Investigations of the marine lysogenic bacterium H24. I. General description of the phage-host system

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ABSTRACT: General features of the marine lysogenic bacterium H24 are described. The bacterial strain was isolated from a sample of North Sea water in 1978. After several consecutive transfers on seawater agar slants spontaneous plaque-formation was observed in 1981 When H24 was grown in bouillon the cultures were found to contain plaque forming units (PFU) at any number between zero and 10^9 ml^{-1}, indicating that spontaneous plaque formation was due to mutational events. Cultures with the highest contents of PFU hardly differed in turbidity from cultures lacking PFU. These observations are ascribed to pseudolysogeny, i.e. immunity of cells against the phage present. When a cured derivative, H24(L10), became available the wild-type phage φH24 residing in H24 was isolated and shown to re-lysogenize H24(L10). It also enabled differentiation between virulent and non-virulent mutants. Mutants of φH24 were found to induce pseudolysogeny. Upon streaking on nutrient agar, material from colonies of pseudolysogenized cells produced various kinds of colonies representing clones of fully sensitive cells, of pseudolysogenized cells, or of mixtures of both. This and accompanying papers report the first intensive studies of a marine lysogenic bacterium.

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

INTRODUCTION

For decades it was assumed that bacteriophage concentrations in seawater samples were too low to be of ecological significance. However, since the discovery of abundances of viral particles ranging between about 10^5 and 10^7 ml^{-1} reported by Bergh et al. (1989), more attention has been focused on marine virology and considerable progress made in acquiring knowledge on this topic. Nevertheless, we still do not know how—or whether at all—specific phage-host systems (PHS) are maintained in nature for extended periods of time.

Several possible mechanisms for PHS maintenance, among them stability of infectiousness of phage particles, have been considered but rejected as being unsuited to maintenance in the long run (Moebus 1992c, Suttle & Chan 1992). At present, close attention is being given to lysogeny, i.e. the inheritable ability of bacterial cells to produce phages whose genetic information they harbour.

Unfortunately, there is almost no information available concerning lysogeny in marine bacteria. Hastings et al. (1961) isolated a temperate phage from luminescent bacteria, and Rambler & Margulis (1979) were able to induce phage production with ultraviolet light in a red pigmented marine vibrio (*Beneckea gazogenes*). Recently Jiang & Paul (1994) reported the occurrence of mitomycin C-inducible lysogeny or bacteriocinogeny in 22 of 51 bacterial isolates collected from estuarine and coastal oceanic water as well as from benthic invertebrates. These authors pointed to the main barrier in lysogeny research, the general lack of suitable indicator strains, which in their case impeded differentiation between lysogenic and bacteriocinogenic bacteria.

Another approach to the involvement of lysogeny in marine phage production was presented by Wilcox & Fuhrman (1994), who followed the production of phage and bacteria in cultures set up with 0.2 and 0.02 µm filtrates inoculated with portions of the respective seawater sample pre-filtered through 0.6 µm filters. They found phage production to depend on minimum concentrations of phage and bacteria, but no indication of spontaneous or light-induced phage production by lysogenic bacteria.

The marine bacterium H24 described in this and subsequent papers was isolated in 1978 from a seawater sample collected near Helgoland, Germany, and used as host bacterium of 4 phage strains detected from the same sample by means of enrichment culture (Moebus 1980). Several years later H24 was found to be a lysogen now able to survive in the presence of 10^{10} ml^{-1} of spontaneously produced, principally destructive mutants of the resident phage strain.

This paper describes the general features of the presently unique marine phage-host system and presents a working hypothesis based on pseudolysogeny, a transient state of immunity, to explain most of the findings. Subsequent papers will deal with investigations performed to test and corroborate the hypothesis. They especially concern the development of pseudolysogeny and the possible ecological implications of the respective traits of H24.

MATERIALS AND METHODS

Media. Seawater mixture (SM) consisting of 75% aged seawater and 25% distilled water was used for preparing the following 3 media. Seawater agar (SWA) contained (in 1 l of SM) 5 g Difco peptone, 1 g Difco yeast extract, 0.01 g FePO$_4$, and 15 g Difco agar; pH adjusted to 7.6. Soft seawater agar (SSWA) was the same as SWA, but with only 6 g agar l^{-1} and FePO$_4$ omitted. Seawater bouillon (SWB/5) was prepared from 1 g Difco peptone, 0.2 g Difco yeast extract, and 0.01 g FePO$_4$ l^{-1} of SM. A fourth medium, beef extract solution (BE) contained 3% Difco beef

extract in distilled water. Media were autoclaved for 20 min at 121°C.

Bacteria and bacteriophages. Available strains are listed in Table 1. The bacterial strain H24, henceforth referred to as H24$_{wt}$, was isolated in 1978 from a seawater sample collected near Helgoland (North Sea). It was found to belong to the *Vibrionaceae* (Moebus & Nattkemper 1983). Several lysogenic derivatives of H24$_{wt}$ have been isolated in the meantime. They differed mainly in regard to the readiness of cells to release wild-type phage φH24. After becoming available, the cured derivative H24(L10) was exclusively used for preparation of phage stocks. H24$_{wt}$ and its various derivatives are maintained on SWA slants, stored in the refrigerator.

The bacteriophage strains, derived either from seawater samples or from H24$_{wt}$, are maintained as high titer stocks prepared by plate elution (Moebus 1980) and stored in the refrigerator. Specifications of available phage strains are presented in Table 1. Phages isolated with H24$_{wt}$ from seawater samples but unrelated to φH24 are given as φH24/x, with x representing a number. The wild-type phage present in H24$_{wt}$ will be referred to as φH24. Mutant strains of φH24 are designated as φH24-x. Mutants of φH24 may be either virulent (vir) or non-virulent (non-vir) (Table 1). Both mutant types cause plaques on H24(L10). In contrast, on H24$_{wt}$ only vir mutants can do so. Their plaques produced with H24$_{wt}$ generally differ greatly in size and appearance from those generated with H24(L10).

