.25 ml incu-

bated at 25°C and 1 rpm in tubes of 18 mm in diameter were used. During later experiments still culture instead of roll culture, and 20 instead of 25°C, was employed because of more reliable growth at the lower nutrient concentrations tested (Moebus 1996a, b). Subcultures were always set up by mixing 4.5 ml SSW with 0.5 ml of appropriately diluted YEP solution and 0.25 ml of the preceding culture diluted according to the expected or desired CFU titer. Dilution of YEP was in distilled water, that of culture in SSW.

RESULTS

The aim of this investigation was to elucidate the influence of nutrient concentration on mutation of wild-type phage ϕH24 and to follow growth of, and phage production by, $H24_{wt}$ under nutrient limitation. Accordingly, at the beginning the reproduction of colony forming units (CFU) and the production of plaque forming units (PFU) under various nutritive regimens were studied.

Influence of nutrient concentration and dilution on occurrence of mutant PFU

During this part of the investigation cultures were grown at 25°C and 1 rpm. Results of preliminary experiments indicated that special attention has to be paid to the adaptation of $H24_{wt}$ to low nutrient concentrations. (At that time it was still unknown that some of the problems encountered can be avoided by still culture, as described by Moebus 1996a.) Therefore, it was decided that a series of subcultures relatively rich in nutrients would be used as a basis for the essential experiments. Henceforth this will be referred to as Series 1. Each of 63 subcultures was incubated for 24 h in SSW with 600 mg l^{-1} of organic nutrients from YEP and inoculated from the preceding one with a dilution factor (DF) of 2.1×10^6. In this way an initial titer of between 68 and 185 CFU ml^{-1} was attained. As shown in Fig. 1, not before about 50 subcultures, each including about 21 generations, was an equilibrium attained between the yields in CFU and PFU within each period of 24 h. At that point, initial titers of subcultures varied only between 121 and 138 CFU ml^{-1}.

With the cell suspension used to set up Series 1, a second series (Series 2) of subcultures was started which differed from Series 1 by having the lower nutrient concentration of 60 mg YEP l^{-1} and by a lower dilution factor of 2.1×10^5 between subcultures. Fig. 2 presents a comparison between results obtained with Series 1 and 2. Only in the final rise in log(ΔPFU/ΔCFU) seen with Series 2 were vir mutants involved (see Fig. 3).

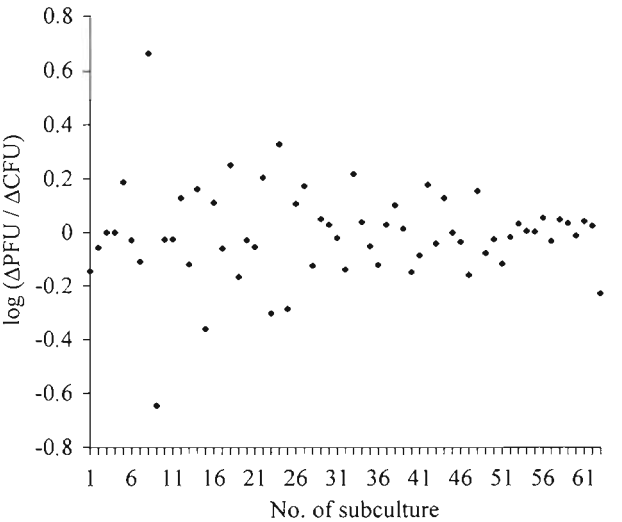

Fig. 1. Changes in the relation of increases during 24 h (Δ) of plaque (PFU) and colony forming units (CFU), as observed with 63 subcultures of Series 1. $H24_{wt}$ was grown in SSW enriched with organic nutrients (1 part yeast extract + 5 parts peptone) to 600 mg l^{-1} and incubated at 25°C and 1 rpm for 24 h. Dilution factor between subcultures: 2.1×10^6

In Series 1', branching off subculture #3 of Series 1, SSW enriched to 600 mg YEP l^{-1} and a dilution factor of 2.1×10^4 was employed. The results are depicted in Fig. 4. As found with Series 2, the rapid increase in vir mutant concentration during subculture 28 and the following ones caused breakdown of the $H24_{wt}$ population. With the last subculture a distinct increase in the final CFU titer, as compared with the last but one, was

