STUDIES ON THE HOST-VIRUS RELATIONSHIP IN A LYSOGENIC STRAIN OF BACILLUS MEGATERIUM[1]

I. The Relationship between Growth and Bacteriophage Production in Cultures of Bacillus megaterium

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Received for publication July 27, 1951

One of the most controversial questions of the host-virus relationship is the significance of lysogenic strains of bacteria. The source and method of release of the free bacteriophage found in such cultures have remained obscure, as has the mechanism whereby virus was transmitted from cell to cell.

The term "lysogenic" has unfortunately been used in two senses. Some workers have used the term to describe any association between bacteriophage and bacteria which allows both to survive in successive transfer. Many such cultures, which are lysogenic by this definition, are themselves subject to occasional attacks of lysis. From such cultures as these, it is relatively easy to obtain colonies which are bacteriophage-free and are either resistant or sensitive to the original bacteriophage (D'Herelle, 1926). Evidence has also been obtained indicating that the bacteriophage in such cultures may also give rise to mutants which are able to lyse the normally resistant parent (Burnet and Lush, 1936).

With this type of lysogenic culture, it seems probable that the free bacteriophage found in a filtrate could arise from lysis of sensitive bacteria, with an occasional virus mutant appearing which can attack and lyse the normally resistant cells (Delbrück, 1946).

Recently Delbrück (1946) has defined true lysogenicity as not involving lysis of the host cell. A lysogenic strain of *Bacillus megaterium*, isolated and described by Den Dooren de Jong (1930, 1931), seemed to be truly lysogenic. Spores from this culture can be heated to a temperature sufficient to inactivate free bacteriophage and will still germinate and give rise to lysogenic colonies. The culture can be washed free of demonstrable virus and will still give rise to phage carrying clones. Lysis of washed cells with lysozyme does not yield bacteriophage, yet the titers of free phage in a broth culture of the organism reach 10^9 per ml (Den Dooren de Jong, 1930; Gratia, 1936; Wollman and Wollman, 1936, 1938, 1939; Cowles, 1950). Virus-free colonies of this culture have not been obtained by the usual plating techniques, and spontaneous lysis of the culture has not been reported. This culture appeared to be a very stable lysogenic culture and seemed well suited for an investigation of the mechanisms involved in the phenomenon of lysogenicity.

[1] Aided by a grant from the Jane Coffin Childs fund for Medical Research.

[2] This paper is based on this author's Doctorate thesis deposited in the Yale University library.

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During the period in which the experiments dealing with the mechanism of release of bacteriophage were being completed (those experiments involving single cell or spore isolations), Lwoff and Gutmann, in France, published a series of papers dealing with the same phenomenon (Lwoff and Gutmann, 1949*a*, *b*, *c*; 1950*a*). In these particular studies our observations confirm those of Lwoff and Gutmann.

MATERIALS AND METHODS

I. *Cultures.* The lysogenic organism used throughout this study was *Bacillus megaterium*, strain 899, or its mutants. The indicator strain of *B. megaterium*, lysed by the bacteriophage of *B. megaterium*, was obtained from Dr. A. D. Hershey and was designated as strain PR3E. Bacteriophage, unless otherwise noted, was obtained by inoculating the lysogenic strain into broth and filtering the culture, after growth, through a Chamberland L5 filter. This filtrate could be stored under refrigeration for at least 6 months with no significant loss in titer.

II. *Media.* Preliminary experiments indicated that the clearest and most easily countable plaques were obtained with a complex medium. The following media were used in all bacteriophage titrations, cell counts, and maintenance of stock cultures.

Broth. Twenty g trypticase (B.B.L.), 6 g yeast extract (B.B.L.), 1,000 ml distilled water.

Agar medium. 18 g agar (B.B.L.), 1,000 ml broth.

III. *Bacteriophage titrations.* The plaque counting technique was used in all experiments. Two-tenths ml of a heavy suspension of strain PR3E cells, washed from an 18-hour slant with about 2 ml broth, was put on the surface of an agar plate. To this, 0.1 ml of the diluted bacteriophage was added, using a serological pipette. The mixture of cells and bacteriophage was then spread evenly over the entire surface of the plate with a sterile glass rod. The plates were dried in an incubator with the covers partially removed, and then incubated for 18 to 24 hours at 35 C. Unless otherwise noted all reported titers are the average of duplicate platings. This technique, if carefully used, gave counts reproducible within a 10 to 15 per cent range.

IV. *Micromanipulation techniques.* The techniques and apparatus for single spore and cell isolations will be described in the section devoted to this portion of the work.