Methods. Bacterial cultures were grown in a culture roller at 25°C and 1 rpm, with SWB/5 used throughout. Cultures and subcultures used in experiments were

Table 1. Bacteria and phages. p.m.: number of particles measured. Vir: virulent

(A) Bacterial strains

Designation	Description	Appearance
H24$_{wt}$	Lysogenic wild-type strain	Large white colony (~2 mm diam. after 2 d 25°C)
H24(L3)	Lysogenic derivative of H24$_{wt}$	As before
H24(Lg)	As before, multiple resistant against all available phage strains except H24/21	Colony grey, knob-like (~1 mm diam. after 2 d 25°C)
H24(L10)	Non-lysogenic derivative of H24$_{wt}$	Large white colony as of H24$_{wt}$

(B) Bacteriophage strains

Designation	Source	Type/halo	Family	Measurement of particles (nm) Head (width/height)	Tail (length/diam.)	p.m.
φH24/1	Seawater	Vir / +	*Podoviridae*	58 / 58	15 / 20	10
φH24/2	Seawater	Vir / +	*Siphoviridae*	50 / 54	99 / 10	11
φH24/21	Seawater	Vir / –	*Podoviridae*	59 / 63	~10 / ~17	6
φH24[a]	H24$_{wt}$	Temperate	*Myoviridae*	50 / 55	92 / 20	10

[a]Mutants of phage φH24 were derived either from H24$_{wt}$ or H24(L3) and are designated as follows:
    Vir type: φH24-1, φH24-4, φH24-5, φH24-9, φH24-11, and φH24-12
    Non-vir type: φH24-2, φH24-3, φH24-6, φH24-7, φH24-8, φH24-10, and φH24-13

generally run for 24 h each, and pre-warmed SWB/5 was employed with the latter.

For the determination of plaque-forming units (PFU), Adams' (1959) double-layer method was used, preferably with plates containing a bottom layer of 10 ml SWA. In special cases, however, plates with a bottom layer of 20 ml SWA had to be employed (see 'Results'). Cultures of indicator bacteria were incubated for 18 h and used in 0.2 ml aliquots per SSWA overlay (2.6 ml each). H24$_{wt}$ was used to enumerate virulent mutants (PFUvir) of wild-type phage ϕH24, and H24(L10) to detect any type of PFU (PFU$_\Sigma$) produced by H24 strains.

Where H24$_{wt}$ had to be used in PFU titrations, the following procedure was found to greatly minimize the chance of producing cultures with high PFU contents: Before set-up of experiments, 3 to 4 consecutive daily streaks on SWA were made and freshly grown, 2 d old colonies of about 2 mm in diameter exclusively used as inoculum of roll cultures. Such cultures generally contain no, or only a few, vir mutant PFU (PFU$_{vir}$) ml^{-1} so that it is possible to work with only one such culture per test without too much risk of producing PFU titrations which cannot be reasonably evaluated. With H24(L3) no method of comparable reliability was found.

Numbers of colony-forming units (CFU) were determined by spreading suitably diluted cell suspensions on SWA (10 or 20 ml SWA per plate). If necessary, 0.1 ml anti-phage serum (AS) diluted in SM was spread on SWA prior to use of the plates (AS-SWA). Low speed centrifugations were run for 30 min at 4650 × g and about 8°C. Filtrations were done with Sartorius cellulose nitrate filters (0.15 µm pore size) washed with 1 ml of BE before use.

To detect cured (de-lysogenized) bacteria, perfect looking colonies of H24$_{wt}$ or H24(L3) grown on SWA or AS-SWA were isolated by loop and the material suspended in 1 ml of SM each. The suspensions were transferred by means of a 20-point inoculator to (I) SWA, (II) a double-layer plate with the SSWA layer seeded with H24(L10), and (III) SWA plated in advance with 0.1 ml of high titer phage stock of ϕH24-1. Steps I and II were made with the same inoculator, step III with a separate one. Step I secured material for repetition of the test, if necessary; steps II and III were done to test for lysogeny. Tests performed in this way only rarely produce false negative results, which with little effort can be detected. On the other hand, plaques occurring in spots produced in step II invariably indicate lysogenicity of the tested material.

Serum preparation. Anti-phage serum (AS) was prepared by inoculating a rabbit twice with the non-virulent mutant ϕH24-2. Prior to use portions of 20 to 30 ml of serum were repeatedly treated with large numbers of cells of H24(L10) to remove residual antibodies active against the bacteria and finally sterilized by filtration.

Electron microscopy. Phages were prepared, and micro-photographs produced, as described by Frank & Moebus (1987).

Photography. Photographs of colonies and plaques were taken with a Wild Photomakroskop M400 on Agfapan 25 film.

Abbreviations. Besides those already mentioned, abbreviations compiled in Table 2 will be used in this and the subsequent papers.

RESULTS

During the investigations of Moebus & Nattkemper (1981) and Moebus (1983) several hundred bacterial isolates derived from seawater samples were screened by spot-tests for bacteriophage sensitivity. With about half a dozen strains, conspicuously disturbed lawns were observed. The disturbances were caused by small turbid plaques (diameter 0.1 to 0.3 mm) which were detected only due to being present in very large numbers, as well as to the fact that large portions of the bacterial lawn remained unused in the spot-test.

Preliminary tests performed at that time revealed that the presence of bacteriophages in the respective cultures probably could not be related to contamination. It was found that purification of the bacteria by additional consecutive streaks on SWA in principle did not change the ability of these bacteria to cause plaque formation. However, the frequency of plaque forming units in liquid cultures of these bacterial strains varied between zero and perhaps 10^5 PFU ml^{-1} or even more. This was observed with bacterial isolates producing only one type of colonies as well as with isolates which,

Table 2. Uncommon abbreviations used in this and consecutive papers

LWC	Large white colonies produced by H24$_{wt}$, H24(L3) and H24(L10) in the absence of infective phage, about 2 mm in diameter after growth for 2 d at 25°C on SWA
SGC	Small grey colonies produced by H24$_{wt}$, H24(L3) and H24(L10) in the presence of high concentration of suitable mutants of the wild-type phage ϕH24, consisting of pseudolysogenized, i.e. immune, cells which regain phage sensitivity upon complete removal of phage
TTP	Tiny turbid plaques with diameter 0.2 to 0.6 mm depending on H24 derivative, produced by wild-type phage ϕH24 released by lysogenic cells in the presence of non-lysogenic indicator strain H24(L10) during plate incubation

in streaks of material taken from single colonies, always gave rise to more than one colony type Plaque formation generally was observed with all of the different colony types, with the numbers of PFU also varying from culture to culture

From these preliminary findings it was concluded that the bacteria in question probably were lysogenic and that the observed plaques were due to spontaneous mutations of the phage genome residing in the cells.

With strain H24$_{wt}$, derived in 1978 from a seawater sample taken near Helgoland, 4 phage strains differing in plaque morphology and yield of progeny phages per plaque were obtained from the same seawater sample. Phage strains ϕH24/1 and ϕH24/2 produced plaques of 3 to 5 mm in diameter consisting of a small clear centre surrounded by a large turbid halo, but differed in other aspects. e.g in the size of the clear centre and its relation to the width of the halo as well as by the number of progeny phage eluted from plaques of equal size They were insensitive to the AS produced against mutant phage ϕH24-2. By electron microscopy these strains were observed to differ morphologically from each other as well as from ϕH24 and its mutants. The remaining 2

phage strains were later identified as being related to ϕH24, and discarded.