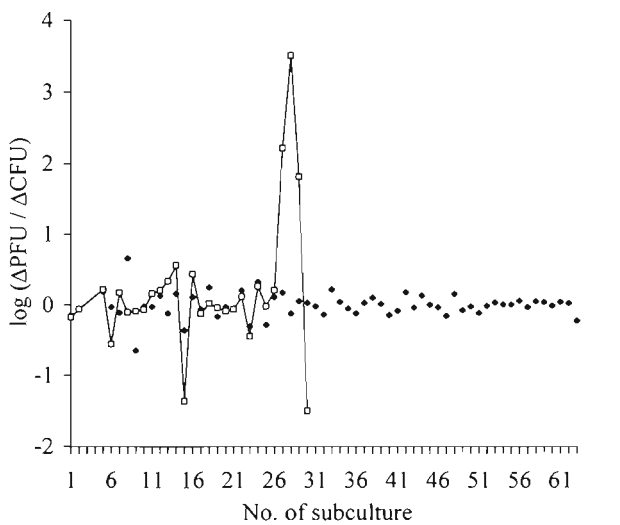

Fig. 2. Changes in the relation of increases during 24 h (Δ) of PFU and CFU, as observed with 63 subcultures of Series 1 (◆) and 29 subcultures of Series 2 (□). Subcultures of Series 2 in SSW with 60 mg l^{-1} of organic nutrients after dilution of 2.1×10^5 in between. Other conditions as in Fig. 1

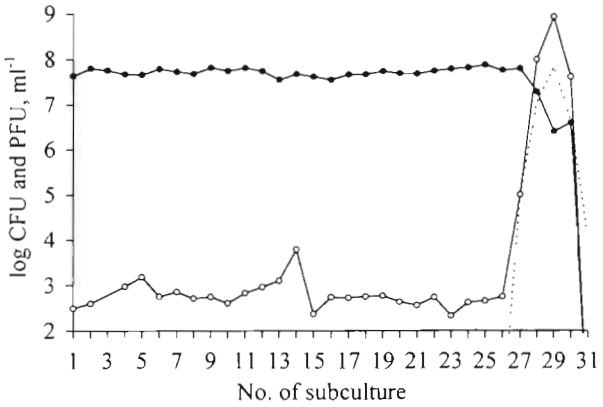

Fig. 3. Series 2 of subcultures grown in SSW with 60 mg l^{-1} of organic nutrients at 25°C and 1 rpm for 24 h. Dilution factor between subcultures: 2.1×10^5 Final concentrations of CFU, (—●—) and PFU. (—○—) PFU_{Σ} as detected with host H24(L10); (-----) vir mutant PFU

observed which was not due to the growth of phage resistant CFU. Instead, it is ascribed to pseudolysogeny.

The early findings obtained with Series 1, 1', and 2 made it clear that the high dilution factors employed for inoculation of subcultures hindered the gradual build-up of PFU populations, as observed in series of subcultures using dilutions of only 1/100 (Moebus 1997b). With vir mutations the situation obviously changed completely as long as they occurred early during a subculture, thus making sure that the next subculture received a few vir mutant units as either 'cellular PFU' or free virions (Figs. 3 & 4). The consequence was always very rapid population build-up.

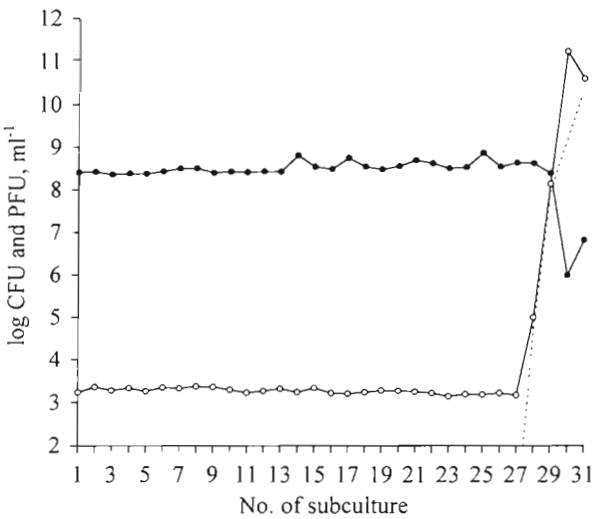

Fig. 4. Series 1', branching off subculture #3 of Series 1, grown in SSW with 600 mg l^{-1} of organic nutrients at 25°C and 1 rpm for 24 h. Dilution factor between subcultures: 2.1×10^4 Other information as in Fig. 3

These observations were confirmed by tests performed with subculture #8 of Series 1, which at the end of incubation was found to contain 3.56×10^3 of PFU_{vir} and of 5.18×10^3 PFU_{Σ} ml^{-1}. Due to the high dilution between subcultures #8 and #9, the PFU_{vir} were diluted out and remained without consequences in Series 1. Subculture #8, after storage in the refrigerator, was repeatedly used to initiate series differing in organic nutrient concentration and dilution factor between subcultures. Most of these series were finished after 2 to 4 subcultures because of nutrient concentrations that were too low, sometimes in combination with dilutions in between that were too high, but in most cases because of the steady increase of the titer of PFU_{vir} taking place in subculture #8 during storage. This is illustrated in Fig. 5. Although the cells probably became pseudolysogenized during storage, after dilution into subcultures they rapidly fell victim to vir mutants.

To check the influence of DF and YEP concentration on the development of mutant populations more accurately, 15 series of subcultures were inoculated from subculture #34 of Series 1 according to the scheme shown in Table 1, which also presents selected results.