EXPERIMENTAL RESULTS

I. *The relationship of lysogenic B. megaterium growth to bacteriophage release.* Preliminary experiments indicated that the titer of bacteriophage in growing *B. megaterium* cultures was proportional to the number of cells in the culture. This experiment was conducted to compare the growth of the lysogenic bacterium with its production of bacteriophage.

An inoculum of 0.1 ml of a 1/100 dilution of an 18-hour broth culture of *B. megaterium*, washed twice in M/15 phosphate buffer, was added to a 500-ml Erlenmeyer flask containing 100 ml of broth, and the culture was incubated at

37 C in a shaker. Samples were removed at intervals and diluted in chilled broth for both cell and bacteriophage titrations. It is not necessary to remove the lysogenic cells in titrating for the free bacteriophage with this system. The lysogenic cell gives rise to a plaque with a small colony in its center, in contrast to the clear plaque formed by free bacteriophage. Cell counts were made by placing 0.1 ml of suspension on the surface of an agar plate and spreading the inoculum over the entire surface of the plate. Colonies were counted with a Quebec Colony Counter after 24 hours' incubation at 35 C. All counts are the average of four replicate platings.

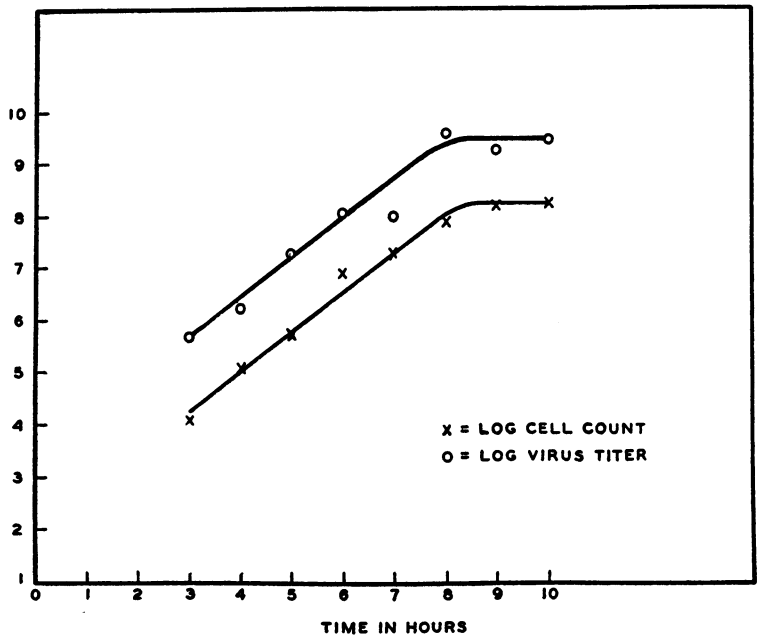

Figure 1. Cell counts and bacteriophage titers during logarithmic growth phase of *Bacillus megaterium*.

The results of this experiment are shown in figure 1. The ratio of bacteriophage to bacteria, as taken from the growth curves, is about 25/1. This calculation was made on the assumption that the bacterial counts determined from the colonies represented the actual number of viable cells in the culture. However, *B. megaterium* is a chain-forming organism, so that colonies arising on agar plates develop from more than one cell. To correct this bacteriophage/bacteria ratio it was necessary to determine the average number of cells per chain in a growing culture of *B. megaterium*. This was done by removing samples from a culture, grown under conditions identical with those used in establishing the growth curves, and making stained preparations from them. The slides were examined under oil and the average number of cells per chain determined. The results are presented in table 1. It can be readily seen that the average chain of *B. megaterium*, grown under these conditions, consists of 1.9 cells. This introduced

a correction factor for the original cell-virus ratio, lowering it to approximately 13/1.

From the growth curves presented in figure 1 it is apparent that the free bacteriophage titer in a shake culture of *B. megaterium* is directly related to the number of cells in the culture. Calculation of the generation time for bacteria and bacteriophage gave 22.4 and 23.2 minutes, respectively. It seemed then that the production of bacteriophage by the lysogenic *B. megaterium* could be a function of cell division or multiplication. The results, however, do not indicate the manner in which the virus is released from the cells. The experimental growth curves could have been obtained by any of three methods of virus release from lysogenic cells. (1) Virus could be released from each cell in the culture by a method other than lysis. Each cell would have to release an average of 13 particles to give the obtained growth curves. This would be true lysogenicity as defined by

TABLE 1

The average number of cells per chain in Bacillus megaterium

Distribution

AGE OF CULTURE IN HOURS	NUMBER OF UNITS CONTAINING						TOTAL CELLS	NO. CHAINS COUNTED	X
	1	2	3	4	5	6 Cells			
4½	6	62	9	4	0	0	173	81	2.1
5½	76	241	27	5	1	1	670	351	1.9
6	23	75	6	5	0	0	211	109	1.9
6½	87	269	19	3	0	1	700	379	1.8
Totals.....	192	647	61	17	1	2	1754	920	1.9

Trypticase-yeast extract broth shaker culture at 35 C.