Fig 1 shows the morphological types of phages found with H24$_{wt}$ so far ϕH24/1 and ϕH24/21, isolated in 1978 and 1988, respectively, are *Podoviridae* that differ distinctly in their tail structures. While the short compact tail was seen in all intact particles of ϕH24/1, observations made on ϕH24/21 point to the existence of a more complex structure. It seems to consist of 2 parts, one attached directly to the head, and in shape being as broad as it is short, and the other, extending from it, reminiscent of the tube protruding from contracted sheets of *Myoviridae* (Fig. 1e). These 2 phage strains differ also in that ϕH24/21 can produce plaques with a lysogenic mutant of H24$_{wt}$, H24(Lg), which forms grey colonies and is resistant against all other available H24 phages. (Multiple resistance was found with all phage-resistant mutants of H24$_{wt}$ investigated, whether lysogenic or not.) The virions of ϕH24 and its mutants are visually indistinguishable. Therefore, only ϕH24 is shown, each one particle fully intact and with contracted sheath (Fig. 1d & e, respectively).

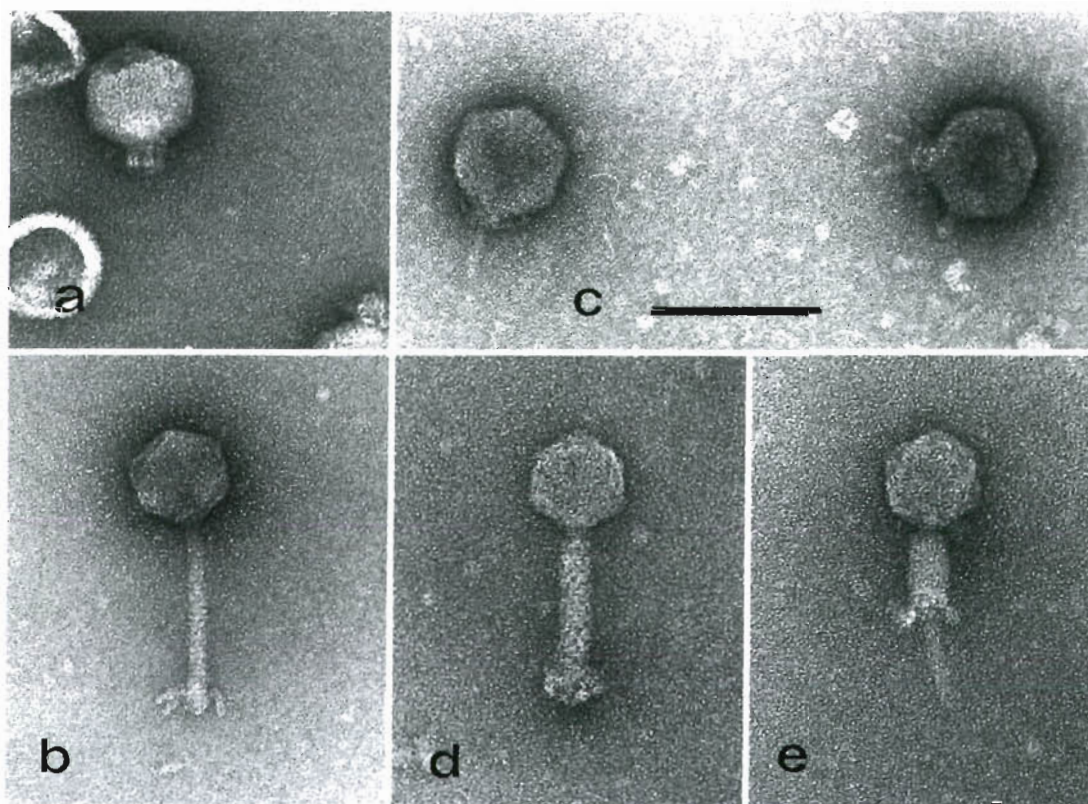Some of the more or less varied plaques of mutant phage produced with H24(L10) as host are shown in Fig 2

Fig 1 Particles of phage strains (a) ϕH24/1, (b) ϕH24/2, (c) ϕH24/21, and (d, e) ϕH24. Scale bar = 100 nm. Photographs by H. Frank
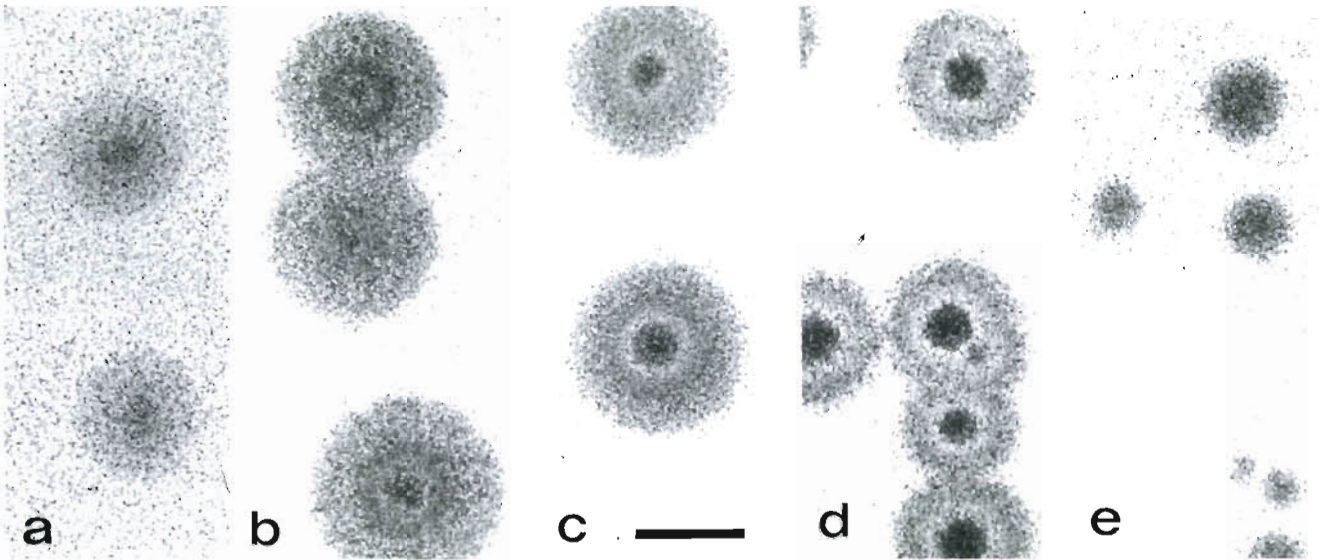
Fig. 2. Plaques produced with host H24(L10) by temperate strain (a) φH24, (b) non-virulent strains φH24-2, and (c) φH24-3, and the virulent strains (d) φH24-4 and (e) φH24-5. Scale bar = 1.0 mm

