STUDIES ON LYSOGENESIS

I. The Mode of Phage Liberation by Lysogenic Escherichia coli[1]

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The stable association of a bacteriophage with a bacterial strain, known as lysogenesis, has received scarce attention in the last ten years, especially by comparison with the remarkable progress made during the same period in the study of common bacteriophage infection followed by lysis of the infected cell (see a summary in Benzer *et al.*, 1950). Only recently Lwoff and coworkers (1950 *a,b*), working with *Bacillus megatherium*, have succeeded in defining more precisely the lysogenic condition. In this paper we report observations on a lysogenic strain of *Escherichia coli* that carries more than one detectable type of phage.

MATERIAL AND METHODS

The lysogenic strain of *Escherichia coli*, strain "Li," is presumably the same strain studied in detail by Bordet and Renaux (1928) and by Bordet and Bordet (1946). The phage liberated by this strain is active on a strain of *Shigella dysenteriae*, strain "Sh." Both bacterial strains were obtained through the kindness of Dr. J. Lederberg. A streptomycin resistant strain "Sh/s" was developed from "Sh" by serial transfers in the presence of increasing concentrations of dihydrostreptomycin sulfate (Eli Lilly & Company).

"Li" was grown on "bacto" nutrient broth with 0.5 per cent NaCl. "Sh" was grown in LB medium: bacto tryptone 1 per cent, yeast extract 0.5 per cent, NaCl 1 per cent, glucose 0.1 per cent, in H_2O; pH adjusted to 7.0 with 1 N NaOH. "Sh/s" can grow both in the presence and in the absence of streptomycin, which was usually employed in a concentration of 10 μg per ml.

The standard techniques for phage work, as described by Adams (1950), were followed except for the following points. In using the soft agar technique for assaying phage on strains "Sh" or "Sh/s", the bottom layer consisted of LB agar (LB medium plus 1 per cent agar and 2.5×10^{-3} M $CaCl_2$), and the top layer consisted of 0.6 per cent nutrient agar enriched with 0.5 per cent yeast extract. "Sh" or "Sh/s" from 24 hour aerated cultures was used in amounts of 0.2 ml per plate as plating bacteria. Phage stocks consisted of lysates of "Sh" or "Sh/s" prepared either in LB medium plus 2.5×10^{-3} M $CaCl_2$ or on LB agar (plate technique), centrifuged and filtered.

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RESULTS

Titration of free phage. A basic problem in studies on lysogenesis is the titration of free phage present in cultures of lysogenic bacteria. If a phage assay is done on a lysogenic culture by plaque count, the lysogenic bacteria multiply on the assay plate and produce new phage, giving origin to colony-centered plaques, whose number is proportional to the concentration of bacteria but independent of the free phage concentration in the plated culture. Previous workers have assayed the free phage by selectively killing the lysogenic bacteria with heat or by removing them by centrifugation. We used a more rapid method that employs streptomycin to kill bacteria prior to plating for free phage assay on a streptomycin resistant indicator strain "Sh/s". The phage produced by *E. coli* "Li" can stand at least 500 μg of streptomycin added to a 2 ml agar tube without loss of titer. A comparison of the free phage titers obtained by the streptomycin technique and by centrifugation shows excellent agreement, as demonstrated in table 1.

We must remember that, whatever the technique used, the phage titers are proportional to, but not necessarily identical with, the absolute number of

TABLE 1

Free phage titers in Escherichia coli "Li" cultures

FREE PHAGE TITER, PLAQUES PER ML		RATIO "BACTERIA"/"FREE PHAGE"
Streptomycin technique	Centrifugation technique	
3.2×10^6	3.8×10^6	570
2.9×10^4	3.3×10^4	6400
5.5×10^5	4.6×10^5	3500
1.1×10^6	9.5×10^5	870

active phage particles, since the "efficiency of plating" (ratio "plaques"/"active particles") is not known *a priori*, although it is presumably constant for a given phage preparation under standard conditions.

Production of phage by growing E. coli "Li." The streptomycin technique makes it easy to follow quantitatively the production of phage during growth of a lysogenic strain. The results of such an experiment with "Li" are shown in figure 1. During the central part of the logarithmic phase, the ratio "bacteria"/"free phage" is constant, i.e., the rate of bacterial multiplication and the rate of phage production are identical. At the beginning and at the end of the logarithmic phase the ratio "bacteria"/"free phage" changes, an indication that the rate of free phage production depends on the physiological conditions of the bacteria.

