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Investigations of the marine lysogenic bacterium H24. II. Development of pseudolysogeny in nutrient-rich broth culture

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ABSTRACT: Development of pseudolysogeny by 3 strains of H24 was investigated in nutrient-rich liquid cultures. At first, H24(L10), a cured derivative of the lysogenic wild-type strain $H24_{wt}$, and the non-virulent mutant ϕH24-2 of the wild-type phage ϕH24 residing in $H24_{wt}$ were used as a model phage-host system. Attempts to separate phage from pseudolysogenized cells failed due to abundantly present 'cellular PFU'. These release phage particles only after removal of the pseudolysogeny-inducing agent and do so for a period of time. Therefrom it is concluded that phage production in 'cellular PFU' was halted at various stages during the development of pseudolysogeny, depending on time of infection. Observations made with 2 lysogenic H24 strains during the course of several successive cultures are in general agreement with results obtained with the model phage-host system. The nature of the pseudolysogeny-inducing agent remains unknown. Based on information from the literature, the possibility of polysaccharide depolymerase being involved is discussed.

KEY WORDS: Marine · Bacteria Lysogeny Pseudolysogeny

INTRODUCTION

The main features of the marine lysogenic bacterium H24 as revealed during laboratory investigations were presented in the preceding paper (Moebus 1997). Spontaneous development of lytically active broth cultures and the formation of small grey colonies (SGC) from such cultures which upon streaking on seawater agar split into SGC and large white colonies (LWC) were among the most challenging features. It was found that spontaneous production of virulent mutants of a phage harboured by wild-type strain $H24_{wt}$ or its lysogenic derivative H24(L3) caused the lytic activity of cultures and that formation of SGC depended on the presence of high concentrations of phage mutants. The ability of H24 to survive in the presence of potentially lethal phage was explained by the development of transient immunity called pseudolysogeny.

Soon after isolation of H24(L10) it was found that, in the presence of excessive concentrations of phage ϕH24-1, a virulent (or vir) mutant of the wild-type phage ϕH24 residing in $H24_{wt}$, or of ϕH24-2, a non-virulent (or non-vir) mutant, this cured derivative of $H24_{wt}$ develops the same unusual type of SGC as observed with $H24_{wt}$ under comparable conditions (Moebus 1997). The development of pseudolysogeny in populations subjected to massive phage attack was found to be highly important for the progress and outcome of the laboratory investigations. Without an understanding of the circumstances under which pseudolysogeny develops, other characters of H24 might be difficult, if not impossible, to elucidate. The present paper reports observations made with regard to the development and features of pseudolysogenic populations during broth culture.

The agent inducing pseudolysogeny remains unknown and accordingly is called 'factor X'. The possible nature of 'factor X', based on the literature regarding so-called capsule phages (Lindberg 1977), will be discussed.

MATERIALS and METHODS

Media. 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 (1997).

Bacteria and bacteriophages. Strains as listed by Moebus (1997) were used.

Methods. With exceptions given under 'Results', methods described by Moebus (1997) were employed. Strain H24(L10) in combination with ϕH24-2 was used as a model phage-host system (PHS) to elucidate certain features of psydolysogenic cultures. For comparison, strains $H24_{wt}$ and H24(L3) were employed to produce pseudolysogenic cultures by spontaneous occurrence of mutant phage. H24(L3) differs from $H24_{wt}$ by its stronger inclination to produce mutants of ϕH24, which apparently are not different from those produced by $H24_{wt}$.

H24(L10) and $H24_{wt}$ also served as indicator strains. H24(L10) was employed to enumerate virulent (vir) and non-virulent (non-vir) plaque forming units (PFU_{Σ}), and $H24_{wt}$ served as indicator of vir mutant PFU (PFU_{vir}). In the latter case precautions were taken as described by Moebus (1997).

For electron microscopic studies of cells, one of the following procedures was used: (1) Pseudolysogenic cells were gently spread on agar covered by a film of collodion through which the liquid was sucked into the agar. Pieces of the film then were prepared for electron microscopy by coating with carbon and gold. (2) Cells were collected at varying incubation time from model PHS cultures run to induce pseudolysogeny, fixed with glutaraldehyde, embedded in agar, postfixed with 1% OsO_4 for 60 min, dehydrated in an ethanol series and embedded in Epon. Thin sections were stained with 2% uranyl acetate in 70% ethanol for 60 min and checked for the presence of phage and phage-like structures. Electron micrographs were taken with a Siemens Elmiscope IA operating at 80 kV with a nominal magnification of 40 000×.

Special terms. Cultures grown to induce pseudolysogeny in the model PHS will be referred to as induction culture (IC). 'Excess PFU' relates to PFU whose origin and character was initially unknown. 'Cellular PFU' indicates cells either infected from outside or harbouring the mutated genome of ϕH24 which do not release phage before being fixed in SSWA overlay, i.e. during plaque formation. 'Surplus phage' relates to free virions released by 'cellular PFU' when the concentration of the pseudolysogeny inducing agent 'factor X' is reduced below the necessary minumum.

RESULTS

State of pseudolysogeny in the model phage-host system [H24(L10):ϕH24-2]

The advantage of a model PHS is the experimenter's freedom to determine when and how many phage particles of which kind should attack the respective bacteria. This was extensively used with the present model PHS.

Mixed cultures of bacteria and phage in SWB/5 at first develop in the usual way: uninfected cells multiply and infected ones generate new phage particles until after some time lysis of cells causes partial clearing of the culture. Soon after the minimum in CFU is attained, the final level of about 10^{10} PFU ml^{-1} is reached. In its presence a cell population develops consisting of between 98 and 100% pseudolysogenic cells which form LWCs typical of H24(L10) when spread on SWA seeded with anti-phage serum (AS-

Table 1 Influence of initial PFU concentration on the course of induction cultures. Selected results. Initial concentration of H24(L10) was 1.8×10^7 CFU ml^{-1}. Cultures incubated at 25°C and 1 rpm. Platings for CFU and PFU determinations after 3, 6, 9, 12, and 24 h (1st expt) and after 3, 6, 9, 13, and 25 h (2nd expt) of incubation. When necessary, SWA was seeded with anti-phage serum prior to use in CFU platings

Initial PFU ml^{-1}	CFU minimum CFU ml^{-1}	CFU minimum Reached after (h)	PFU near maximum PFU ml^{-1}	PFU near maximum Reached after (h)	Final CFU ml^{-1}	Final PFU ml^{-1}	Final PFU/CFU
0 (1st expt)					8.0×10^8		
0 (2nd expt)					9.4×10^8		
2×10^7	5.6×10^5	3	$\sim 10^{10}$	6	4.7×10^8	1.3×10^{10}	28
2×10^6	5.9×10^5	6	$>1.5 \times 10^{10}$	6	3.5×10^8	1.9×10^{10}	54
2×10^5	4.8×10^5	6	2.9×10^{10}	9	3.2×10^8	2.7×10^{10}	84
2×10^4	4.0×10^5	6	3.4×10^{10}	9	3.6×10^8	3.6×10^{10}	100
2×10^3	5.5×10^6	9	$\sim 4 \times 10^{10}$	12	4.1×10^8	4.2×10^{10}	102
2×10^3	7.8×10^6	9	4.6×10^{10}	13	4.0×10^8	5.7×10^{10}	142
2×10^2	1.9×10^7	13	3.3×10^{10}	13	3.5×10^8	5.4×10^{10}	154
2×10^1	3.2×10^7	13	3.1×10^9	13	2.1×10^8	4.1×10^{10}	195
2×10^1	2.8×10^7	13	7.7×10^9	13	2.3×10^8	5.4×10^{10}	234
About 2	2.9×10^7	13	5.8×10^9	13	2.1×10^8	5.8×10^{10}	252
2	2.5×10^8	13	7.1×10^8	13	3.3×10^8	4.6×10^{10}	139
2	2.1×10^8	13	8.5×10^8	13	2.8×10^8	4.3×10^{10}	154

SWA). Other rarely observed colony types invariably were found to be built of genetically resistant cells.