Only with Series 11 (Table 1) were high concentrations of PFU_{vir} observed, which in subcultures #3 and #4 exclusively were represented by small turbid plaques assumedly originating from 'cellular PFU_{vir}'. With subculture #5, however, the vast majority of PFU were represented by the larger clear plaques typical of ϕH24-1. From the high CFU titer it is concluded that pseudolysogeny developed in this subculture. In sum, these results correspond with those found with $H24_{wt}$ grown in SWB/5 and 1/100 dilutions between subcultures (Moebus 1977b).

With each of 9 subcultures of Series 12 to 43 vir mutants always were near or below the level of detec-

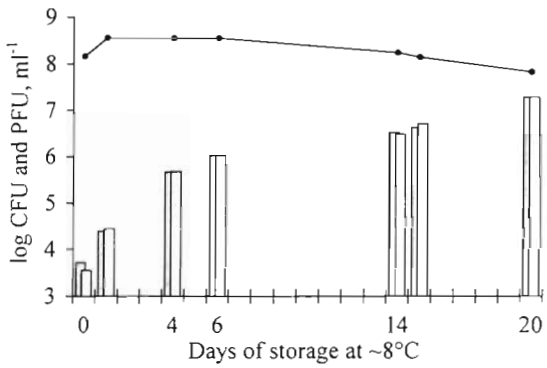

Fig. 5. Subculture #8 of Series 1. Changes in concentrations of CFU (—●—) and PFU (columns) during storage in the refrigerator (~8°C). Shaded columns: PFU_{Σ} as detected with host H24(L10); open columns: vir mutant PFU

Table 1 (A) Designations of subcultures set up from subculture #34 of Series 1. (B) Data concerning growth of $H24_{wt}$ and occurrence of mutants of phage ϕH24 in relation to nutrient concentration and dilution between succeeding subcultures. Concentrations of colony (CFU) and plaque forming units (PFU) as observed after 24 h of incubation at 25°C and 1 rpm in synthetic seawater enriched with yeast extract-peptone solution (1:5 parts, YEP) to the designated level. Selected data. Findings obtained after 48 h of incubation presented in parentheses by symbols: = : no change, ↑: significant increase

A. Subcultures

Dilution factor (DF)	YEP concentration (mg l^{-1}) 600	60	6	0.6
210	11	21	31	41
2100	12	22	32	42
21000	13	23	33	43
210000	14	24	34	no

B. Growth of $H24_{wt}$

Series	YEP mg l^{-1}	Dilution factor	Sub-culture	Non-vir	Vir	CFU
11	600	210	1	2.2×10^3	<10	3.3×10^8
			2	2.3×10^3	<10	3.6×10^8
			3	$\sim 3 \times 10^4$	$>10^4$	3.4×10^8
			4	8.5×10^6	1.5×10^6	3.5×10^8
			5	2.7×10^{10}	2.6×10^{10}	$\sim 5 \times 10^7$
34	6	210000	1	2.3×10^2	<10	$>2 \times 10^6$
			2	3×10	<10	5.4×10^5 (=)
			3	<10	<10	8×10^3 (↑)
			4	<10		<10 (=)
41	0.6	210	1	5×10	<10	1.1×10^6
			2	6×10	<10	$>10^6$
			3	18×10	<10	$\sim 10^6$
			4	5×10		7.9×10^5
			5	8×10		9.4×10^5
			6	13×10		8.5×10^5
			7	3×10		1.3×10^6
			8	7×10		6.5×10^5
			9	10×10		9.4×10^5
42	0.6	2100	1	2×10	<10	5.8×10^5 (=)
			2	<10	<10	2.9×10^5 (↑)
			3	<10	<10	8×10^4 (↑)
			4	<10		2.5×10^4 (↑)
			5	~10		4.5×10^4 (↑)
			6	3×10 (=)		6.5×10^4 (↑)
			7	<10 (↑)		1.2×10^4 (↑)
			8	<10		1.1×10^4
			9	<10		1.8×10^4
43	0.6	21000	1	3×10	<10	2.5×10^5 (↑)
			2	<10	<10	7.5×10^4 (↑)
			3	<10	<10	7×10^2 (↑)
			4	<10		2×10^2 (↑)
			5	<10		<10 (=)

tion. Regarding the numbers of non-vir PFU and CFU, variation was found to be small and more or less independent of the DF in Series 12 to 24. With 600 mg YEP l^{-1} about 2.0×10^3 non-vir PFU ml^{-1} and 2.5×10^8 CFU ml^{-1} were counted. The corresponding numbers for series with 60 mg YEP l^{-1} were about 4×10^2 PFU and 4 to 6×10^7 CFU ml^{-1}.

With 6 mg YEP l^{-1} only (Series 31 to 34) somewhat greater variability between subcultures was found, which was more pronounced in regard to PFU than CFU. Independently of DF the latter varied between 2.5×10^6 and 9.6×10^6, with the maximal count possibly being an overestimation by about 20% as calculated from the second highest value. In contrast, the PFU ranged between 50 and 160 ml^{-1} in Series 31, between 50 and 300 ml^{-1} in Series 32, and between 70 and 2200 in Series 33.