Delbrück (1946). (2) Virus could be released from certain cells in the culture by a method other than lysis. Certain cells might continually synthesize and release virus during growth or division. (3) Virus could be released by the lysis of certain cells formed at a constant rate by the normally resistant parent cells.

II. *Bacteriophage release by the lysogenic B. megaterium.* It was felt that the method of virus release in this lysogenic system could be discovered by the use of micromanipulation techniques. The isolation of single virus-free cells or spores of *B. megaterium* in microdrops of broth, microscopic observation during the growth of these isolates, and titration of the microdrops of broth for free bacteriophage at intervals during cellular growth and division should demonstrate the processes involved in the production of virus by this lysogenic culture.

In general, the micromanipulation techniques introduced by Barber (1904) and expanded by Gee and Hunt (1928) were followed. Basically they involve the transfer, by means of a micropipette, of a microdrop containing a single spore or cell onto the underside of a supported coverslip. The micropipette is controlled by means of a micromanipulator. The usual techniques of micromanipulation employed in bacteriology for the purpose of obtaining cultures arising from single cells or spores were modified to suit the needs of these experiments.

One of the most difficult and vital points in isolating single cells has been the preparation of the coverslips on which the isolations are made. To the authors' knowledge, the use of plastic coverslips in lieu of the usual glass slips was first suggested by Pomper (1949), and the success of these experiments was greatly facilitated by his suggestion. "Turtox" plastic coverslips, 24 mm by 50 mm, no. 2 thickness, eliminated the tedious and difficult greasing of glass coverslips described by Gee and Hunt (1928). The plastic coverslips are washed in warm water, rinsed in distilled water, and air dried. They can then be mounted directly on the top of a moist chamber, sealing the edges with vaseline. Sterility may be obtained by exposing the washed coverslips to ultraviolet light for a few minutes.

TABLE 2

Single cell growth observations on Bacillus megaterium

ISOLATION	NO. CELLS IN CHAIN	TOTAL NO. CELLS GROWN OUT	LYSIS OBSERVED	FREE BACTERIOPHAGE DEMONSTRATED
1	3	16	−	−
2	2	12	−	−
3	2	9	−	−
4	2	>35	−	−
5	2	30	−	−
6	2	19	−	−
7	2	>34	−	−
8	4	12	−	−
9*	4	>49	+	+
10	2	30	−	−
11*	2	10	+	+
Totals 11	—	>256	2 Isolations 9 Negative	2 Isolations 9 Negative

* See figures 2 and 3 for details.

All micropipettes were drawn by hand over a microburner as described by Gee and Hunt (1928).

Preliminary experiments demonstrated that the usual method of discharging and filling micropipettes by mouth pressure or suction did not allow sufficient control of the microdrop. A special threaded syringe was constructed and connected to the micropipette by means of polyethylene catheter tubing. The delicacy of control provided by this instrument proved to be excellent, and microdrops of any desired size could readily be made.

Stock suspensions of spores were prepared by washing them from cornmeal extract agar slants with M/15 phosphate buffer, pH 7.4, centrifuging, and rewashing them 5 times in additional buffer. Cell suspensions were prepared from 12 to 14-hour broth cultures and washed 5 times in phosphate buffer. Bacteriophage titrations of the supernate from the fifth washing of either preparation showed titers of less than 200 per ml.

Single spores or cells were isolated in microdrops of 4× normal strength broth and observed microscopically for growth, division, and lysis. When the number