Early investigations without a non-lysogenic indicator strain available

The ability of H24$_{wt}$ to form plaques spontaneously was not recognized before 1981, when an investigation of the taxonomy of phage-sensitive bacteria was performed (Moebus & Nattkemper 1983). At that time a liquid culture, inoculated with material taken from a freshly prepared slant, produced a confluently lysed lawn, although the culture appeared perfectly well grown. With material from the same slant, a streak was obtained with only a few colonies resembling those typical for strain H24$_{wt}$, i.e. large white colonies (LWCs) of about 2 mm in diameter after 2 d at 25°C. The majority of single colonies were much smaller (0.5 to 1.0 mm in diameter), grey, somewhat glassy (small grey colonies, or SGCs), often containing white material distinctly arranged. In addition, a number of white colonies showed various degrees of lytic attack. Fig. 3 presents examples of such colony types. Note that the LWCs in Fig. 3a show no sign of cross-infection by phage present in very large numbers in the SGCs, as observed with other LWCs in the same plate. These are rather untypical observations, which sometimes were also made with streaks prepared with SGC material. (For explanation see below.)

Further streaks performed with portions of the various colony types produced results as follows: Material from LWCs always gave rise to the same type of colony. The same was true when the material was taken from a white colony showing symptoms of lysis as long as the lytic zone was avoided during removal. If this was not the case, the resulting streak would contain a mix-

ture of the various colony types. A similar result would be found after streaking material taken from SGCs. Repeated streaking of respective material always would give the same results.

Suspensions prepared with the SGC material, when plated with strain H24$_{wt}$ as host, were always found to contain large numbers of PFU, much in contrast to suspensions of LWCs which rarely had more PFU than the culture of the host alone. The occurrence of SGCs apparently depended on the presence of phages able to transform cells which, in the absence of these phages, would give rise to LWCs.

The positive correlation between PFU concentration and the occurrence of SGCs became obvious when cultures inoculated with cells of large white colonies were repeatedly sub-cultivated. Determinations of CFU and PFU performed at the end of each subcultivation revealed that, in comparison to the preceding subculture, the titer of PFU as well as the portion of small grey (and lytic white) colonies had increased. Differences in turbidity of the various sub-cultures remained small as long as the PFU titer was not considerably higher than 10^7 ml^{-1}.

Summarizing these observations, it was concluded that SGCs either consist of cells most or all of which produce phages spontaneously without being destroyed or, more probably, develop in the presence of phages able to transform cells which, in the absence of these phages, would give rise to LWC. The last-mentioned interpretation was preferred, since it would help to explain the occurrence of LWC when SGC were streaked on SWA.

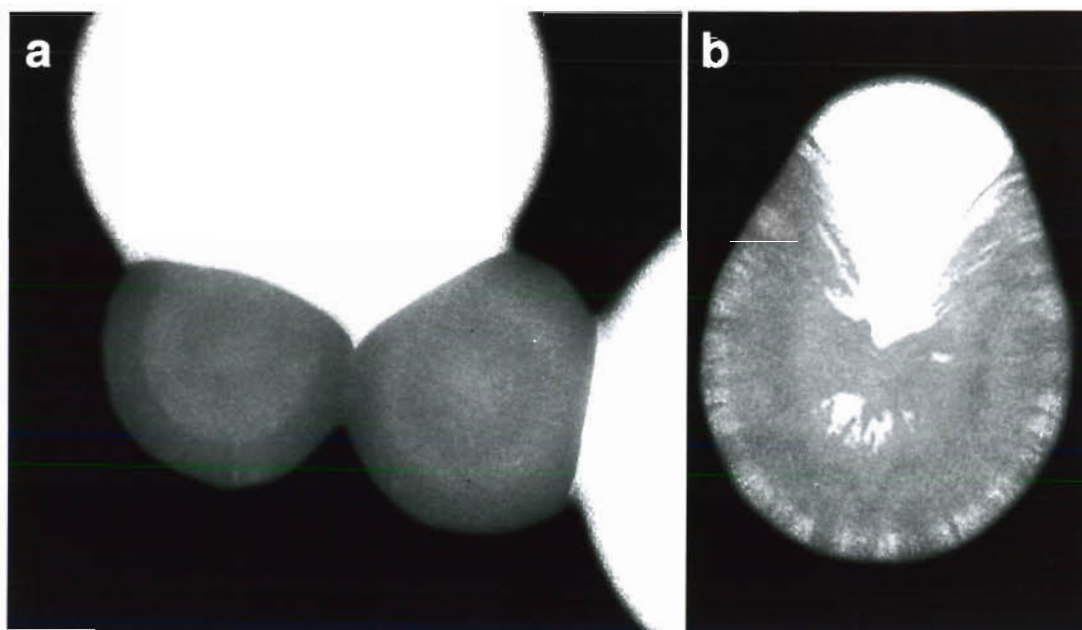The dependence on abundantly present phages for SGCs to occur was confirmed when cells of LWC were

Fig. 3. Colonies of H24(L3), grown on SWA with sub-optimal concentration of anti-phage serum after plating from a culture containing about 2.5×10^{10} PFU ml^{-1} of virulent mutants and about 9×10^7 CFU ml^{-1} The large white colony shown in (a), top, measured 1.5 mm in diameter and the small grey colonies 0.8 mm (left) and 0.9 mm (right). The mixed type colony in (b) measured 1.2 by 1.6 mm

incubated on SWA seeded in advance with more than 10^8 PFU per plate from 'lysates' prepared from lytically active sub-cultures. These 'lysates' most probably contained more than one type of mutant phage; however, they differed in the predominant mutant type as indicated by differences in plaque size observed with H24$_{wt}$. With 2 such 'lysates', each containing about 90% of the predominant phage type, identical results were obtained: After incubation of cells from LWCs on plates seeded with phage only SGC were found, their numbers being in reasonable agreement with the various dilutions of the cell suspensions plated. With both 'lysates' it was found that only about 0.15% of the LWC cells grew to colonies, which were exclusively SGCs. On phage-free SWA only LWCs (= 100%) developed from the LWC cells.

When cells from SGCs were suspended and plated on SWA seeded with phage from the aforementioned 'lysates', only SGCs were again found. However, in these cases apparently almost 100% of the cells were able to develop to SGCs. On phage-free SWA a mixture of SGCs, LWCs and lytic LWCs was found, with the portion of SGCs decreasing with increasing dilution of the cell suspension. The latter findings are in general agreement with observations made with streaks of SGC material.