The time lapse until lysis, of course, depends on the number of phages inoculated initially. The maximal titer of PFU is reached earlier, and remains lower, the higher the initial PFU titer, but afterwards it remains constant until the end of incubation after 24 h. The titer of pseudolysogenic cells at this time is relatively unaffected by the initial phage titer and ranges between 2×10^8 and 6×10^8 CFU ml^{-1} (about 30 to 50% of the CFU titer of a phage-free control culture). This type of culture grown to induce pseudolysogeny will henceforth be called induction culture (IC). Results of a series of ICs employing various sizes of phage inoculum are compiled in Table 1. In the 3 cultures started with only about 2 PFU ml^{-1}, the reduced chance of early infection strongly influenced the development of the cultures.

The survival of genetically sensitive cells is attributed to pseudolysogeny and its development ascribed to the action of 'factor X'. The concentration 'factor X' reaches in an IC should be higher the more cells became involved in phage production. Their number depends on the CFU concentration attained before the culture succumbs to cell lysis and on the degree of the breakdown. This again should greatly depend on the amount of phage inoculated initially, relative to the initial CFU concentration. Therefore, the concentration of 'factor X' was expected to be the lowest in cultures started with high initial PFU titers, and to increase with decreasing initial PFU concentrations until an optimum may be reached. If so, pseudolysogenic cells grown in a culture started with a low phage titer should be better protected during subcultivation from phage infection than cells grown in a culture started with a high phage titer.

For the subcultivation experiment (results in Fig. 1), ICs with initially 2.7×10^7 CFU ml^{-1} were inoculated with φH24-2 to concentrations of 2.0×10^7 and 2.0×10^3 PFU ml^{-1}, respectively, and incubated for 24 h. After dilution of the ICs in pre-warmed SWB/5, changes of CFU and PFU in subcultures (SCs) were followed for another 10 h. Dilution factors between ICs and SCs were 2, 4, 6, 8, 10, and 20.

The results obtained during this experiment agree with expectations presented above. Pseudolysogenized cells grown in the IC started with high initial PFU titer (Fig. 1a), at first produced new PFU, but later multiplied. Their protection against phage infection was less well developed than that of the cells grown in the other IC, resulting in small initial losses of CFU (and PFU) after dilution for more than factor 2 followed by late increases in CFU concentration. According to the working hypothesis the CFU increases became possible when pseudolysogeny again was fully effective due to newly produced phage and 'factor X'.

In contrast, the cells from the IC started with low phage concentration (Fig. 1b) immediately resumed multiplication which ceased when 'factor X' assumedly was reduced to a critical concentration. This must have been due to 'consumption' by newly produced cells and the lack of appreciable phage production during the first 5 h of subcultivation. The differences between the subcultures of both series obviously did not depend on the PFU concentration present at the beginning of incubation but on the physiological status of the cells.

To answer the question whether pseudolysogenized cells are protected by 'factor X' from within and/or from outside, the following experiment was performed. Pseudolysogenized cells were used either unwashed or washed twice in SWB/5 in the centrifuge at low speed. The pelleted cells were stored in the refrigerator, while about 97% of the PFU present were removed from the spent IC medium by high speed centrifugation (2 h at $39000 \times g$ and 4°C). Then the unwashed cells were suspended in high speed supernatant (HSS) and the washed cells in SWB/5, and both suspensions were diluted in the respective medium to attain a concentration of about 5×10^8 CFU ml^{-1}. H24(L10) cells grown in SWB/5 without phage were diluted in SWB/5 to the

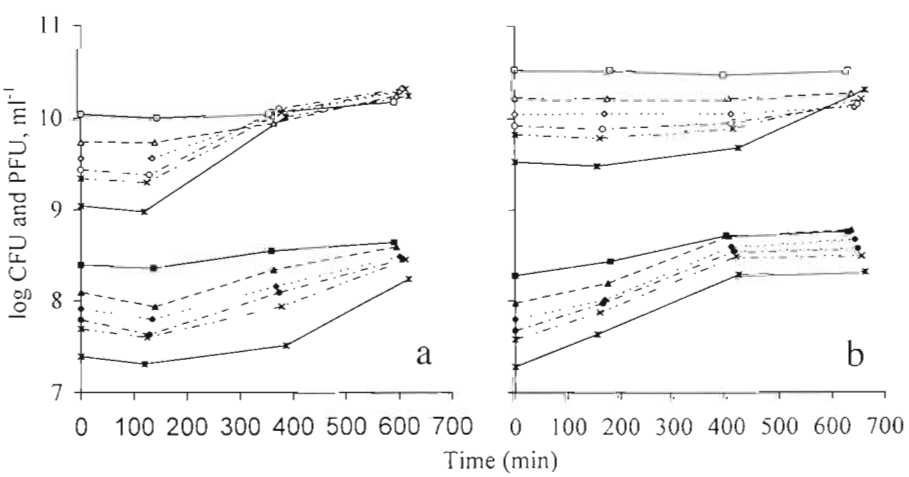

Fig. 1 Development of colony- (CFU, lower group of curves) and plaque-forming units (PFU, upper group of curves) during subculture (SC) dependending on initial PFU titer in induction cultures (IC). Initial PFU titer ml^{-1} in IC was 2×10^7 (a) and 2×10^3 (b). Incubation of SCs (7 ml each in tubes) in SWB/5 at 25°C and 1 rpm. Dilution factor between IC and SC was 2 (■, □), 4 (▲, △), 6 (◆, ◇), 8 (●, ○), 10 (×), and 20 (∗). CFU titrations on SWA with anti-phage serum

same CFU concentration and used as control. Finally, for subcultivation samples of 7 ml each were prepared in SWB/5 containing between 5 and 50% of HSS, but constant numbers of CFU

The results found with HSS used at concentrations of 5, 10, and 50% are presented in Fig. 2. The most important observations made in this experiment were:

(1) The initial number of CFU in the suspension of washed cells (Fig 2b) used to inoculate the samples was considerably less than expected (44% of the titer of unwashed cells, Fig. 2a). Since under the experimental conditions only about 1% of CFU will be lost by decanting the low speed supernatant, the loss of more than 50% of CFU during the washings and (probably) the storage period must be ascribed to infection by phage.

(2) This assumption is in agreement with the observation of initially higher rates in PFU increase in the samples containing washed pseudolysogenized cells, as compared with those prepared with unwashed ones. With the latter (Fig. 2a), during the first 3 h of incubation, the PFU concentration increased by factors ranging between only 1.1 and 2.2. In contrast, with washed cells the PFU titer increased in less than 3 h by factors ranging between 4 and 30, inversely correlated with the HSS concentration of the respective sample. Note that after 200 min PFU concentrations had to be the same in the subcultures since these were inoculated with the same numbers of cells including infected ones.

(3) Concerning CFU, only in the samples containing 50% of HSS (closed squares in Fig. 2a, b) was an appreciable difference between the course of events found. In the sample with unwashed cells the number of CFU increased for about 3 h and remained almost the same during the next 7 h. In contrast, with washed cells similar observations were made as with all other samples containing lower amounts of HSS: after about 3 h the increase of CFU was interrupted by a phase of CFU decrease lasting for about 3 h.