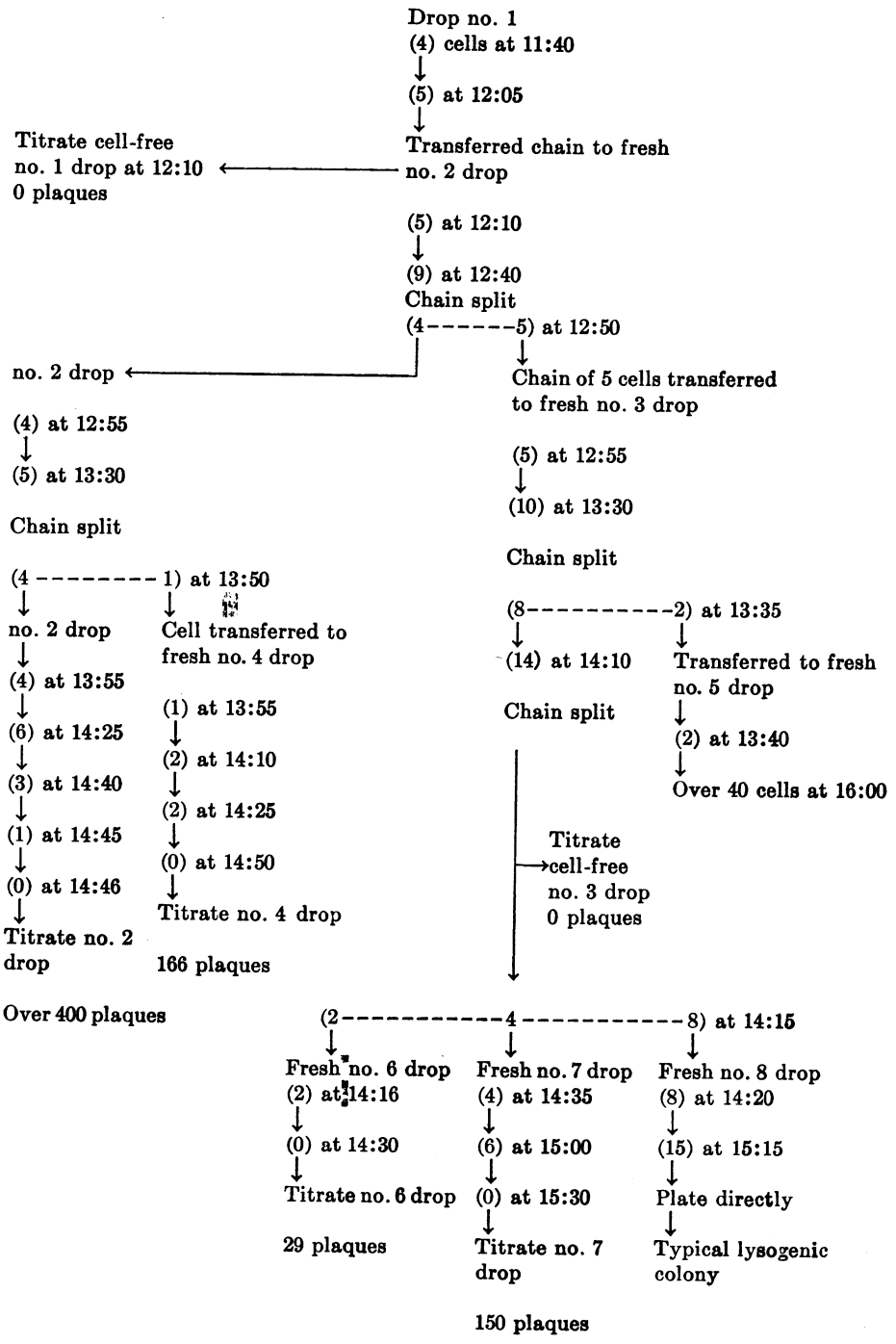

Figure 2. The release of bacteriophage by *Bacillus megaterium*

of cells in a drop reached 12 to 15, it became very difficult to observe each cell carefully. If the daughter cells failed to separate following formation, they could sometimes be separated by alternately taking them into the micropipette and expelling them. When the chain had split, either naturally or by mechanical breakage with a pipette, each separate unit was transferred to a fresh microdrop, and the original microdrop, now cell-free, titrated for bacteriophage. This