Data for the remaining Series 34 to 43 are presented in Table 1. In these series the DF was too high and/or the nutrient concentration too low, resulting in 24 h CFU concentrations often far below the possible maxima as observed after incubation for another 1 or 2 d. Respective observations made after 1 additional day of incubation are indicated by symbols in parentheses.

Due to the last-mentioned observations, subculture #13 of Series 42 was used twice to inoculate new subcultures: subculture #14 after the usual incubation period of 24 h, and subculture #13a after 48 h of incubation, initiating a sub-series. The combined results are presented in Fig. 6. During prolonged incubation of subculture #13, not only did appreciable increases in CFU and PFU occur, but in the ensuing Subseries 42/13 relatively high final titers were found up to subculture #13e, although the incubation period again was only 24 h. If this observation was due to development of a population better-adapted to the low nutrient concentration during the 2 d incubation of SC #13, one has to conclude that the adaption was unstable.

Finally, parallel series of subcultures were run to elucidate the problem of individuality in the course of the development of subculture series. The results are shown in Fig. 7. Subculture #52 of Series 1 was used to initiate 3 subseries each with dilutions of 210 (series A to C) and 2100 (series D to F), respectively, between subcultures in SSW enriched to 600 mg YEP l^{-1}

Employing DF 210, a more or less gradual increase in PFU_{Σ} was observed, with PFU_{vir} having little if any influence on the trend of the increase in PFU_{Σ} as long as the concentration of PFU_{vir} remained below 10^6 ml^{-1}. This is also true with regard to final CFU concentra-

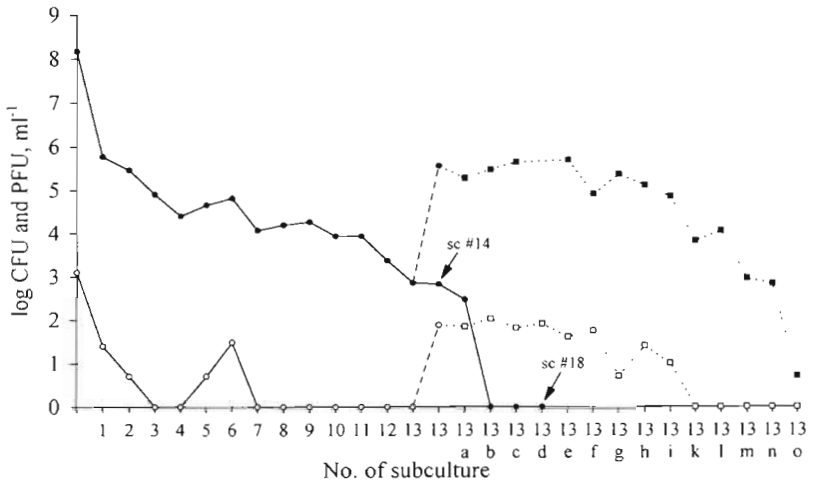

Fig. 6. Series 42, branching off subculture #34 of Series 1, and sub-series 42/13. Influence of length of incubation period on the development of CFU (●, ■) and the occurrence of PFU (○, □) when $H24_{wt}$ was grown in SSW with 0.6 mg l^{-1} of organic nutrients at 25°C and 1 rpm. Dilution factor between subcultures: 2100. Subculture #13 of Series 42 (●, ○) was incubated for 2 d to start sub-series 42/13 (■, □). Thereafter the previous schedule was resumed

tions. Under this regimen PFU_{vir} never became a majority of PFU before reaching maximal concentration (Fig. 7a, c). In subsequent subcultures, however, PFU were almost exclusively represented by PFU_{vir}. The comparatively weak reduction in final CFU concentration in series A (Fig. 7a) is ascribed to late occurrence of 'cellular PFU_{vir}' and to at least partial protection by pseudolysogeny.

The 3 series of subcultures run with DF 2100 led to markedly different results. In 2 of these series (Fig. 7d, e), no gradual increase in PFU_{Σ} at all was observed during the first 7 subcultures, but in all 3 series PFU_{Σ} increased to maximal values within 1 or 2 subcultures, which is much more rapid than in the series using DF 210. Furthermore, in each of the series D to