As deducible from Fig. 2a, b, the removal of 'factor X' during washes in SWB/5 resulted in appreciable loss of protection by pseudolysogeny and of over 50% of CFU grown in the IC. Surviving washed pseudolysogenized cells, however, were shielded against phage attack to a much greater extent than non-pseudolysogenized cells (Fig. 2c). Fully sensitive cells gained little if any protection by 'factor X' present in the medium from the beginning of subcultivation, but were almost completely protected following the release of first generation progeny phage. From this it was concluded that at least part of the protective effect of 'factor X' is established inside the cells, and this remains essentially unaffected by washes.

(4) The most startling observation in this experiment concerns the concentration of PFU in the suspension of washed cells. Since the 2 washes of the cells in 10 ml of SWB/5 each should have resulted in a combined dilution of non-sedimenting matter by about 10^{-4}, the PFU concentration of the IC (5.6×10^{10} ml^{-1}) was expected to be reduced accordingly. Instead it was found to range between 3 and 5×10^{8} PFU ml^{-1}, which is at least a factor 100 higher than expected.

The last finding, in combination with that mentioned under (2), triggered investigations aimed at finding a method to separate CFU and PFU Such a method would be indispensable for an array of experiments regarding the reaction of pseudolysogenized cells to phage and vice versa.

First, the separation of PFU from pseudolysogenic cells by successive washings in the centrifuge was checked. To enable accurate estimates of the minimum dilution factors attained with washings, centrifuge tubes with the pellets only and after re-suspending the cells were weighed on an electronic balance. By ignoring the biomass of cells present in the pellets, minimum dilution factors of up to 200 were determined. Based on these factors, the PFU titers to be expected under the condition that phages would be washed out (= 'expected PFU') were calculated.

Whether the cells were washed in SWB/5 or in seawater mixture (SM), after suspending the pelleted cells

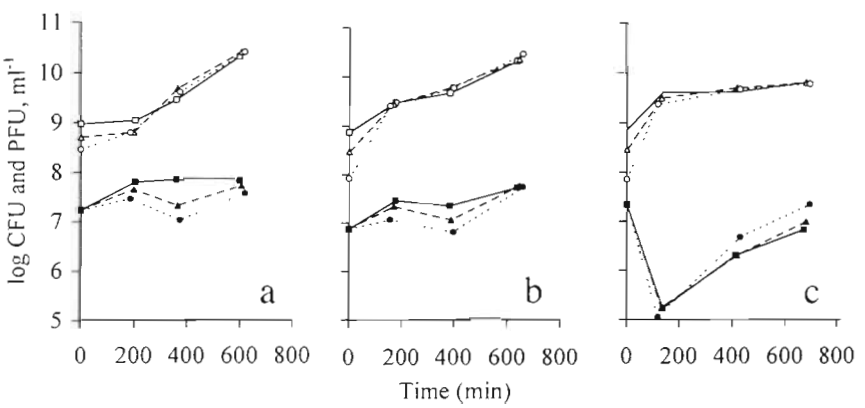

Fig. 2. Development of CFU (filled symbols) and PFU (open symbols) during subculture (SC) depending on treatment of cells pseudolysogenized before subcultivation, as compared with sensitive cells. (a) pseudolysogenized cells not washed; (b) pseudolysogenized cells washed twice in SWB/5; (c) cells not pseudolysogenized. For other details of methods see text. SC in tubes at 25°C and 1 rpm. Medium for SC: (●, ○) SWB/5 with 5% high speed supernatant ϕHSS); (▲, △) with 20% HSS; (■, □) with 50% HSS. CFU titrations on SWA with anti-phage serum

in fresh medium the PFU titer was found to be higher than that of CFU, in early experiments even after the fifth washing. However, in SM practically no decrease in CFU was observed, while with cells washed in SWB/5 considerable loss of CFU due to infection occurred. Results of such an experiment are shown in Fig. 3.

The same observations were made in a number of experiments: each time pseudolysogenic H24(L10) cells were pelleted and resuspended in fresh medium, the actual PFU titer by far exceeded the expected one. This observation was attributed to phage set free from the cells during centrifugation. This interpretation is supported by the fact that with supernatants decanted after each centrifugation, PFU titers were found which not only decreased with the number of successive runs in the centrifuge but always were lower than the PFU titers determined with resuspended pellets of the same run. From this one has to conclude that part of the PFU was sedimented together with the CFU, i.e. consisted of 'cellular PFU' which must be the source of 'excess PFU'

For several reasons it remained questionable whether 'excess PFU' were released via lysis from infected cells which, of course, could be present in induction cultures, or by desorption from immunized cells. Most important were 3 findings:

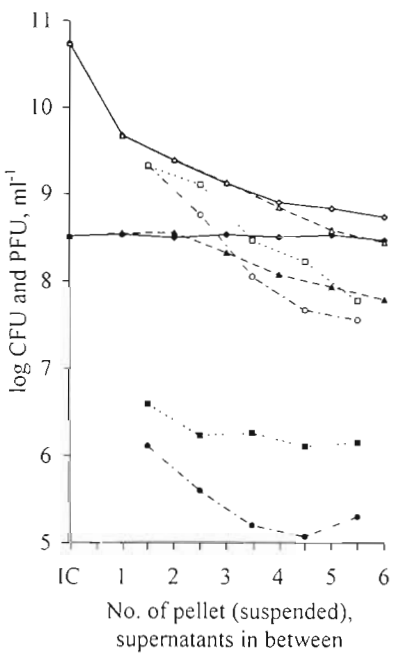

Fig. 3. Results of an attempt to separate pseudolysogenized cells from PFU by washes in the centrifuge. Filled symbols: CFU; open symbols: PFU. (▲, △) suspensions, cells washed in SWB/5; (◆, ◇) suspensions, cells washed in SM; (●, ○) supernatants, cells washed in SWB/5; (■, □) supernatants, cells washed in SM. CFU titrations on SWA with anti-phage serum

(1) The turbidity of induction cultures and phage-free controls hardly differed, but from equal volumes of both types of cultures the pellets of pseudolysogenized cells were much smaller than those of normal H24(L10) cells. From this it was concluded that in ICs infected cells (i.e. cellular PFU) could be present in low quantities only. Direct counts supported this conclusion by agreement with CFU titers.

(2) Treatment of pelleted, unwashed pseudolysogenic cells with anti-phage serum (AS) resulted in a firm clot which broke into large pieces only when forcefully agitated. After centrifugation of this specimen, the supernatant, according to expectation, contained few PFU. Upon repeated washings of the AS-treated cells in SWB/5, the solidity of clots slightly decreased. At the same time the PFU concentration in supernatants increased from about 10^2 ml^{-1} to about 10^5 ml^{-1}. Clotting of pseudolysogenic cells by AS was slightly reduced after 1 to 4 washings (in SWB/5) before AS-treatment, but increased again after more washings (up to 8). The latter findings, obtained in an experiment lasting for 8 h, were probably due to the release of phage by cells which were infected soon after removal of the spent IC medium.

(3) Sensitive cells of H24(L10), when allowed to stand for 20 min at 25°C to adsorb phage φH24-2 present in 100-fold concentration, upon treatment with AS for 10 min formed a clot during centrifugation. The clot was less compact than that observed with unwashed pseudolysogenic cells and could be broken into relatively small pieces by forceful agitation. In contrast, sensitive cells treated with AS for the same time, but before phages were added to attain 100-fold concentration of CFU and with centrifugation started 10 min later, formed an easily suspendable pellet just as untreated sensitive cells did.

