Spontaneous Lysis in Lysogenic Staphylococci.* (27978)

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Cultures of lysogenic organisms contain free infectious phage because of a low rate of spontaneous lysis. Bertani(1) described a method of assaying for free phage in lysogenic cultures of *Shigella* in which streptomycin was employed. He demonstrated that the titer of free phage in the supernatant of centrifuged cultures was the same as the titer obtained when an equal sample of the culture was directly plated with a streptomycin resistant indicator strain on a streptomycin containing medium. Although Bertani found good agreement in work with *Shigella,* when the technic was adapted to the study of lysogeny in staphylococci(2), considerably higher titers were often obtained from direct assay of lysogenic cultures on antibiotic media than from the assay of supernatants of lysogenic cultures. Several possibilities were investigated to explain this difference in numbers.

Materials and methods. Viable cell numbers (colony forming units) were determined by spreading suitable dilutions of broth culture on agar plates with a glass rod. Aliquots of broth cultures were centrifuged for 10 min at 2000 RPM and the supernatant plated to determine the amount of free phage present. The titers of plaque forming units (PFU) resulted from plating suitable dilutions of whole culture on antibiotic containing medium with antibiotic resistant indicator cells.

All plating was done on trypticase soy agar which contained 0.004 M $CaCl_2$. A suitable dilution of either penicillin or streptomycin was added to the plates used for PFU counts. Samples for free phage and PFU counts were inoculated into melted soft agar with 0.1 ml of the diluted indicator strain and overlaid on the plates. Soft agar consisted of nutrient broth, 0.7% agar, and 0.004 M $CaCl_2$. Trypticase soy broth was used to grow cultures and to make serial dilutions. Indicator strains for phage counts were grown overnight on trypticase soy agar slants. The slants were scrubbed with 2 ml broth and diluted 1:10 before use. Plates for phage counts were incubated at 30°C. All other incubations were at 37°C.

The staphylococcal typing phages and propagating strains were generously supplied by Dr. J. E. Blair (Hospital for Joint Diseases, N. Y.). All other strains were obtained from routine clinical specimens.

Results. It was originally observed in screening for lysogeny that higher plaque titers were obtained when drops of culture were spotted on antibiotic resistant indicator strains seeded on antibiotic medium than when supernatants of the centrifuged culture were spotted. For a more quantitative evaluation, the following experiment was performed. A lysogenic strain (PS 53), sensitive to penicillin and streptomycin, was grown in broth and samples were assayed at hourly intervals for cell count, free phage, and plaque forming units. A penicillin and streptomycin resistant indicator strain, 5040 Lt was used for the latter 2 assays. Typical results with penicillin are given in Fig. 1. It can be seen that the PFU titer is approximately equal to the free phage titer until adsorption of free phage by cells becomes appreciable after which the PFU titer can increase to as much as a hundredfold higher than the free phage. Repeated experiments have shown that phage adsorption becomes significant when a cell density of $0.5\text{-}1.0 \times 10^8$ is reached. Results with streptomycin were similar to those obtained with penicillin although of lower magnitude (Fig. 2).

It is not likely that penicillin or streptomycin acts as inducing agent since the PFU and free phage titers are approximately equal for the early logarithmic growth phase. This possibility was tested, however, with a modification of the single burst technic(1) and gave negative results.

Since PFU concentration is the sum of free phage and cell associated phage, it is apparent that the latter cell-associated phage is responsible for the divergence in titer between free

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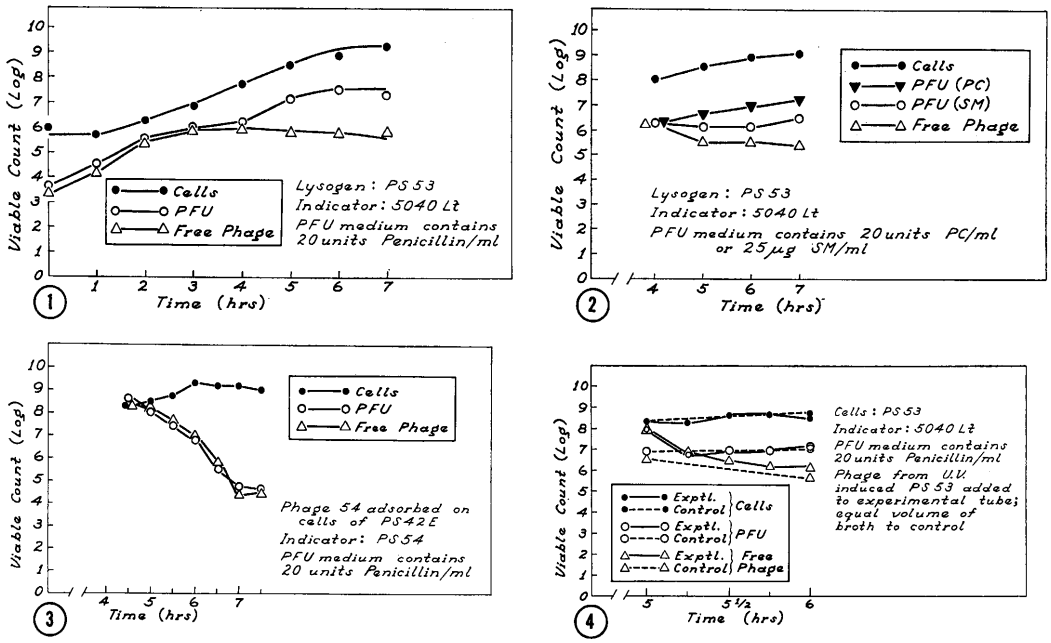