The decrease of the portion of SGCs and concomitant increase of that of LWCs (lytic plus non-lytic ones) with increasing dilution was also observed when cultures of H24$_{wt}$ rich in PFU were tested for CFU and PFU. Although the numbers of colonies in plates inoculated after low dilutions of the cultures could only be estimated, the results of quite a number (>60) of tests can be summarised as follows:

(1) In most cases the estimated numbers of colonies observed with dilutions ranging from 10^{-1} to 10^{-3} agreed fairly well with the dilution, although often colony numbers tended to increase slightly with increasing dilution.

(2) With 6 cultures very similar and unusually low numbers of colonies were found after dilutions in the lower range, whereas the colony counts made after higher dilutions were more or less in agreement with the dilutions. For example, about 5000 colonies each were estimated for a culture diluted both 50- and 500-fold, but 1000, 109, and 12 colonies were counted on the plates inoculated from the next 3 dilutions (each increasing by factor 10).

(3) There was no recognizable correlation between the concentration of CFU and PFU. With one culture containing 9×10^9 PFU ml^{-1} about 5×10^8 CFU ml^{-1} were found, but with another culture containing almost 2×10^9 PFU ml^{-1} only about 9×10^5 CFU ml^{-1} were counted. This observation can without doubt be ascribed to the fact that mutation is an unpredictable statistical event. Mutations of the wild-type phage occurring early during incubation must have quite different consequences compared to mutations taking place toward the end of cultivation.

From these observations it was concluded that formation of SGCs, as well as production of apparently

healthy liquid cultures of H24$_{wt}$ which produced confluently lysed lawns, depended on the presence of large numbers of mutant phage and was due to the development of pseudolysogeny which is induced by material, henceforth called 'factor X', concomitantly released with phage by lysing cells.

Further investigations were severely hampered by the lack of an indicator strain such as a non-lysogenic derivative of strain H24$_{wt}$. Several attempts to isolate cured derivatives failed. In the course of these experiments it was found that H24$_{wt}$ cannot be induced by UV-treatment.

At that time the inclination of H24$_{wt}$ for spontaneous plaque formation was known to vary to some extent among cell lines which differed slightly in the appearance of their colonies. (One of these lines, found to produce the least numbers of spontaneous plaques, was used for PFU titrations during the aforementioned investigations.) To elucidate those differences observed between LWCs an extensive experiment was performed which unexpectedly led to the detection of a cured cell line.

A SGC was streaked on SWA to produce LWCs of which 10 were isolated for the experiment. Upon repeated sub-cultivation on SWA and in SWB/5 it became obvious that the 10th colony isolated for the experiment consisted of cured cells. This line, H24(L10), since then is used as indicator strain for phages released by H24$_{wt}$, and another line, H24(L3), observed to produce plaques spontaneously more often than any of the other cell lines tested, became the most intensively investigated derivative of H24$_{wt}$.

Investigations employing indicator strain H24(L10)

With strain H24(L10) available, the wild-type phage φH24 could be isolated and tested for its ability to lysogenize H24(L10). Cells of H24(L10) not only were lysogenized by φH24 but also retained the capacity for spontaneous plaque formation. Furthermore, soon it became obvious that freshly grown LWCs of H24$_{wt}$ or of any of its lysogenic derivatives, such as H24(L3), generally contain numerous PFU representing non-virulent mutants of φH24, but only a small portion, if any, of virulent mutants. The same is true for broth cultures inoculated with material from freshly grown LWCs. However, in rare cases large portions of vir mutants were found, too. These observations firmly supported the interpretation of spontaneous plaque formation as being due to mutation of the phage genome residing within H24$_{wt}$.

An investigation of the DNAs of H24$_{wt}$ and φH24, employing methods compiled by Sambrook et al. (1989) and improvements thereof, confirmed the clas-

sical prophage status of φH24, i.e. its DNA as being integrated into the genome of H24$_{wt}$ (C. Schütt, Biologische Anstalt Helgoland, pers. comm.).

Early attempts to isolate a number of φH24 mutants of different plaque morphology revealed that 2 types of non-vir mutants, φH24-2 and, to lesser degree, φH24-3, occur by far most often. Regarding vir-mutants, φH24-1 is the most frequent type, whose plaques are indistinguishable from φH24-2 plaques when both phages are propagated on H24(L10). These findings indicated that—as in the case of phage λ (e.g. Sly et al. 1971)—probably more than one mutation is necessary to produce a virulent mutant of φH24.

Among the observations made soon after H24(L10) became available was the following: When broth cultures of low PFU content or suspensions prepared with material of lysogenic LWCs were tested for PFU, the number of plaques per plate with increasing dilution did not decrease, but increased until a maximum PFU ml^{-1} was reached. With increasing dilution, however, the appearance of the plaques changed considerably.

For example, a suspension prepared from a perfectly healthy looking LWC of 2 mm in diameter in 1 ml of SM, containing about 10^8 CFU ml^{-1}, with H24(L10) caused the development of about 1000 plaques when used undiluted. These plaques vary greatly in size (from 0.2 to about 2 mm in diameter) and structure (rather clear to more or less turbid, or clear centre with one to several turbid zones). With the suspension diluted by factor 10, several hundred plaques per plate were found, with the portion of small plaques considerably increased. Among the larger plaques about the same types were present as observed with the suspension used undiluted. Further dilution of the suspension by factor 10 resulted in plates with 2000 to 3000 tiny turbid plaques (TTP), all but a few ranging between 0.2 and 0.4 mm in diameter In such plates a zone devoid of TTP could often be observed around large plaques as shown in Fig. 4. After additional dilutions, plates with TTP only were obtained, the number of TTP per plate gradually decreasing until after dilutions in excess of 10^{-4} a constant number of PFU per colony was attained. Table 3 presents the results of a test performed with an LWC of H24(L3).