From these findings it was concluded that the strong reaction with anti-phage serum possibly was due to large numbers of virions attached to pseudolysogenic cells which, however, should be able to desorb as infectious particles and be observed as 'excess PFU'. Electron microscopic observations, however, did not support this speculation. Most inspected cells showed no adsorbed phage particle, and with a few cells only one adsorbed phage was seen.

Consequently infected cells had to be assumed to be the source of 'excess PFU' as well as 'excess PFU' themselves and henceforth these will be referred to as 'surplus phage' and 'cellular PFU', respectively. Furthermore, since 'surplus phage' observed during early washes could not be due to infection occurring soon after removal of the spent IC medium, it was concluded that 'cellular PFU' are cells with the process of phage reproduction arrested at different stages, the latter depending on time of infection before 'factor X'

reached critical concentration. Following removal of 'factor X', in such cells phage production would be resumed leading to phage release over an extended period of time.

Additional results of the experiment presented in Fig. 3 support this view. After the 5 washes in SM or SWB/5 the cells were suspended in SWB/5 and incubated at 25°C and 1 rpm. With cells washed in SM, a reduction of CFU counts by about one-third occurred before CFU began to increase after about 100 min of incubation, and the increase in PFU concentration was delayed for about 30 min. In contrast, with cells washed in SWB/5 the PFU titer immediately started to increase accompanied by insignificant reduction in CFU concentration which increased after about 60 min.

Obviously part of the CFU washed in SM became accessible to phage attack when resuspended in nutrient-rich SWB/5. Since, however, newly infected cells would not release phage before 2 h of incubation, the increase of PFU after only 30 min in the sample with SM-washed cells and the immediate release of phage by cells washed in SWB/5 indicate the presence, at least, of building components of phage, which remain unassembled in infected cells as long as 'factor X' remains effective.

To test these assumptions, thin sectioned cells from developing ICs were investigated electron microscopically. Mature phage were observed only with samples taken between 5.5 and 6.75 h of incubation. From samples of fully pseudolysogenic ICs, in at least 50% of the cells numerous small electron-dense bodies were found, mainly at the edge of the cell's nucleoid (Fig. 4). Less numerous larger electron-dense bodies, sometimes resembling heads of mature phage particles in size, are regarded as artifacts. They were found in infected cells collected at any time during induction culture as well as in uninfected cells of H24(L10) and of another marine bacterium, $H17^{epe}$, used for comparison.

Regarding the numerous small bodies, it remains an open question whether they are related to phage. Their number per cell may fit such an assumption; however, their presence in so large a portion of inspected cells poses a problem that could not be solved. This investigation, at least, demonstrated, that mature phage are not present inside of cells collected from ICs between 12 and 24 h of incubation but did not rule out the presence of building components of phage in the cells investigated.

At this stage of the investigation it was unmistakably clear that 'cellular PFU' from ICs are the source of 'surplus phage' which, however, are released only after sufficient removal of 'factor X'. During a larger series of washes, 'cellular PFU' should increasingly become enriched relative to free phage. Furthermore, since only about 1% of cells are lost by decanting supernatants, the PFU concentrations in supernatants and filtrates prepared from them should be (almost) identical. One important question remained: Will the con-

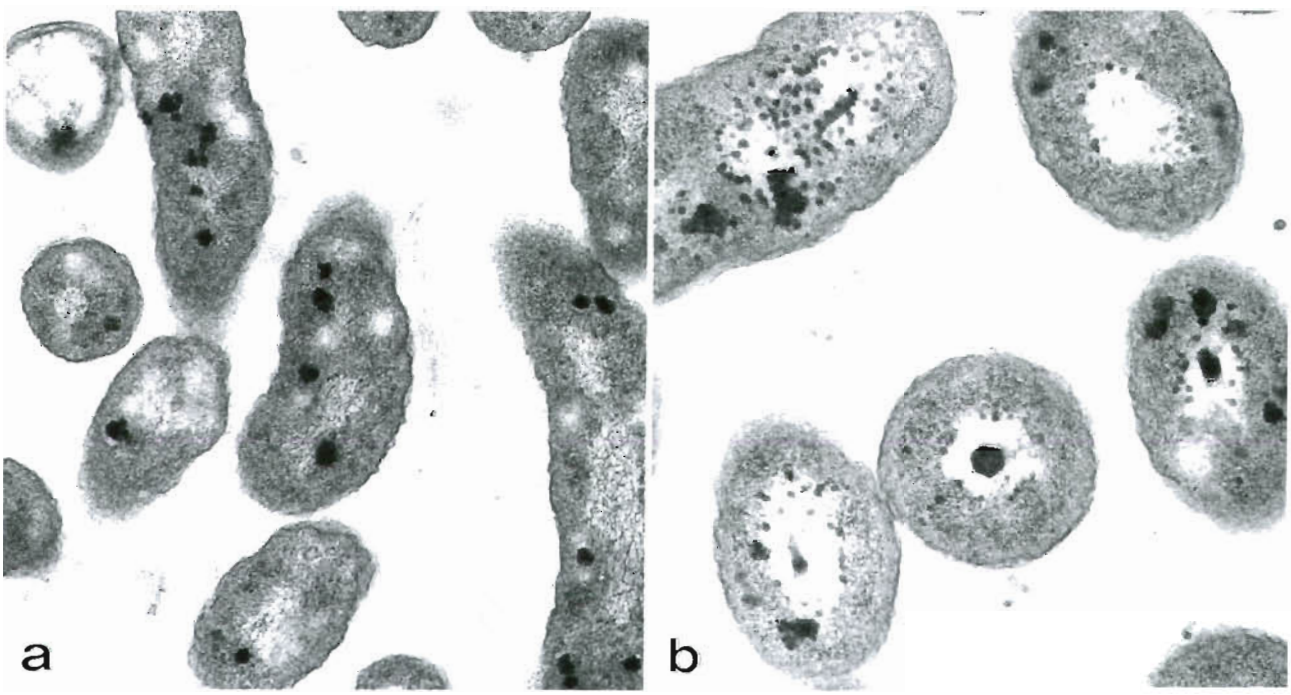

Fig. 4. Sections of cells of H24(L10). (a) H24(L10) not infected; (b) H24(L10) 24 h postinfection with ϕH24-2. Photographs by H. Frank

centration of CFU in a larger series of washes finally exceed that of 'cellular PFU'?

In a series of experiments concerning the aspect of 'expected PFU' versus 'cellular PFU', washes in the centrifuge as well as over filters were tested. With the latter, perfect agreement between 'expected PFU' and PFU concentration in filtrates was found. However, due to higher dilution factors washing by centrifugation was preferred. To enhance the clarity of results, it was decided to concentrate the cells present in the IC used prior to washes. For technical reasons the enrichment factor was 2.4. That this procedure would not interfere with the general outcome of the experiment was known beforehand from investigations with 10-fold enriched pseudolysogenized cells. However, while interpreting the results one had to keep in mind that higher dilutions had to be used in platings of PFU and CFU, which in some cases had important consequences. For example, because of the increased proportion of bacteria to virions the percentage of lytically damaged colonies in platings on SWA was reduced, as compared to experiments performed without enrichment of cells.