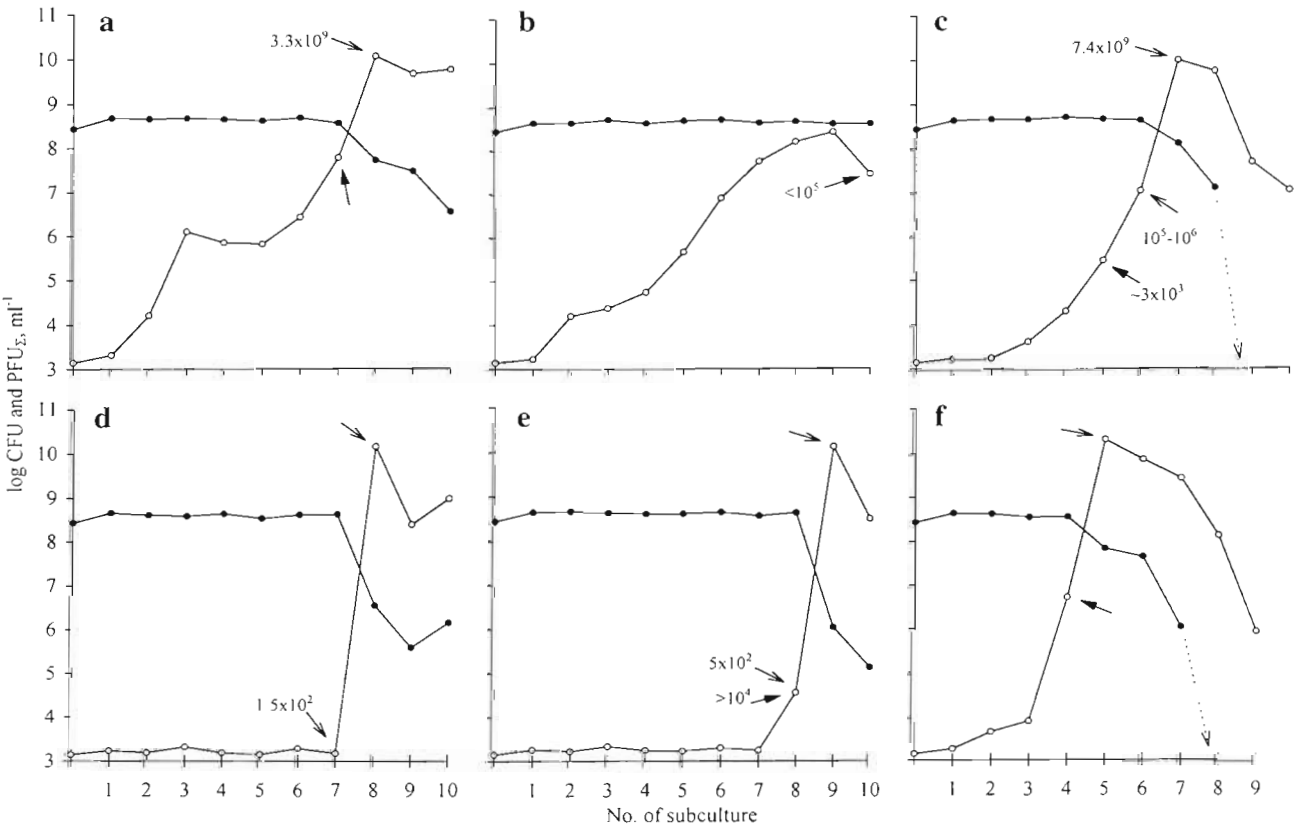

Fig. 7. Individual course of events in parallel series of subcultures branching off subculture #52 of Series 1. $H24_{wt}$ grown in SSW with 600 mg l^{-1} of organic nutrients at 25°C and 1 rpm. Dilution factor between subcultures: 210 (a–c) and 2100 (d–f), respectively. (●) CFU as determined by plating on SWA with anti-phage serum when necessary. (○) PFU titrated with hosts H24(L10) for PFU_{Σ} and with $H24_{wt}$ for virulent mutant (PFU_{vir}). Arrows with filled or open heads: subculture with first observation of PFU_{vir} represented by small turbid large clear plaques, respectively. Arrows without data added: no discernible difference between numbers of PFU_{vir} and PFU_{Σ}

F the concentrations of over 10^{10} PFU_{vir} ml^{-1} are factors of 3 to 7 higher than the highest values found in series A to C.

From decreases in final CFU concentrations observed in series A to F it is concluded that immunity by pseudolysogeny generally was incomplete at the best. Since the development of pseudolysogeny in series D to F was slowed by higher dilution between subcultures, the explosive increase in free vir mutant phage in these series was very probably due to lacking or minimal immunity. The gradual increase of free non-vir mutant phage in series A to C essentially strengthened immunity of cells by pseudolysogeny and, at the same time, suppressed the development of PFU_{vir} populations (whether as 'cellular PFU' or as free phage), if only for some time.

Summarizing the results of this part of investigations, the following points can be made:

(1) The occurrence of mutants of phage ϕH24 is promoted by high nutrient concentration of the culture medium. However, it remains an open question whether increased incidence of mutant phage in nutrient-rich media indicates higher mutation rates due to shortened generation time, i.e. hastened DNA replication, as predicted by the copy error hypothesis (Kubitschek 1970).

(2) In general, vir mutants are preceded by non-vir mutants. Depending on relatively low dilution (1/200 and less) between consecutive cultures in liquid medium, non-vir mutant phage become enriched. The release of non-vir mutant phage, at least for some time, seems to hinder the production or the release of vir mutant phage before the cells concerned are transferred into fresh medium. This effect depends on pseudolysogeny which not only prevents infection but also the release of phage (Moebus 1977b).