The plaques formed by the phage on "Sh" show a wide range of morphological variation, from almost completely clear plaques, 1 to 4 mm in diameter, to tiny turbid plaques, 0.2 to 0.3 mm. Single plaque isolations prove these two types to breed true (figures 2 and 3). Strain "Li," therefore, produces at least two genetically different phages. Twenty single colony isolations from strain "Li"

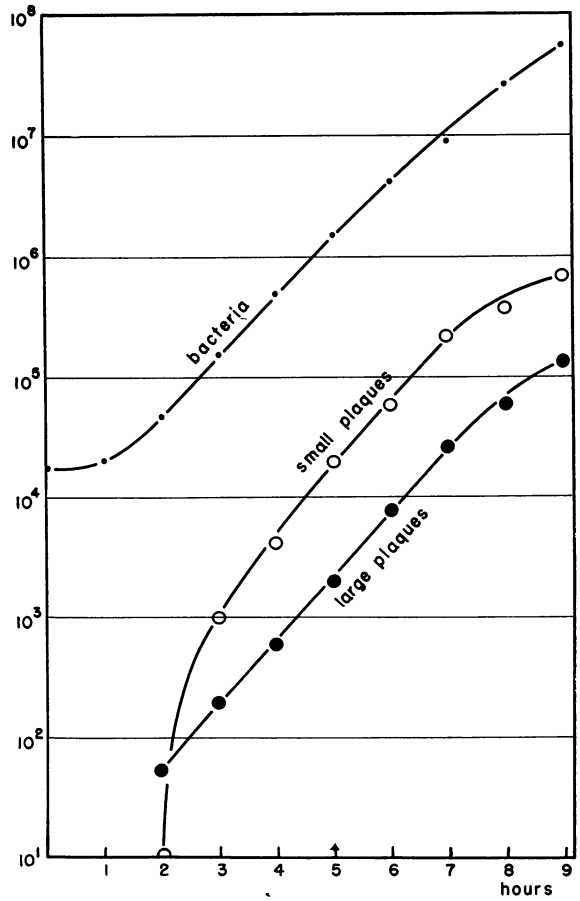
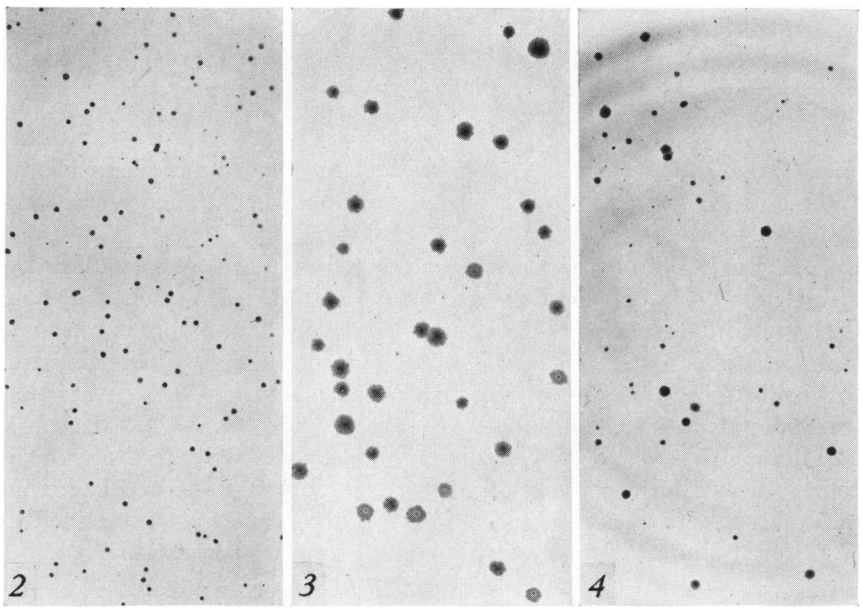


Figure 1. Free phage titer in a growing culture of *Escherichia coli* "Li" in nutrient broth at 37 C. Ordinate: bacterial colony counts and phage plaque counts per ml. The free phage was eliminated from the inoculum by centrifugation.



Figures 2 to 4. The plaques of the three phages liberated by *Escherichia coli* "Li". *2.* Phage *P1*. *3.* Phage *P2*. *4.* Phage *P3*. 1.2 ×.

were examined and all found able to produce both phage types. These are only the two extreme morphological plaque types. In the growth experiments (figure 1) the plaques were scored arbitrarily as either large or small; the counts revealed that the ratio of large to small plaques remained constant during the central part of the logarithmic phase (approximately 1:8), but shifted in favor of the large plaques at both ends of this phase. The relative frequency of liberation of the various phage types is evidently dependent on the physiological condition of the bacteria.

Mode of phage liberation. Phage liberation by lysogenic bacteria might be a continuous process, for example, a secretion of one phage particle after another by growing cells. Or it might be a discontinuous process, consisting of the sudden liberation of many phage particles by one cell (burst), probably accompanied by cell lysis. Burst liberation with lysis was proved for *Bacillus megatherium* (Lwoff and Gutmann, 1950*a*), in which case the rate of phage liberation was so high that the lysis of cells that liberate phage could be observed under the microscope.