Cells from an IC were washed 7 times (corresponding to 8 runs of the centrifuge) in SM pre-cooled to 8°C. For CFU titrations, AS-SWA was used with all cell suspensions and supernatants, but SWA from the second wash on. Fig. 5 presents some of the results. Fig. 5a compiles findings obtained with cell suspensions. Note that the increase in CFU (open columns) from IC to C1 was due to the 2.4-fold enrichment. The black columns in Fig. 5b, presenting the results obtained with supernatants, are the same as in Fig. 5a.

From Fig. 5a it can be seen that the PFU concentration (black columns) decreased relatively steeply as long as it was greater than the CFU concentration (shaded and open columns). After the third wash (C4) approximate equilibrium existed between PFU and CFU concentrations on the one hand (Fig. 5a) and between PFU concentrations in the cell suspension and the supernatant on the other hand (Fig. 5b). During following washings the CFU concentration in cell suspensions became larger than the PFU concentration (Fig. 5a). The latter at the same time increasingly exceeded the PFU concentrations in supernatants (Fig. 5b). Both findings support the interpretation of 'excess PFU' being a mixture of 'cellular PFU' and 'surplus phage'. The fact that identical PFU concentrations in supernatants and corresponding filtrates proves these PFU to be non-cellular ones. Finally, even after more than 3 washes the concentration of free phage in supernatants and filtrates from them remained much higher than would be expected in the case of successful wash-off according to single as well as cumulated dilution factors (see Fig. 6). From this one

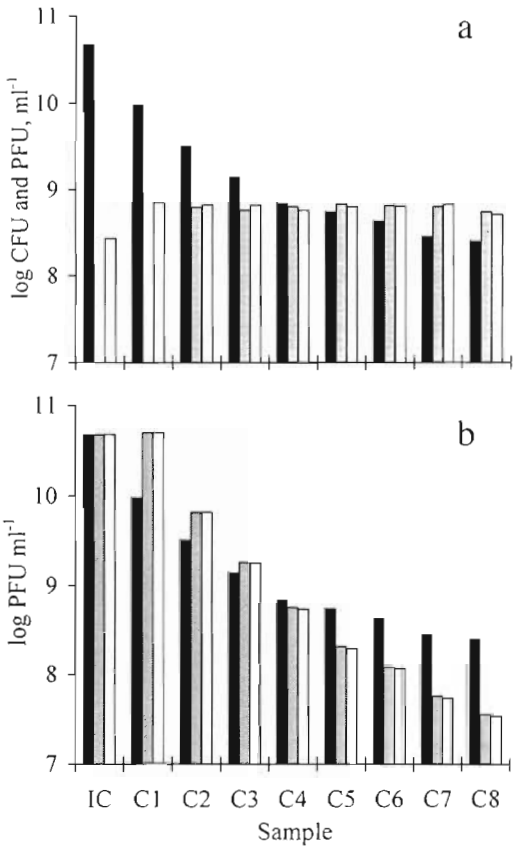

Fig. 5. Effect of 7 successive washes in cold (8°C) seawater mixture in the centrifuge on components (CFU, 'cellular' PFU, and free phage) of IC. Cellular matter of IC enriched 2.4 times before washes (= C1). (a) Concentrations of PFU (black columns) and CFU in suspensions. Open columns: CFU on SWA with anti-phage serum; shaded columns: CFU on plain SWA. (b) PFU concentrations in suspensions (black columns, same as in a), in supernatants (hatched columns), and in filtrates of them (open columns). Filtration through Nuclepore filter of 0.2 µm pore size

has to conclude that 'cellular PFU' during all washes released 'surplus phage'.

Additional important information gained from these experiments concerns the effectiveness of 'factor X'. As already pointed out in the context of the experiment presented in Fig. 2, 'factor X' obviously protects pseudolysogenized cells even if it is removed from outside the cells by repeated washing. With only 2 washes, at least, it could not be washed from the cells. In the present series of experiments the same was found after 7 washes:

(1) Although it must be assumed that the concentration of extracellular 'factor X' during successive washes is rapidly reduced (cf. Fig. 6 for cumulated dilution factor), no appreciable loss in CFU was observed during 7 washes, a procedure lasting for almost 4 h (Fig. 5a). Obviously the pseudolysogenized cells essentially remained protected against infection by phage.

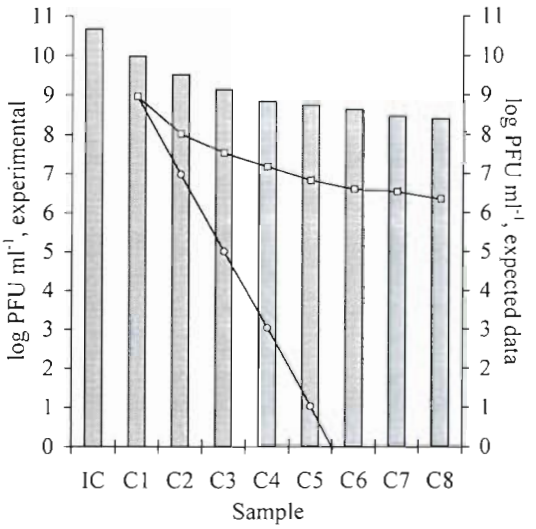

Fig. 6. Comparison of experimental and calculated data of concentrations of PFU after 7 consecutive washes in seawater mixture in the centrifuge (same data as for Fig. 5a). Columns: experimental, (□) expected PFU titer for single dilution factor; (○) expected PFU titer for cumulative dilution factor

(2) When the pelleted material from an IC (used without enrichment of cells prior to treatment) after 6 washes was suspended in pre-warmed SWB/5 and incubated at 25°C without agitation, the small changes in the concentrations of PFU and CFU found after the first hour can be attributed to technical inadequacy (Fig. 7). In the next hour the PFU increased by a factor of 5.6 and the CFU decreased to 17% of the number found after 1 h (i.e. for one CFU lost one PFU was gained). However, while during the next 2 h the PFU continued to increase, by factors of 8.5 and 3.2, the CFU had already begun to multiply again, if only by factors of 1.2 and 1.35. As indicated by the loss of CFU, pseudolysogenized cells obviously became sensitive to

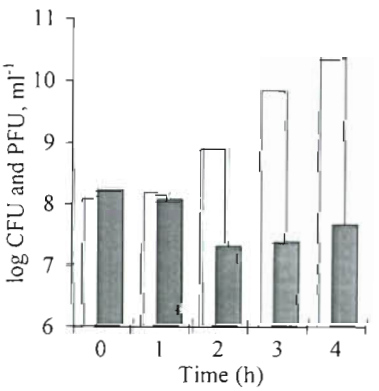

Fig. 7. Development of CFU and PFU in a suspension of pseudolysogenized cells after 6 washes in cold (8°C) seawater mixture in the centrifuge, suspending in pre-warmed SWB/5, and incubation at 25°C as still culture. Open columns: PFU; shaded columns: CFU on SWA with anti-phage serum

phage infection, though for a short time only. Since the latent period of sensitive H24(L10) cells after infection with ϕH24-2 during roll culture at 25°C and 1 rpm is about 2 h, the newly infected cells in this experiment most probably contributed little to the increase in PFU until, at least, the third hour of incubation as still culture.

Experiments with wild-type strain $H24_{wt}$ and its lysogenic derivative H24(L3)

Very much in contrast to the investigation reported above, the outcome of experiments with the lysogenic strains of H24 depended to a great extent on mutation events which are unpredictable as regards the time they take place as well as the type of mutation produced. To follow the build-up of vir and non-vir mutant phage populations, the success of an experiment essentially depends on the use of bacterial inoculums containing as few mutant PFU as possible.