(3) Higher dilution (1/1000 and more) between consecutive cultures prevents gradual build-up of mutant PFU populations as well as pseudolysogenization to a degree sufficient to immunize cells against infection. Hence mutations in ϕH24 immediately become expressed by production and release of mutant phage, which in the case of vir mutants may cause breakdown of the respective bacterial population.

Occurrence of free virions of wild-type phage ϕH24

All experiments presented below were done with still cultures incubated at 20°C. Full size grown plaques of wild-type phage ϕH24 were repeatedly observed to gradually increase in number during succeeding subcultures at nutrient concentrations of 6 and 0.6 mg YEP l^{-1} used in earlier experiments. These increases were always short-lasting and insignificant in terms of numbers, which, for example, over 2 to 4 subcultures increased from 100 to 300 ml^{-1}. However, they might be of significance in regard to the basic question initiating the investigation of H24: How are phage-host systems maintained in nature?

The observed increases in numbers of plaques of ϕH24 were probably triggered by the transfer of $H24_{wt}$ cells from spent into fresh medium either directly or via resumed bacterial growth. Therefore, in experiments started after 36 and 32 previous subcultures grown at 6 and 0.6 mg l^{-1} of organic nutrients, respectively, the influence of nutrient additions on the release of ϕH24 was tested.

Several sets of 3 subcultures with 6 and 0.6 mg YEP l^{-1}, respectively, were used to check whether the increase in the number of ϕH24 plaques was coupled with nutrient addition as such or with bacterial growth initiated by nutrient addition. When nutrients were added, the volume of sufficiently diluted YEP stock was adjusted according to the volume of aliquots withdrawn from the cultures for preceding tests. All information gathered points to a tight correlation between bacterial growth and plaque production. This relates to both nutrient concentrations employed. One should, however, take into account that the effect of nutrient addition at the level of 0.6 mg l^{-1} is rather weak.

Fig. 8 presents results found with 2 sets of 3 parallel subcultures grown in SSW with 6 mg YEP l^{-1}. As already stated, the effect of nutrient addition strictly depended on initiation of bacterial growth. It was low in all cultures when nutrients were added after up to 6 d of incubation. In contrast, nitrient addition induced appreciable increase in plaque numbers of ϕH24 when the CFU concentration underwent slight reductions due to starvation. This was found with all cultures shown in Fig. 8a after 13 d as well as with culture #2 after 9 d, and cultures #1 and #3 after 13 d, depicted in Fig. 8b.

Another finding concerns the survival of free ϕH24 virions. As one can see from both panels of Fig. 8, any increase in plaque numbers was immediately followed by their rapid reduction. This even happened after nutrient addition to culture #1 in Fig. 8a.

In an experiment designed to investigate the physical nature of ϕH24 PFU, cultures of about 210 and 100 ml were placed in Erlenmeyer flasks of 500 ml capacity. Here it was observed that the increase in the number of plaques of wild-type phage ϕH24 in SSW with 0.6 mg YEP l^{-1} was not only weaker but also slower than in SSW with 6 mg YEP l^{-1}. Results concerning bacterial growth and PFU concentrations are compiled in Table 2.

Table 3 presents the findings concerning the nature of PFU causing wild-type plaque formation in the experiment described above. They were obtained

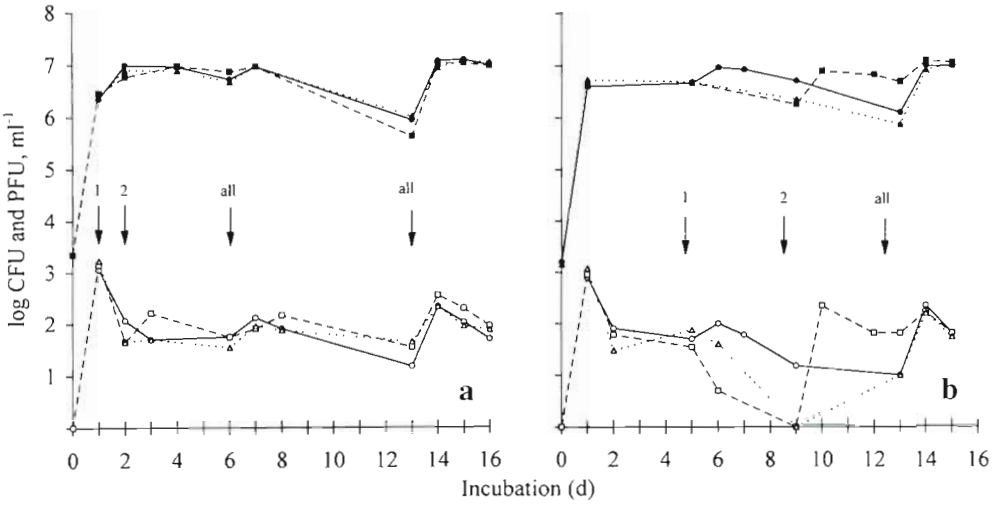

Fig. 8. Growth of $H24_{wt}$ (filled symbols) and occurrence of wild-type phage PFU (open symbols) in synthetic seawater with 6 mg l^{-1} of organic nutrients from YEP. Two sets of 3 parallel cultures each (a and b) inoculated with thoroughly adapted $H24_{wt}$ cells. Arrows indicate day of nutrient addition to renew the initial concentration; number above arrow indicates the respective culture in set. (●, ○) #1, (■, □) #2, (▲, △) #3