TABLE 2

Burst liberation of phage by Escherichia coli "Li"

Results from 4 single burst experiments with *E. coli* "Li". A total of 6 tubes gave only one plaque; this was approximately the amount of free phage expected to be carried over with the inoculum. These tubes, therefore, were not included in column 2. Column 3 was calculated from columns 1 and 2, assuming a Poisson distribution of bursts among the tubes.

(1) TOTAL NO. OF TUBES	(2) NO. OF TUBES WITH PHAGE	(3) NO. OF TUBES EXPECTED TO HAVE MORE THAN 1 BURST	(4) PHAGE PARTICLES PER TUBE, RANGE	(5) INOCULUM, BACTERIA PER TUBE	(6) BACTERIA PER TUBE AFTER INCUBATION
77	8	0.42	10–82	330	2172
77	9	0.49	15–129	345	2693
76	5	0.17	10–31	469	2940
78	11	0.75	6–67	188	2845
308 (Tot.)	33 (Tot.)	1.83 (Tot.)	6–129	333 (Av.)	2663 (Av.)

Burst frequency: 1/45,000 bursts per cell per generation.

With *E. coli* "Li," a decision could be reached by means of a modification of the "single burst" experiment, in which we studied the distribution of free phage produced in a number of parallel cultures. If phage is liberated in bursts, experimental conditions should be found in which some of the cultures liberate no phage, while others liberate during the period of examination an amount of phage corresponding to one burst.

A 24 hour culture of the "Li" strain was almost completely freed of free phage by repeated centrifugations and diluted to contain a few hundred bacteria per ml; 0.5 ml amounts were distributed into 80 tubes. The tubes were incubated at 37 C for 2 hours, corresponding to three bacterial generations. A drop of

streptomycin (about 250 μg) was then added to each tube. The whole content of each tube was plated on one plate using "Sh/s" as indicator. The experimental data, reported in table 2, show that liberation of phage by *E. coli* "Li" occurs indeed in bursts.

A remarkable fact came to light in the single burst experiments. The plaques in each burst were morphologically quite homogeneous, so that most bursts could easily be classified as large (22 bursts out of 33, see figure 3) or small (7 bursts, see figure 2) plaque type. The homogeneity of the phage in each burst was checked by picking the smallest plaque from each burst classified as "large plaque type," and the largest from each of the "small plaque type," and replating them on sensitive bacteria. In all cases, the plaques from large plaque bursts again gave large plaques and *vice versa*. A few bursts (4 out of 33) showed a greater variability of plaque size, but did not consist of a mixture of the large and small types; plaques picked from these bursts again gave plaques of various

TABLE 3

Serological and plaque type classification of 13 different isolates of phage liberated by Escherichia coli "Li"

PHAGE TYPE	PLAQUE TYPE	NUMBER OF SINGLE PLAQUE ISOLATES TESTED	k FOR SERUM AGAINST THE SMALL PLAQUE PHAGE, MIN^{-1}		k FOR SERUM AGAINST THE LARGE PLAQUE PHAGE, MIN^{-1}	
			Range	Average	Range	Average
P1	small (<1.0 mm)	4	52–110	75.4	2.2–8.0	5.4
P2	large (1 to 4 mm)	6	5.0–12	8.9	18–26	22.0
P3	medium size	3	−1.0–0.3	−0.5	3.1–9.2	6.3

In addition, an antiserum against an isolate of type *P3* was prepared; when tested against three phage isolates, one representative of each type, the following k values were obtained: 2 on *P1*, 10 on *P2*, and 72 on *P3*.

size, intermediate, on the average, between the two extreme types. The phage from two of these bursts, analyzed serologically (see next section) was found to belong to a third type, not easily recognizable by plaque morphology when admixed with the other phages produced by the "Li" strain (see figure 4).

Types of phage produced by E. coli "Li." A preliminary serological classification of the various phages found the "Li" strain was attempted. From 13 bursts in the experiments previously reported, 13 single plaques, including all three morphological plaque types, were sampled; a lysate was made from each plaque and filtered. Two of these lysates, one a typical small plaque phage (*P1*), the other a typical large plaque phage (*P2*), were injected into rabbits to produce homologous antiphage sera. The 13 lysates were tested for inactivation by antiserum as follows. Diluted serum and phage were mixed at 37 C; the final serum dilutions were 1:320 for the serum against the small plaque phage and 1:100 for the serum against the large plaque phage. Controls were made with

sera from the same rabbits before injection. Each mixture was assayed twice for phage at different times. The tests were repeated three times and the survival ratios were averaged. From the inactivation data, the neutralization rate k (min^{-1}; Hershey *et al.*, 1943) was calculated for each serum against each lysate; the results are summarized in table 3. Cross-reactions were present, but in the main the lysates fell into three categories well correlated with plaque type. The differences in the values of k within each category are probably not significant.