Knowing that colonies of $H24_{wt}$ and H24(L3) may contain free phage (Moebus 1977), it was checked whether growing colonies on SWA seeded with anti-phage serum (AS-SWA) was advantageous. This was not the case. However, it was found that the concentration of mutant PFU in cultures of $H24_{wt}$ after 18 h at 25°C and 1 rpm generally was lower than that in H24(L3) cultures. The precautions taken in preparation of cultures of $H24_{wt}$ to be used for titrations of virulent PFU (Moebus 1997) proved to be useful even with H24(L3) for starting experiments comprising a series of subcultures.

The purpose of experiments using liquid cultures was to answer the following questions: (1) Is a pattern discernible in regard to the appearance of the various types of mutant phage, as was suggested by previous observations? (2) Will the phenomenon of 'cellular PFU' releasing 'surplus phage' be encountered, as in the case of the model PHS [H24(L10):ϕH24]?

At first, 4 identically treated cultures of H24(L3) were used, each one inoculated with a 2-d-old colony (2.0 to 2.5 mm in diam.) by means of a loop. The initial PFU concentration, as determined with H24(L10), ranged between 9.3×10^2 and 1.77×10^3 ml^{-1}. The concentration of vir mutant PFU was below 10 PFU ml^{-1}.

Each line of cultures consisted of the primary culture and 4 successive subcultures with 1/100 dilution in between. At the end of each 24 h incubation, turbidity readings at 660 nm were taken and CFU (on AS-SWA) and PFU were titrated, using H24(L10) and $H24_{wt}$ as hosts. The cultures then were centrifuged at low speed and the pelleted cells washed twice in pre-cooled seawater mixture (SM) under the same conditions. Supernatants were tested for PFU only.

With each 2 of the 4 culture series (groups A and B) similar observations were made depicted in Fig. 8 by representative examples. Until the end of the second subcultures no significant differences between the 4 series were observed. Most important is the finding of practically identical PFU concentrations in supernatants of second subcultures and of the 2 washes of their cells. The latter also is true of both the third subcultures of group A (Fig. 8a). This pattern changed decisively for the supernatants produced with the third subcultures of group B (Fig. 8b) and with all the fourth subcultures: supernatants produced from the cultures contained as many PFU as the cultures themselves and only about 10 times more PFU than the supernatants of the 2 washings.

In accordance with the observations made with the model PHS [H24(L10):ϕH24-2], these changes in the pattern of PFU concentrations in the cultures on the one hand and the supernatants on the other are interpreted as follows: Before equal numbers of PFU were found with subcultures and supernatants produced from them, the majority of PFU were present as 'cellular PFU'. They were sedimented by centrifugation just as normal CFU and portions of about 1% discarded with the supernatants. At that time free phage particles were present at concentrations slightly higher than that of 'cellular PFU'. Nearly constant PFU numbers in the supernatants of the respective culture and the 2 washings prove that free phages particles were not released in appreciable amounts. This changed abruptly when PFU concentrations of $>10^{10}$ ml^{-1} were attained in the third subcultures of group B and in all of the fourth subcultures. Then the supernatants of the cultures contained as many PFU as the cultures themselves, indicating that the majority of PFU were free phage.

Concerning the release of 'surplus phage', no clear-cut conclusion could be drawn from the above results. The reduction in PFU numbers by repeated washing was considerably less pronounced than observed with the model PHS (see Figs. 5 & 6), sometimes even lacking completely. From this one has to conclude that either the number of 'cellular PFU' in cultures con-

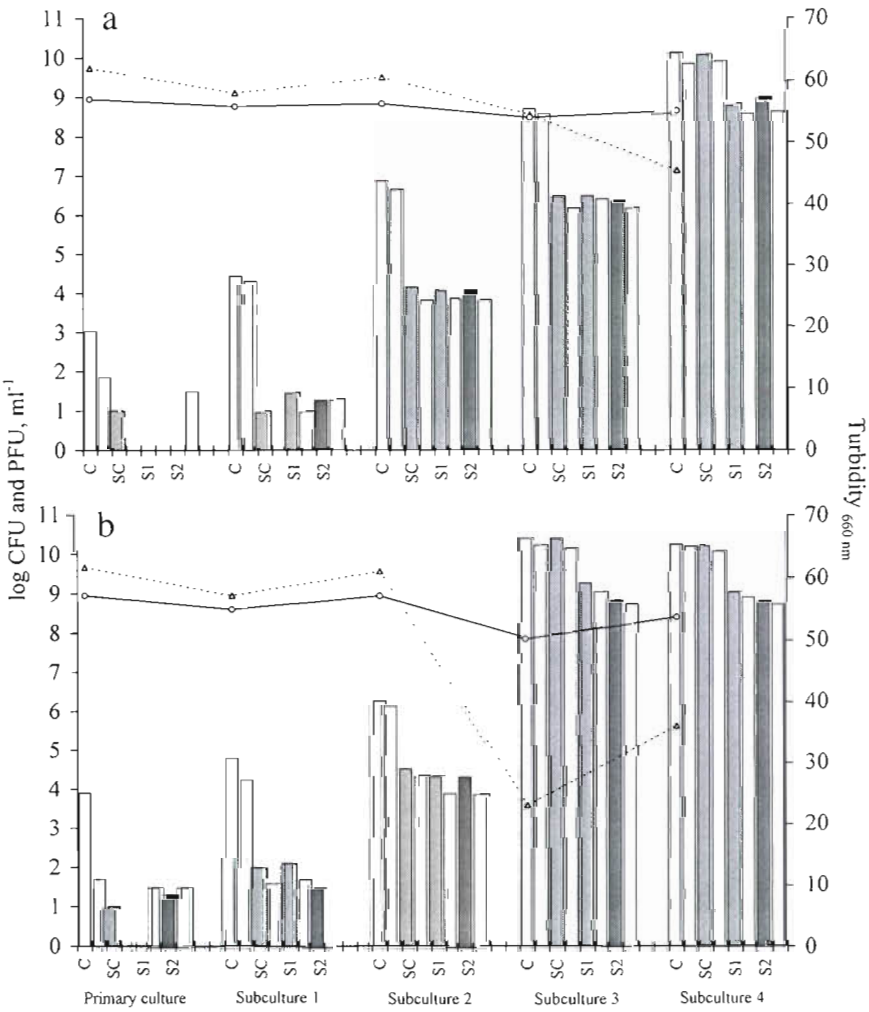

Fig. 8. H24(L3). Development of CFU and PFU during 5 consecutive cultures of 7 ml SWB/5 each, incubated in culture tubes (18 mm diameter) for 24 h at 25°C and 1 rpm. (a) and (b) present typical results found with each 2 out of 4 culture series. Each primary culture was set up with the material of one colony grown on SWA for 2 d at 25°C. Dilution between cultures: 1/100. (o) CFU and (Δ) turbidity readings at 660 nm at the end of each 24 h incubation period. Shaded columns: PFU_{Σ} as determined with H24(L10); open columns: vir mutant PFU only, detected with $H24_{wt}$. C: culture, S: supernatant of culture (SC) and of first (S1) and second (S2) wash in seawater mixture (8°C)

taining $>10^{10}$ PFU ml^{-1} was much larger than ever attained in cultures of the model PHS and their portion equal to or even larger than that of CFU, or more 'surplus phage' were released.