by filtering 5 ml portions of each of the 4 cultures through Sartorius cellulose nitrate filters of 0.15 µm pore size. Filters were washed with 1 ml of 3% BE solution each either before or after filtration. Filtrates and washes then were titrated for PFU with H24(L10). Obviously the PFU were retained in unwashed filters until being eluted by BE. From these results no doubt remains that with SSW cultures containing the lowest amounts of YEP tested, the majority of wild-type phage plaques observed were initiated by free virions. The data are also in agreement with earlier observations of short-lived peak concentrations of ϕH24 PFU.

In filtrates produced with filters washed with BE prior to use, the free virions survived storage in the refrigerator much better than in the culture from which the respective filtrate was prepared. This corresponds with the aforementioned observations made with cultures to which nutrients were added during incubation. The survival of free virions may have been supported by the BE that unavoidably became part of the filtrate. However, much greater importance is attached to the general ability of temperate phage to adsorb to, and to infect, cells bearing the genetic information of a homologous phage. Usually, this has no consequence for the infected cell's survival but eliminates the infecting virion.

Table 2. Growth of $H24_{wt}$ and occurrence of plaques of wild-type phage ϕH24 during incubation at 20°C as still culture (in Erlenmeyer flasks of 500 ml capacity). Synthetic seawater with 6 and 0.6 mg l^{-1} of yeast extract-peptone solution (1:5 parts, YEP). Culture designations: 6/210 and 6/100 = 210 or 100 ml with 6 mg YEP l^{-1}; 0.6/210 and 0.6/100 = respective volumes with 0.6 mg YEP l^{-1}. Cultures inoculated from 36th and 32nd subculture, respectively, at respective nutrient concentration. Titrations of CFU and PFU on daily scale. bld: below level of detection; nt: not tested

Time (d)	CFU ml^{-1}	PFU ml^{-1}	CFU ml^{-1}	PFU ml^{-1}
	6/210		**0.6/210**	
0	1.8×10^3	bld	5.6×10^2	bld
1	7.0×10^6	1.6×10^3	5.5×10^4	bld
2	7.4×10^6	~60	1.1×10^6	2.4×10^2
3	7.8×10^6	~80	6.8×10^5	~90
8	2.1×10^6	nt	3.0×10^5	nt
12	9.2×10^5	nt	1.4×10^5	nt
16	2.5×10^5	bld	1.1×10^5	bld
	6/100		**0.6/100**	
0	1.8×10^3	bld	5.6×10^2	bld
1	6.6×10^6	1.7×10^3	3.8×10^4	bld
2	7.4×10^6	~35	9.8×10^5	3.3×10^2
3	5.7×10^6	~35	6.4×10^5	~75
8	2.2×10^6	nt	2.4×10^5	nt
12	4.5×10^5	nt	1.6×10^5	nt
16	7.0×10^4	bld	4.0×10^4	bld

Table 3. The physical nature of wild-type PFU was determined by filtration of $H24_{wt}$ culture through Sartorius cellulose acetate filters of 0.15 µm pore size. Filters washed with 3% BE solution either before or after filtration of 5 ml of culture. Same cultures as in Table 2. PFU ml^{-1} of filtrate or washes, respectively. bld: below level of detection

Age of culture (d)	Designation of culture	Filter washed with BE: Before	Not washed	After
1 d	6/210	1.27×10^3	~25	3.01×10^3
	6/100	1.28×10^3	~55	3.99×10^3
	0.6/210	~10	bld	<10
	0.6/100	~10	bld	<10
2 d	6/210	~15	bld	~45
	6/100	<10	bld	~45
	0.6/210	2.1×10^2	bld	6.4×10^2
	0.6/100	2.1×10^2	bld	3.4×10^2
3 d	0.6/210	~75	bld	~50
	0.6/100	~40	bld	>100

DISCUSSION

The main features of the marine lysogenic bacterium H24 as revealed during laboratory investigations employing the most commonly used microbiological media have been presented in preceding papers (Moebus 1997a, b). Some or all of these features may be of no importance when H24 exists in its natural habitat. Then its capacity to develop immunity against certain phage types by pseudolysogeny might rarely if ever be used. According to the observations made in the present investigation, the probability of mutations in the genome of phage ϕH24 under natural conditions will also be greatly reduced. However, the observed relation between frequency of occurrence of phage mutants and nutrient concentration points to the dangers of using nutrient-rich media for long-term cultivation of microorganisms whose natural environment is nutritionally poor.