The analysis of the various plaque types found in such a series of plates revealed that almost 100% of plaques found after dilutions of 10^{-2} or higher contained particles of phage φH24 in excess of 99%. In plaques with a diameter of 0.5 mm or more isolated from plates poured with undiluted suspension or after dilution by 10^{-1} and 10^{-2}, a mixture of progeny phages was generally found, irrespective of the type of plaque isolated. This can probably be attributed to the inability to avoid contamination by phage from neighbouring plaques. However, with the exception of fully
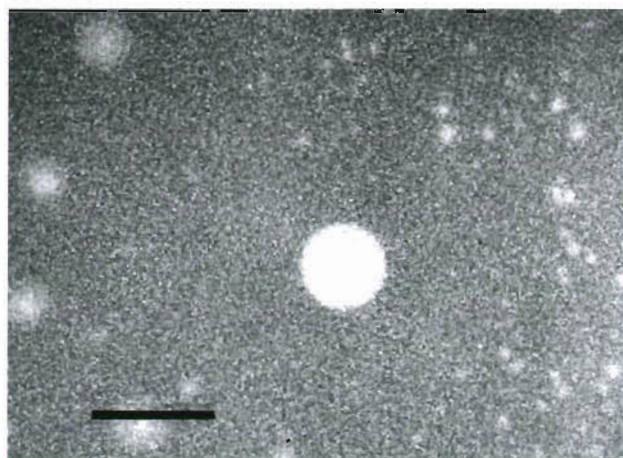
Fig. 4. Inhibition zone for tiny turbid plaques around a large mutant plaque. Scale bar = 2.0 mm

developed φH24 plaques, in all plaques isolated from such plates progeny phages representing at least one phage mutant were found, generally in excess of 50%.

The following hypothesis was based on these observations: Most of the large plaques (diameter 1 mm and more) found in plates poured with cell suspension after dilution up to 10^2 are caused by free phage particles mainly representing mutant phage. The majority of smaller plaques observed under these conditions are ascribed to mutant 'cellular PFU', i.e. cells which start to release mutant phage during plate incubation. The TTP found in plates poured with higher dilutions of the

cell suspension are assumed to originate from H24$_{wt}$ cells which during plate incubation spontaneously start to release wild-type phage φH24. As indicated by inhibition zones around large plaques, the development of TTP is influenced by material, possibly by (or part of) 'factor X', set free during plaque formation. This material is assumed also to be responsible for the inverse relation between TTP numbers and dilution factor in the lower range of dilutions as documented in Table 3.

In investigations regarding TTP, it was found to be essential to employ plates with 20 ml of SWA since in plates with bottom layers of only 10 ml of SWA the tiny plaques containing (almost) exclusively phage φH24 developed only at the edge of the plates.

Regarding the aforementioned inhibition of TTP formation in the vicinity of large plaques, it must be mentioned that sometimes a few φH24 plaques of intermediate size were found within the inhibition zone. It is speculated that H24$_{wt}$ or H24(L3) are able to release phage φH24 at least as long as inhibition is not fully developed. This inhibition is thought to be due to pseudolysogenization of cells which prevents phage adsorption.

Quite similar turbid plaques of about 0.5 mm in diameter can be seen when H24$_{wt}$ is grown in liquid culture and double-layer plates are prepared with H24$_{wt}$ as indicator. These plaques do not increase in number with increasing dilution of the culture. Of course, phage eluted from such plaques are vir mutants. Most of them produce clear plaques of about 1 mm in diameter, such as those found with φH24-1. During successive subcultivations small turbid plaques generally occurred in advance of the larger clear ones. From this it is concluded that most small turbid plaques observed with H24$_{wt}$ as indicator, as in the case of TTP of φH24, originate from H24$_{wt}$ cells which release vir mutant virions rather late during incubation in SSWA overlays. This conclusion is restricted only by the fact that vir mutants exist which under all circumstances produce small but relatively clear plaques on H24$_{wt}$, such as vir mutant φH24-5 (Fig. 2e). Mutants of this type, however, seem to occur rarely.

With H24(L10) as indicator, observations made with broth cultures of H24$_{wt}$ or H24(L3) are similar to those made with cell suspensions prepared from LWCs of these strains. However, there is one striking difference to be pointed out: When broth culture is

Table 3. Strain H24(L3). Plaque formation in dependence of dilution before double-layer platings with H24(L10). A colony grown on SWA during incubation for 2 d at 25°C (2.0 mm in diameter, 8.93×10^7 CFU) was cut from the plate, its material suspended in 1 ml of seawater mixture, and the suspension titrated for CFU and PFU. Plaque type I: diameter between 0.5 mm and up to 2 mm, more or less clear, mostly with sharp edge, encloses vir and non-vir mutants as well as large plaques of φH24. Plaque type II: diameter up to 0.4 mm, after weak dilution mostly plaques of mutant phage, after dilution of 10^2 or higher TTP containing almost exclusively φH24. nc: not counted

Dilution factor	Plate 1 Type I	Plate 1 Type II	Plate 2 Type I	Plate 2 Type II	Mean Type I	Mean Type 2	PFU detected in colony
0	127	>790	156	>690	141	>740	>8800
2	84	626	96	551	90	588	13560
5	81	299	74	319	77	309	19300
10	29	474	32	466	30	470	50000
20	14	1189	20	511	17	1350	273400
50	9	~3440	3	~2920	6	~3180	~1590000
100	0	~2900	0	nc	0	~2900	~2900000
200	0	~3400	4	nc	2	~3400	~6800000
500	0	~2080	0	nc	0	~2080	~10400000
1000	0	1158	0	1368	0	1263	12630000
2000	0	803	0	725	0	764	15280000
5000	0	366	0	424	0	395	19750000
10000	0	199	0	227	0	213	21300000

used undiluted or diluted by a factor up to 10, higher numbers of large plaques and a much smaller portion of plaques with a diameter of less than 0.5 mm will be found than with suspensions prepared from LWC. This obviously is due to larger numbers of free mutant phage in broth cultures than in freshly grown colonies.

In consequence of the observation made with single colonies and of the large qualitative differences between stock cultures kept on SWA slants, the influence on lysogenic LWCs of storage in the refrigerator was investigated. Plates with 20 ml SWA were seeded with material from an LWC to grow between 50 and 80 colonies per plate during 2 d of incubation at 25°C. After various periods of storage at 8°C, pieces of agar bearing a single colony were cut out and transferred into 2 ml of seawater mixture. The suspended material was titrated for CFU and PFU with H24(L10) as indicator of PFU. The suspensions then were centrifuged, the supernatants filtered through BE-washed Sartorius filters, and the filtrates titrated for PFU.

Typical findings are presented in Table 4. With freshly grown LWCs it was found that free phage were outnumbered by CFU by a factor of about 10^5 Furthermore, free virions accounted for between less than 10 to about 50% of PFU_Σ. These patterns, however, changed considerably during storage of the plates in the refrigerator. It must be emphasized that none of the tested colonies showed the faintest symptom of lysis.

However, the considerably reduced correspondence between colony diameter and CFU number found with colonies stored for the longer periods of time, as compared with colonies tested before storage, implies that lytic processes take place during storage.