FIG. 1. Free phage and plaque forming units (PFU) produced by a lysogenic staphylococcal strain.
FIG. 2. Comparison of PFU titer on penicillin (PC) and streptomycin (SM).
FIG. 3. Phage adsorption and inactivation by resistant host cells.
FIG. 4. Effect of added homologous phage on free phage and PFU titers.

phage and PFU. The data suggest that the differences might be proportional to the amount of adsorbed phage. Adsorption was not readily reversible, however, since repeated washings or treatment in a Waring blendor for as long as 2 minutes did not result in release of infectious adsorbed phage from cells.

Although phages adsorbed to cells are not readily removed by mechanical means it is conceivable that infectious phage could be desorbed under the influence of antibiotic treatment. This hypothesis was first tested in a non-lysogenic system by the following experiment. A culture of PS 42E (penicillin sensitive, immune to typing phage 54) was grown to a titer of 10^8 cells/ml at which time an equal number of phage 54 particles were added. The free phage and PFU titers were then determined for a 3½-hour period with PS 54 (penicillin resistant) as an indicator. The results (Fig. 3) indicate that adsorption and inactivation are concomitant and that penicillin could not effect the release of infectious phage adsorbed to cells. It is of interest to note that the same rate of adsorption and inactivation occurred when this experiment was performed on heat killed cells of PS 42E.

A similar experiment of adding homologous phage to the lysogenic system gave results illustrated in Fig. 4. The added phage, obtained by ultraviolet induction of a culture of PS 53, was adjusted to give a cell:free phage ratio of 1 at the time that the PFU and free phage titers diverged significantly. A control culture, without added phage, was assayed simultaneously. It is evident from the results that phages adsorbed to cells are inactivated immediately, as shown by the simultaneous decrease of PFU and free phage titers during the 15 minutes after phage addition, and also by the fact that the PFU titer is not increased above the titer of the control culture. The cell-associated phage responsible for the high PFU concentration is therefore unrelated to the readsorbed phage.

The most likely remaining explanation for this phenomenon would be that the sensitive lysogens are not inactivated immediately by the antibiotic and considerable growth and spontaneous induction and lysis can occur before the cells are inactivated. If this be the

TABLE I. Effect of Antibiotic Concentration on PFU Assay of a 5 Hr Culture of PS 53 (Indicator strain is 5040 Lt).

Antibiotic	Concentration	PFU/ml*
Penicillin	5 units/ml	1.3×10^7
	10 "	1.1×10^7
	20 "	7.8×10^6
	50 "	6.4×10^6
Streptomycin	10 μg/ml	7.1×10^6
	25 "	9.3×10^6
	50 "	7.0×10^6
	100 "	5.7×10^6

* Free phage titer was 4.5×10^6/ml.

case then increasing the antibiotic concentration should, by increasing the killing rate of cells, cause a reduction in the PFU titer. This was confirmed by assaying for free phage and for PFU at several levels of penicillin and streptomycin. The results in Table I of samples from a 5 hr culture do show the decrease in PFU with increasing antibiotic level and the tendency to approach the value for free phage.

Discussion. The observation that PFU titer is higher than free phage is not surprising and has been noted previously(3,4). The magnitude of the change, as much as one hundredfold, is quite unexpected, since these authors suggest that only cells late in the lytic cycle are capable of releasing phage in the presence of bactericidal concentrations of antibiotic. The similarity between the PFU titer and the projected free phage titer (if adsorption had not occurred) suggested that the antibiotic may cause the release of adsorbed phage. This is unlikely since adsorbed phage is rapidly inactivated. The most likely explanation would then be that the antibiotic permits a limited amount of multiplication and lysis to occur in the lysogenic cell. Kreuger *et al.*(5) have shown that penicillin allows phage production to occur in exposed staphylococci. However, to account for lack of divergence in the younger culture, one must either postulate a combination of burst size and rate of spontaneous lysis such that the PFU does not appreciably increase before free phage readsorption, or else an increasing rate of spontaneous lysis with increasing age of culture. This same lack of divergence would indicate that any penicillinase produced by the resistant indicator strain is not significant for permitting further multiplication and lysis of the lysogenic cell.

The hypothesis of limited growth and lysis in the presence of antibiotic, particularly streptomycin, would not be in agreement with the findings in other organisms and may suggest that staphylococcal cellular functions are not as rapidly inactivated as those of other organisms by penicillin and streptomycin. Certainly, the streptomycin method of free phage assay should be carefully evaluated before adopted for new lysogenic systems.

Summary. Cultures of lysogenic staphylococci yielded significantly higher plaque counts when plated directly on streptomycin or penicillin with antibiotic resistant indicator strains than when supernatants of centrifuged cultures were assayed. This occurs at cell concentrations that permit significant readsorption of free phage, which are inactivated upon adsorption. The probable cause for this divergence in titers is discussed.

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