Lysis of E. coli "Li" *from without.* Attempts by many authors to liberate phage by artificial disruption of lysogenic bacteria by means of lysozyme, penicillin, infection with an unrelated phage, or mechanical grinding were unsuccessful (Burnet and McKie, 1929; Rountree, 1949; Boyd, 1950; and Lwoff and Gutmann, 1950*a*). Lysis from without (Delbrück, 1940) was tried with *E. coli* "Li." Phage T6 was added to a growing culture of the "Li" strain (7×10^7 cells per ml) at a multiplicity of about 200. The free phage liberated was assayed at 4, 8, and 12 minutes after addition of phage T6. No significant increase in titer was observed although clearing of the culture was noticeable after the first 5 minutes.

DISCUSSION

As mentioned in the previous section, all attempts to liberate phage by artificial disruption of the lysogenic cells have been unsuccessful. This indicates that no mature infective phage is normally present in lysogenic bacteria. It has, therefore, been assumed by many authors (see Lwoff and Gutmann, 1950*a*) that phage is carried by lysogenic bacteria in a condition or state (*prophage*) that allows multiplication, but does not allow infection of susceptible cells. The homogeneity of phage type within each burst reported in this paper for *E. coli* "Li" offers additional evidence for the correctness of this assumption. It has been shown, in fact, that when a cell of the "Li" strain liberates, for instance, the large plaque phage, no plaques of the other types appear in the burst, although all cells of the strain can give rise to bacterial clones producing three kinds of phage.

To assure equal distribution of prophages to the daughter cells at each division, there must either exist a mechanism that is somehow connected with the process of cell division or the number of prophages must be so high that their random distribution at cell division does not cause any important shift in the average number of prophages per cell. Assuming the second alternative to be more likely and considering the mode of liberation of phage as described in this paper, one is faced with two main possibilities:

1. Liberation of phage by a lysogenic cell is caused by "maturation" of all or several of the prophages present in the cell, so that each prophage gives origin to one mature, infective phage particle. The process probably starts as a consequence of a particular metabolic condition of the cell. The homogeneity of burst as to the type of phage, reported in this paper, should then be interpreted as due either to specificity of the responses of the different prophage types to these particular conditions, or to some interference between the different types (Delbrück and Luria, 1942; Delbrück, 1945).

2. Liberation of phage is the consequence of a "change" in the state of one of the prophages, which multiplies and gives origin to a clone of mature and infective phage particles. The homogeneity of burst is a direct consequence of this hypothesis, whenever, as would be the case with *E. coli* "Li," the frequency of the change that initiates phage liberation is low.

We hope to be able to test soon these two hypotheses experimentally. At any rate, the nature of the assumed "metabolic condition" of the cell or "change" of the prophage remains unknown. All we know today is that the incidence of these changes is influenced by cultural conditions. In *B. megatherium* (Lwoff *et al.*, 1950*b*) and in strain K-12 of *E. coli* (Delbrück and Weigle, personal communication) ultraviolet or roentgen irradiation greatly increases the frequency of phage liberation. In preliminary experiments we could not detect any significant rise in the rate of phage liberation after ultraviolet irradiation of *E. coli* "Li." The difference may be due to strain differences or to cultural factors as yet unidentified.

Another problem raised by our data concerns the genetic relationship between the three phage types carried by *E. coli* "Li." They are serologically different but have some common antigens. On the basis of what is known for other phages, it seems unlikely that the differences observed among the three phages are due to single mutational steps. We may ask whether these phages have derived from a common ancestor that was the first to parasitize *E. coli* "Li," or from different phages that have infected "Li" independently at different times.

Some of the problems raised in the discussion are now under study, using both *E. coli* "Li" and a variant of *S. dysenteriae* "Sh" made lysogenic for phage *P2*. These experiments will be reported in a future paper.

SUMMARY

The lysogenic *Escherichia coli*, strain "Li" (Lisbonne and Carrère), liberates several bacteriophages active on a strain of *Shigella dysenteriae*. The phages can be classified by plaque morphology into three types that are also serologically distinct, although somewhat cross-reacting. Phage is liberated in clusters (bursts), each cluster presumably deriving from lysis of one cell. In the conditions of our experiments, the frequency of bursts was approximately 1:45,000 per cell generation. Phage bursts are always homogeneous as to phage type; no mixed bursts have been observed.

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