Generally, low numbers of PFU are present in freshly grown colonies. However, sometimes hundreds or thousands of PFU can be observed. In such colonies lacking symptoms of phage attack, obviously a high degree of immunization is reached. When this is the case, development of LWCs and SGCs occurring in physical contact without any sign of cross infection (see Fig. 3a) may be possible.

All observations described above were made with H24$_{wt}$ or its derivative H24(L3) when incubated at 25°C. Preliminary experiments were performed with colonies of H24(L3) grown at 10 and 18°C. Strains H24(L3) and H24(L10) grow well at these temperatures and the plaquing efficiencies of phages ϕH24 and ϕH24-1 were found to be equal to those at 25°C when phage stocks and H24(L10) were used. The emergence of phage mutants was not hampered by the lower temperatures. However, formation of TTP was suppressed in plates incubated at 10°C. For example, with the suspension of a colony grown at 10°C and diluted 10^{-2}, 2000 to 3000 TTP were observed in duplicate plates incubated at 25°C, but only 13 and 9 in plates incubated at 10°C.

Of H24(L3) several clones have been isolated which differed in the portion of TTP in relation to CFU when colonies or cultures were tested. Generally, the clones differed in TTP to CFU ratio by a factor of about 10. These clones are rather stable genetically, since the trait in question is maintained over years. However, there are indications that switching back and forth between higher and lower ratios of TTP to CFU may occur. During an investigation of H24$_{wt}$ under nutrient-limited growth, a cell population developed which produces relatively large TTP with diameters mainly ranging between 0.4 and 0.6 mm.

Before it was known with certainty that H24(L10) is a non-lysogenic derivative of H24$_{wt}$, it was observed that H24(L10) formed small grey colonies (SGC) if plated on SWA seeded with phage from the 'lysates' prepared from highly lytic H24$_{wt}$ cultures, containing more than 1 type of phage mutant, just as found with lysogenic material. This was a temporarily confusing observation, since it could mean that colonies or liquid cultures of H24,

Table 4. Changes in the number of CFU and PFU in colonies of H24(L3) during storage at 8°C. Colonies grown in 2 d at 25°C. Cut out from SWA plates, colony material was suspended in 2 ml seawater mixture each, and tested for $CFU_{(S)}$ and $PFU_{(S)}$ (S: suspension). Filtrates, prepared from supernatants of centrifuged suspensions, were checked for $PFU_{(F)}$ (F: filtrate). Findings obtained after 0, 14, and 28 d of storage are presented. Those found after 7 and 21 d were intermediate to the respective periods of storage. nd: not differentiated, sum: all types of plaques, ϕH24: plaques of wild-type phage

Day of storage	Colony diam. (mm)	$CFU_{(S)}$ ($\times 10^6$)	$PFU_{(S)}$ ($\times 10$) PFU_Σ	ϕH24	$PFU_{(F)}$ ($\times 10$) PFU_Σ	ϕH24
	1.8	46	10	nd	6	3
	2.0	47	50	nd	26	1
0	2.2	62	90	nd	14	10
	2.2	62	50	nd	13	12
	2.3	62	80	nd	14	13
	2.4	85	90	nd	6	2
	3.9	139	1600	1300	140	140
	4.0	153	8300	1000	2090	150
14	4.4	200	13600	1000	2010	120
	4.5	169	1900	1000	530	300
	4.6	180	72500	2100	15510	270
	4.7	176	2500	1300	500	320
	4.5	324	1200	600	293	208
	4.6	191	676000	2000	184000	300
28	5.3	253	1400	1200	393	370
	5.5	308	497000	6000	125000	2000
	5.5	328	1710000	10000	409000	2000
	6.0	236	34000000	?	5000000	?

whether lysogenic or not, contain a certain, if varying, portion of cells that have the disposition to form SGC. This possibility was finally ruled out when suspensions of H24(L10) prepared from single colonies, after dilutions ranging between 1:10 and 1:3000, were plated on SWA seeded with varying amounts of phage ϕH24-2.

Typical results of such an experiment were as follows:

(1) On SWA seeded with ϕH24-2, its concentration decreasing by a factor of 3 from one series of plates to the next between 10^{10} and 3.7×10^8 PFU per plate, quite similar numbers of SGCs developed, ranging between 1 and 3% of the cells suspended from the colony investigated.

(2) With 1.2×10^8, 4.1×10^7, and 1.4×10^7 PFU per plate, the numbers of SGC increased considerably, representing 6, 24, and about 33%, respectively, of the colony's cells.

(3) Within each series of plates seeded with equal numbers of PFU per plate, there was a slight increase in the portion of cells forming SGC with increasing dilution of the cell suspension. For example, on plates seeded with 3.3×10^9 PFU, 1.9, 2.5, and 2.8% of the colony's cells formed SGC when plated from the dilutions 1:300, 1:1000, and 1:3000, respectively. On plates containing 1.2×10^8 PFU each, the respective percentages were 3.9, 5.5, and 6.0.

(4) In plates mentioned in (1) to (3), no LWCs were found except a few grown outside the area infested with phage, most of them showing symptoms of phage attack.

(5) Very much in contrast to titrations of the same material on plain SWA, unusually large differences between colony counts for parallel plates were often observed on SWA seeded with phage.

Such observations are contrary to the assumption that distinct cells present in a colony or culture are able to form SGCs in the presence of phage. Instead, they indicate that any of the cells present can do so, depending on their survival (i.e. remaining uninfected), until conditions in its environment are established that either make surviving cells inaccessible to phage infection or protect infected cells from the usual consequences of phage reproduction. The large differences in colony counts between parallel plates are obviously due to differences in the distribution of phage on the agar surface.

The reaction of H24(L10) to the available phage mutants (Moebus 1997) was investigated by a combination of liquid culture and plate test. All vir mutants caused development of pseudolysogeny and of SGCs. With non-vir mutants striking differences were observed, placing them into 2 groups. One group includes 3 strains causing the growth of SGCs. In spot tests, the cells pseudolysogenized by these mutants

were found to be insensitive to vir mutants. H24(L10) cells treated with non-vir mutants of the other group are sensitive to vir mutants and form white colonies almost as large as LWCs characterized by a faint star-like pattern and no visible lytic reaction when plated on SWA instead of AS-SWA. The material of such colonies will give uniform streaks on SWA. Since no further investigation of the latter phage-host systems was performed, it cannot be ruled out that the phage mutants in question lysogenized H24(L10).

Little doubt remains that the pseudolysogenic state in H24$_{wt}$, H24(L3) and H24(L10) is not caused by phage genomes residing in infected cells as extra-chromosomal units for a few generations only (carrier state). Although the reason(s) for pseudolysogeny to develop in H24 remain(s) unknown, all observations made so far are in agreement with the hypothesis that, together with newly produced phage particles, an immunizing agent ('factor X') is released from infected cells. Attempts to separate 'factor X' from particulate matter, especially from infective phage particles present in pseudolysogenic cultures, have been unsuccessful.