Another similar experiment compared H24(L3) with $H24_{wt}$. This time the occurrence of vir mutants during each of the successive subcultivation in SWB/5 was investigated. Concentrations of all types of PFUs, using H24(L10) as indicator, were determined only at the end of each of the 24 h incubations. Findings concerning the development of CFU and vir PFU are shown in Figs. 9a & 10a. Figs. 9b & 10b present the results obtained with supernatants prepared from the primary and the following 2 subcultures and the respective 2 washes of cells in SM pre-cooled in the refrigerator. In these figures the concentrations of CFU and PFU found with supernatants (columns) are compared with the respective findings obtained with the respective cultures (solid or broken lines).

Following 1/100 dilution of the preceding culture in fresh SWB/5, with both lysogenic strains of H24 steep increases in the concentrations of PFU_{vir} were observed. They lasted for several hours but slowed down before the CFU populations reached the stationary phase. The strong increases of PFU_{vir} in relatively short periods of time cannot be attributed to production by cells uninfected until the end of the preceding period of cultivation, as might be suggested by the decreases in CFU numbers observed during the first 2 h of subcultivation. If these CFU reductions were due to infection, progeny phage would be released from the infected cells not before 2 h postinfection. Such infections may have contributed at the end of the rise period to the increase in PFU_{vir}, but not at its beginning. Therefore, the most reasonable explanation for the steep increase in PFU_{vir} concentration is the release of phage particles from 'cellular PFU' which came into being by mutation of wild-type phage ϕH24 during the preceding culture period. This mutation probably also initiated the vegetative cycle of phage reproduction, which, however, did not result in phage release.

This interpretation of the results is based on the following observations: When aliquots of subcultures withdrawn after 6 h of incubation were subjected to washes in the centrifuge, the numerical distance between PFU_{vir} in the culture and its supernatant was considerably smaller than that for CFU in both samples. This can be seen with the second subculture of H24(L3) (Fig. 9b) and with both subcultures run with $H24_{wt}$ (Fig. 10b). In contrast, with aliquots taken after 24 h of incubation from the same subcultures the numerical distances for CFU and PFU_{vir} between the culture and the respective supernatant was about the same. (One may read this directly from Figs. 9b and 10b with a ruler measuring the distances between column and respective solid or dashed line.) These differences can only be explained by considerable portions of free phage among PFU_{vir} in aliquots withdrawn after 6 h, while in aliquots taken after 24 h of incubation the vast majority of PFU_{vir} sedimented with the CFU, i.e. they were 'cellular PFU_{vir}'. This interpretation is supported by the general, if only weak, trend of decreasing numbers of PFU_{vir} in supernatants of culture and washes, found with samples taken after 6 and 10.5 h of

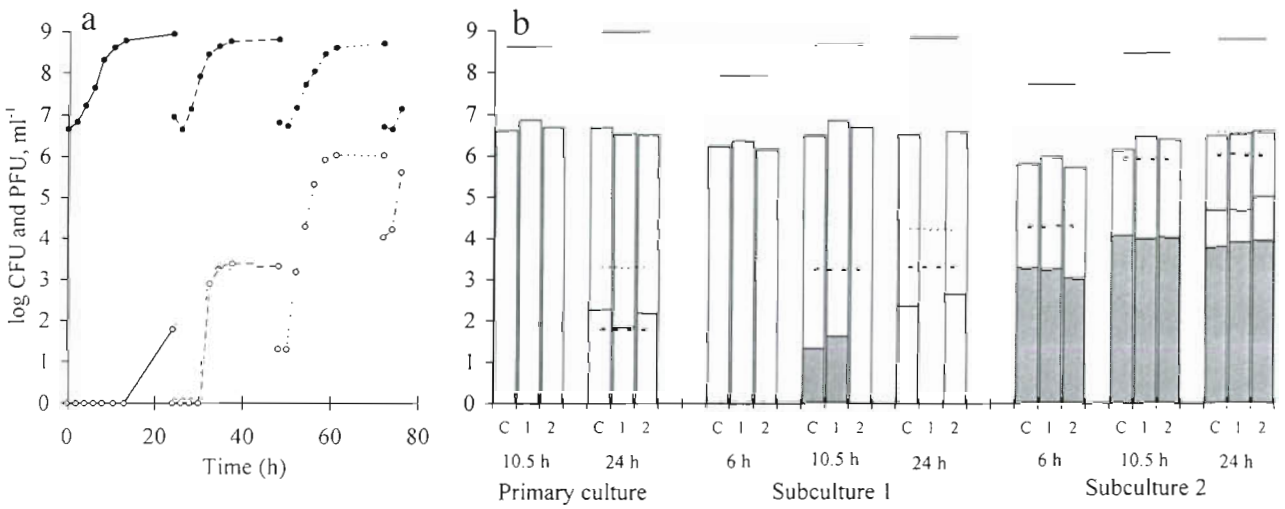

Fig. 9. H24(L3). Development of CFU and PFU in consecutive cultures of 30 ml of SWB/5 each (in 200 ml culture flasks), incubated at 25°C and 1 rpm. Primary culture was set up with material from a colony grown on SWA for 2 d at 25°C. Dilution between cultures: 1/100. For centrifugations, samples of 2 ml each were withdrawn. (a) (●) CFU; (○) vir mutant PFU. (b) Columns: findings with supernatants; solid and dashed lines: results with corresponding culture. Open column and solid line: CFU; grey column and thin dashed line: PFU_{Σ} as determined with H24(L10); shaded column and thick dashed line: vir mutant PFU only, as detected with $H24_{wt}$. C: supernatant of culture; 1 and 2: supernatants of first and second wash, respectively

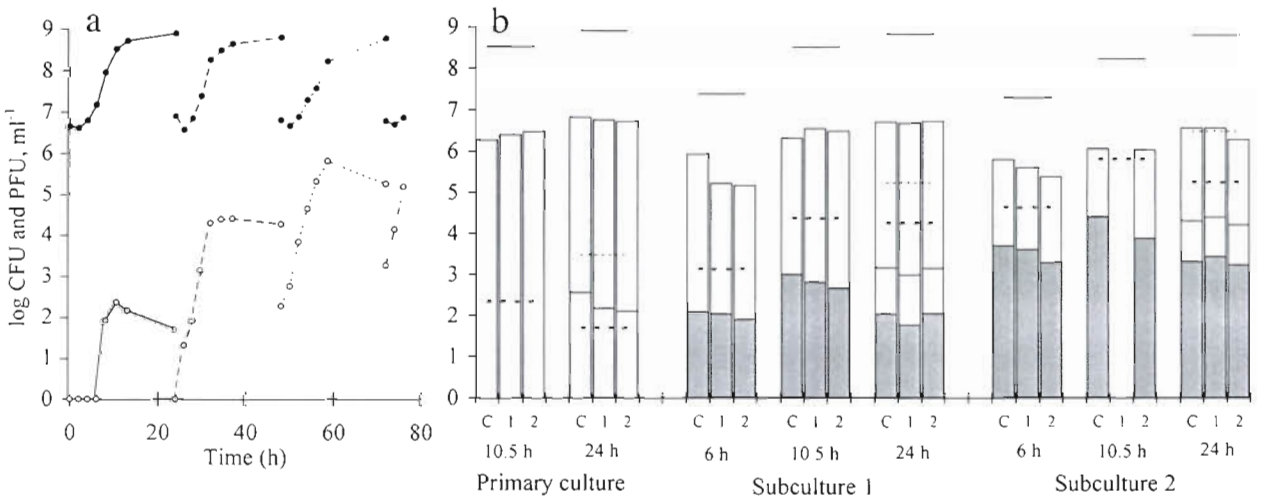

Fig. 10. $H24_{wt}$. Development of CFU and PFU in consecutive subcultures. Other information as for Fig. 9

incubation. In contrast, with samples taken after 24 h and tested in the same way, no decrease in PFU_{vir} was found.