These dangers are all too well known to people using microorganisms on an industrial scale. Problems caused by spontaneously occurring phage in cultures used as starters in cheese-making are of enormous economical interest, to mention only the best known example.

With regard to $H24_{wt}$ the phenomenon of spontaneous plaque formation offers the opportunity to gain some insight into processes that contribute to the occurrence of, and variation in, marine phage-host systems. Based on the results presented in this paper, the chances of phage mutants arising that may infect and be reproduced by bacteria differing from $H24_{wt}$ are regarded as limited but existent. When the experiment summarized in Table 3 was performed, a few mutant plaques (below 1% of plaque counts) were observed in the filtrates of cultures set up with 6 mg YEP l^{-1}.

The chances of wild-type phage ϕH24 meeting with a host cell that is unsuited to establishing lysogeny but would reproduce it cannot by estimated. From this point of view the present findings of low numbers of free virions of ϕH24 are of no bearing. They are, however, of interest with regard to the problem of inducibility of lysogenic bacteria by natural factors.

In the view of this author, organic nutrients, which in most marine environments occur in limiting concentrations, are the most important factor causing the release of free phage by lysogenic bacteria. Of course, what happened during this investigation when the nutrient concentration was increased or replenished cannot be accounted for by induction. Induction caused by various agents leads to high losses in viable cells. By way of contrast, $H24_{wt}$ most actively released ϕH24 particles when its cells were multiplying.

In relation to phage ecology, this would be an economic way to convey the phage's genes to a new generation. When nutrients become available, they will be used by all organisms fit to do so. Therefore, conditions supporting non-specialized bacterial growth provide the best chances of free phage encountering a suitable host cell which in one way or the other—by lysogenization or immediate phage reproduction—will support the survival of the phage strain in question.

Pseudolysogenization was never indicated in experiments performed at the nutrient concentration of 6 mg YEP l^{-1} and below. From this it is reasonable to assume that under natural conditions this trait of $H24_{wt}$ has hardly any chance of becoming effective.

This investigation and the preceding papers dealing with $H24_{wt}$ and its derivatives (Moebus 1997a, b) report for the first time some substantial details about a marine lysogenic bacterium. As fascinating as they may be, they provide only a glimpse of the diversity waiting to be discovered. With another lysogenic bacterium certainly more or less different observations would have been made.

Acknowledgements. I am very grateful to Dipl. Biol. Antje Wichels, Biologische Anstalt Helgoland, and to the anonymous reviewers for critical reading of my manuscripts and making many helpful suggestions.

LITERATURE CITED

Bergh O, Børsheim KY, Bratbak G, Heldal M (1989) High abundance of viruses found in aquatic environments. Nature 340:467–468

Børsheim KY (1993) Native marine bacteriophages. FEMS Microbiol Ecol 102:141–159

Hastings JW, Keynan A, McCloskey K (1961) Properties of newly isolated bacteriophage of luminescent bacteria. Biol Bull (Woods Hole) 121:375

Jiang SC, Paul JH (1994) Seasonal and diel abundance of viruses and occurrence of lysogeny/bacteriocinogeny in the marine environment. Mar Ecol Prog Ser 104: 163–172

Kubitschek HE (1970) Introduction to research with continuous cultures. Prentice-Hall Biological Techniques Series, Prentice-Hall, Inc., Englewood Cliffs, NJ

Moebus K (1987) Ecology of marine bacteriophages. In: Goyal SM, Gerba CP, Bitton G (eds) Phage ecology. John Wiley & Sons, New York, p 137–156

Moebus K (1996a) Marine bacteriophage reproduction under nutrient-limited growth of host bacteria. I. Investigations with six phage-host systems. Mar Ecol Prog Ser 144:1–12

Moebus K (1996b) Marine bacteriophage reproduction under nutrient-limited growth of host bacteria. II. Investigations with phage-host system [H3:H3/1]. Mar Ecol Prog Ser 144:13–22

Moebus K (1997a) Investigations of the lysogenic marine bacterium H24. I. General description of the phage-host system. Mar Ecol Prog Ser 148:217–228

Moebus K (1997b) Investigations of the lysogenic marine bac-

terium H24. II. Development of pseudolysogeny in nutrient-rich broth culture. Mar Ecol Prog Ser 148:229–240

Rambler M, Margulis L (1979) An ultraviolet light induced bacteriophage in *Beneckea gazogenes*. Origins of Life 9: 235–240

Spencer R (1955) A marine bacteriophage. Nature, Lond 175:690

Wilcox RM, Fuhrman JA (1994) Bacterial viruses in coastal seawater: lytic rather than lysogenic production. Mar Ecol Prog Ser 114:35–45

This article was submitted to the editor

Manuscript first received: September 2, 1996
Revised version accepted: January 9, 1997