Finally, the aspect of curing lysogenic H24 is addressed. As reported above, the cured derivative H24(L10) was picked from a streak produced with cells of an SGC. Later on it was found that H24$_{wt}$ or H24(L3) could be cured by treatment in liquid culture with some of the mutants available. The curing effect did not depend on the vir or non-vir character of the mutant phage, but on other, still unknown traits.

The curing of H24(L3) by spontaneously produced mutants of ϕH24 was studied in an experiment performed with 4 series each comprising 5 consecutive cultures. From CFU titrations performed at the beginning of the experiment and at the end of the first subcultures 40 or 60 colonies were isolated and tested as described. All 200 and 240 colonies, respectively, were found to consist of lysogenic material. In contrast, of 80 colonies derived from CFU titrations made at the end of the fourth subculture of each of the four series, the following numbers of cured cell lines were found: 9 and 6 from 2 culture series that reached maximum concentration of PFU$_{vir}$ during the third and fourth subculture, but 16 and 23 from 2 series attaining maximal PFU$_{vir}$ concentration during the fourth subculture only. Obviously cured cells were lost by phage infection when their immunity was weakened by transfer from third to fourth subculture and simultaneous 1/100 dilution into fresh SWB/5.

DISCUSSION

One of the main obstacles in marine bacteriophage research is the low concentration of between 0 and 10

infective particles ml^{-1} in seawater samples generally observed when tested with prospective host bacteria (Moebus 1987, 1992a, b). The highest concentrations reported so far (some 10^4 particles ml^{-1}; Ahrens 1971) were found in the brackish environment of Kiel Bight (German Baltic coast). In samples collected near Helgoland up to 1.5 × 10^3 infective phage particles ml^{-1} were observed; however, such peak concentrations lasted for short periods of time only (Moebus 1992a, b).

The latter observation is in agreement with results of laboratory investigations concerning the viability of free bacteriophage particles in untreated seawater. Findings reported by Moebus (1992c) and Suttle & Chen (1992) indicate that the infectivity of free virions is too rapidly lost for specific phage-host systems (PHS) to be sustained for several months without intermittent phage reproduction.

This situation is impaired by low nutrient concentrations characteristic of most marine environments. It causes reduced production of progeny phage by infected cells as compared to the numbers found with nutrient-rich laboratory cultures. Findings in recent investigations concerning the productivity of 6 PHS under nutrient conditions resembling those typical for North Sea water (Moebus 1996a) are in agreement with the observation of generally very low concentrations of infective phage particles in seawater samples.

In sum, the above-mentioned observations do not provide adequate information on the strategy of maintenance of marine phage-host systems under natural conditions. The situation in marine virology became even more complicated when Bergh et al. (1989) for the first time reported up to 10^7 ml^{-1} of virus particles in seawater as observed by electron microscopy. Meanwhile their findings were confirmed by several other authors.

To explain the enormous difference between the numbers of phage particles observed in seawater with biological methods on the one hand and by electron microscopy on the other, widespread lysogeny among marine bacteria is assumed. Based on what is known about phage production by non-marine lysogenic bacteria, spontaneously or following induction by various means, this assumption is quite reasonable.

Lysogeny is widespread among non-marine bacteria, the frequency of occurrence between members of taxonomic groups ranging between a few and 100%, with the majority of values between about 25 and 65% (for a review see Ackermann & DuBow 1987). Regarding marine isolates, the sole source of information (Jiang & Paul 1994) reports 43% of lysogenic or bacteriocinogenic bacteria among 51 isolates of marine origin. However insufficient this scant information is, it compares well with the results found with non-marine bacteria.

Bratbak et al. (1990), citing Freifelder (1987), who in his book states (p. 106) that 'more than 90 percent of the thousands of known phages are temperate', conclude 'that marine phages in general are temperate and not lytic' Freifelder's (1987) statement obviously is based on electron microscopic observations of phage whose production was induced in non-marine lysogenic bacteria

The conclusion of Bratbak et al. (1990) is in no way supported by observations reported up to now. For the vast majority of observed marine phages nothing is known about their possible ability to lysogenize, given the right host and favourable conditions, or about lysogenic bacteria being their source. And, of course, the large portions of bacteria which are present in marine environments but cannot successfully be cultivated, as well as the many bacterial strains which grow well in laboratory cultures but have not yet been shown to serve as host for marine phage, must be taken into account as sources of viral particles observed electron microscopically.

The isolation of H24 and its cured derivative, H24(L10), was a welcome chance to gain some insight into the biology of a marine lysogenic bacterium. Whether it is a typical or a rather exotic member of marine bacterial populations remains unknown. However, in regard to pseudolysogeny this author assumes that H24 is not an exceptional strain.

With PHS [H3:H3/1] it was found that pseudolysogeny very effectively protected bacterial cells from phage attack (Moebus 1996b). Furthermore, from the appearance of the plaques produced by a large portion of marine phage strains, it was concluded that pseudolysogeny is a rather common characteristic of marine phage-host systems. Under favourable natural conditions this mechanism may support the coexistence of genetically sensitive host bacteria and virulent phage.

Concerning H24, pseudolysogeny certainly is the decisive trait regarding the survival of cells during laboratory studies employing nutrient-rich media. Its observation is rather extraordinary mainly because it is directed against mutants of the phage residing in lysogenic strains of H24. The nature of 'factor X', the hypothetical agent inducing pseudolysogeny, is unknown. With reference to Li (1961) it is assumed to be a substance such as an enzyme (or a mixture of substances) that is released by lysing cells during phage production.

Pseudolysogeny probably also is advantageous in the process of curing lysogenic H24 as it enables genetically sensitive cells to survive in the presence of infective phage. It has long been known that lysogenic bacteria can be cured by treatment with phage. Bertani (1953) reported the curing of Shigella dysenteriae strain Sh(P2), lysogenized by phage P2, by super-

infection with a P2 mutant of intermediate virulence. Later on, the curing effect of superinfection by homo- or heteroimmune phages was reported by several authors. An important role is attributed to competition for insertion sites on the bacterial genome, which with many phage strains are known to be highly specific. Competitiveness, of course, depends on the right genetic condition as shown by Kaiser & Masuda (1970).

The next paper in the series (Moebus 1997) deals with aspects of pseudolysogeny as observed with various derivatives of H24. It will also present a detailed discussion of the possible nature of 'factor X', based on the literature regarding so-called capsule phages reviewed by Lindberg (1977).

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