It remains to elucidate the whereabouts of free vir mutant phage present after 6 h but not, or only at reduced proportions, after 24 h of incubation. First of all, loss of free phage by infection of CFU must be taken into account. Since the concentration of free phage (vir and non-vir) remained rather low even during the second subcultures, the concentration of 'factor X' should have remained low, too. Therefore, infection will have occurred as long as protection of CFU by pseudolysogeny was weak enough, which possibly was the case until the end of each 24 h culture period.

DISCUSSION

The investigations performed with the model system [H24(L10):ϕH24-2] lead to an understanding of observations made with $H24_{wt}$ shortly after it was found to cause spontaneous plaque formation. Pseudolysogeny was shown to be the mechanism by which genetically sensitive cells are protected from attack by potentially damaging phage. This provides an explanation for the occurrence of cultures of $H24_{wt}$ which at the end of incubation look perfectly normal in regard to turbidity, but produce confluently lysed bacterial lawns. Such observations initially were all the more surprising as only a negligible minority of colonies grown from such a culture consisted of genetically resistant cells.

The inability to completely remove PFU from pseudolysogenized cell populations was shown to be due to the presence of 'cellular PFU', the portion of which finally proved to be much greater than expected based on observations made with pelleted cells or in direct counts. The latter barely differed from CFU counts of the respective induction culture, and the volume of pelleted pseudolysogenized cells appeared to be much smaller than a pellet of an equal number of untreated H24(L10) cells. However, according to the results presented in Fig. 5 one has to assume that the portion of 'cellular PFU' in induction cultures may reach about 50% of CFU counts on AS-SWA.

Concerning pseudolysogeny the results found with 2 lysogenic strains of H24 are in general agreement with observations made with the model PHS [H24(L10): ϕH24-2]. This state of immunity to phage mutants released by $H24_{wt}$ or H24(L3) is indicated by the survival of genetically sensitive CFU of these strains in the presence of, at least, 10^{10} infective phage particles ml^{-1}. Any attempt to completely reproduce the results obtained with the model PHS would have required disproportionate efforts. Therefore, at present the answer to the question remains open whether in lysogenic strains of H24 the release of 'surplus phage' by 'cellular PFU' during washes is of the same importance as with the model PHS. The reported findings do not rule out the possibility that 'cellular PFU' in cultures of the lysogenic strains of H24, at least temporarily, attain much larger numbers than in cultures of the model PHS. However, the rapid increases of vir mutant PFU observed after starting a new subculture can be interpreted in terms of release of 'surplus phage'

Taking together all observations made with the many colonies and cultures investigated, there can be no doubt that non-vir mutants generally occur in advance of vir mutants. Accordingly it is assumed that in $H24_{wt}$ at least 2 mutations are necessary to produce a vir mutant, as was found to be the case in phage λ (e.g. Sly et al. 1971).

Pseudolysogenized cells become clotted by antiphage serum (AS). [Note that antibodies active against normal H24(L10) cells have been removed in advance

by treatment of the serum with large amounts of H24(L10) cells.] Since clotting was found not to be due to large numbers of virions adsorbed to pseudolysogenic cells, the question as to the reason(s) for clot formation by AS remains to be answered.

Lindberg (1977), in a review, compiled information on the relationship between bacterial surface carbohydrates and phage adsorption which serves as a clue. Investigations on capsulated bacteria and their phages found that polysaccharide depolymerases (PD) were part of the phage's proteinaceous envelop and that production of PD by the bacteria was under control of the genome of infecting phages. The amount of PD produced by phage-infected cells in soluble form in most investigated phage-host systems was much greater than that bound to phage particles. PD were neutralized by anti-phage serum but not by antibacterial serum. Their activity is often indicated by the large halo which may surround phage plaques. The halo's surface is somewhat sunk in the surrounding bacterial lawn due to reduced volume of the decapsulated cells.

This corresponds perfectly with observations made with $H24_{wt}$ and H24(L10) infected by phages ϕH24/1 or ϕH24/2. Both phages (cf. Moebus 1997) cause large halos around small clear centers. With ϕH24 and its mutants no halos were observed; however, this does not rule out the possibility that ϕH24 is an exopolysaccharide (EPS) specific phage. Lindberg (1977) referred to a number of publications reporting on EPS specific phage causing no halo. The same applies to phage morphology, since EPS specific phages were found among Myoviridae, Siphoviridae, and Podoviridae, to which all of the H24-phages mentioned in this paragraph belong. EPS specific phages that belong to these 3 viral families but infect the same host have also been reported before (Lindberg 1977).

The methods of Duguid and Anthony as given by Gerhardt et al. (1981) were employed unsuccessfully to demonstrate capsule formation by $H24_{wt}$ and H24(L10). However, 2 observations may indicate capsule formation in these host bacteria. Firstly, when pseudolysogenized cells were washed in SWB/5, with each of 4 to 6 washes the pellets increased in size and concomitantly changed from grey to white. Secondly, the consistence of $H24_{wt}$ and H24(L10) colonies containing phage-sensitive cells point to the possible existence of exopolysaccharide, as compared with phage-resistant clones. Material of the latter (rough) type of colonies is less easily suspended than that of phage-sensitive (smooth) type. In this context it is important that genetically resistant clones of H24, whether lysogenic or not, generally were found to be multiply resistant to ϕH24/1, ϕH24/2, and the various mutants of ϕH24. Resistance to phage of non-capsulate bacterial mutants was reported by several authors (Lindberg 1977).

Taking all findings made with H24(L10) and its pseudolysogenized cells together, it appears justifiable to assume that $H24_{wt}$ and its derivatives probably produce extracellular polysaccharide and that ϕH24/1, ϕH24/2, and the temperate phage ϕH24 and (many of) its mutants are so-called capsule bacteriophages. If this hypothesis is correct, then 'factor X' (or part of it) should be a polysaccharide depolymerase. Since this enzyme binds to the cells' surface, it would (1) cause the observed clotting of pseudolysogenized cells treated with anti-phage serum and (2) prevent phage adsorption (by competition) under the condition that PD is an essential part of the phage's component establishing contact with the cell's phage receptor, as indicated in the literature reviewed by Lindberg (1977). Unfortunately, this hypothesis could not yet be tested for validity.

Concerning $H24_{wt}$ and its derivatives, nothing more than the final results as known about events taking place in consequence of the induction and/or establishment of a mutation leading to the production of vir or non-vir mutants of phage ϕH24. The same applies to the genetics of the various mutant phages. Some information, however, could be gathered with regard to the reason why mutations in ϕH24 occur so conspicuously often. This topic is addressed in the subsequent paper.

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LITERATURE CITED

Gerhardt P, Murray RGE, Costilow RN, Nester EW, Wood WA, Krieg NR, Phillips GB (1981) Manual of methods in general bacteriology, 2nd edn. Am Soc for Microbiol, Washington, DC

Lindberg AA (1977) Bacterial surface carbohydrates and bacteriophage adsorption. In: Sutherland I (ed) Surface carbohydrates of the procaryotic cell. Academic Press, New York, p 289–356

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

Sly WS, Rabideau K, Kolber A (1971) The mechanism of lambda virulence II. Regulatory mutations in classical virulence. In: Hershey AD (ed) The bacteriophage lambda. Cold Spring Harbor Monograph Series, Vol 2, Cold Spring Harbor Laboratory, Cold Spring Harbor, p 575–588

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