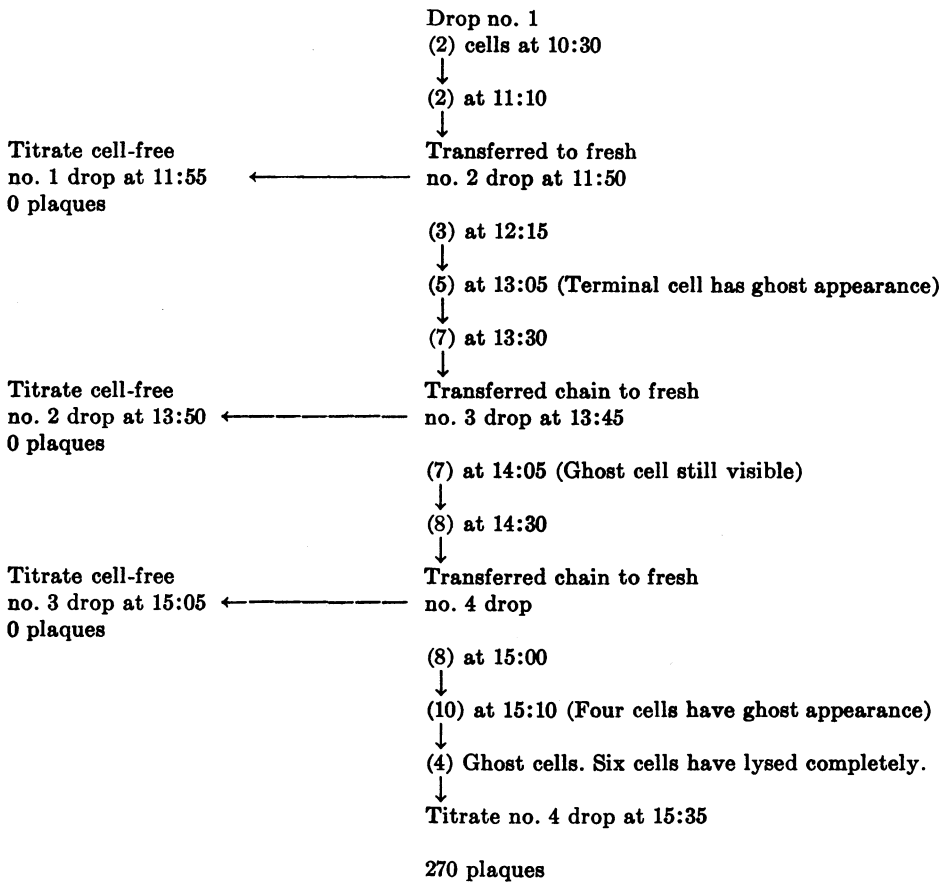

Figure 3. The release of bacteriophage by *Bacillus megaterium*

was accomplished by removing the microdrop in which the cells had been growing with a sterile micropipette and expelling the microdrop onto an agar plate seeded with the *B. megaterium* indicator strain.

The results of all single *cell* growth observations are summarized in table 2. Although 24 individual isolations were conducted with single *spores*, and a total of about 325 cells observed, lysis was not noted, nor was bacteriophage found in any case. Figures 2 and 3 present the details of experiments in which bacteriophage was demonstrated.

DISCUSSION

It is apparent from examination of table 2 that the release of bacteriophage by the lysogenic *B. megaterium* is accompanied by the lysis of certain cells of the culture. The division and growth of more than 550 cells were followed, and in no case was free bacteriophage found except when a cell lysed.

The results shown in figure 2, taken from an experiment in which lysis was observed, demonstrate that cells which are going to lyse, designated susceptibles, are capable of division, even though they are "infected." The formation of the susceptible cells probably occurred in microdrop number 2 at 12:50. At this time there were 9 cells in the drop, in two chains of 4 and 5 cells each. All 4 cells in one chain must have been susceptibles at this time since they and all of their progeny eventually lysed and released virus. Presumably at least one of the cells in the chain of 5 cells was also a susceptible since some, but not all, of these cells or their progeny eventually lysed and released virus.

From the data presented in figures 2 and 3 it is possible to calculate an average burst size for the susceptible cells in a culture of the lysogenic *B. megaterium* which undergo lysis.

From figure 2 it can be seen that a total of 16 cells lysed, releasing over 745 virus particles. From figure 3 it can be seen that a total of 6 cells released 270 virus particles.

$$\text{Average burst size} = \frac{\text{Total virus} = \frac{745}{16} = 46.5}{\text{Total cells lysing} = \frac{270}{6} = 45}$$

A rate of formation of these susceptible cells could now be calculated if the number of daughter cells which one susceptible cell can form, before lysis of all ensues, was known.

The data of figures 2 and 3 are not sufficient to clarify this point with any degree of accuracy, but a very rough order of magnitude may be calculated. From figure 2, it is apparent that one susceptible cell gave rise to 16 progeny before lysis of all ensued. In figure 3, it is seen that another susceptible cell gave rise to 6 progeny before lysis occurred. A mean of 11 susceptible cells, arising from one original, is thus obtained.

Assuming 10 susceptible cells as the average, the rate of formation of susceptibles from the normally resistant parent can now be calculated.

The bacteriophage/bacteria ratio was shown to be in the order of 10/1. The burst size was shown to be about 50. Each susceptible is assumed to form about 10 dependents, also susceptible, before lysis occurs.

Therefore, each susceptible, in effect, gives rise to 500 bacteriophage units (10 cells $\times$ 50 virus particles). Since the bacteriophage/bacteria ratio is about 10/1, one susceptible (S) must be formed for every 50 resistant (R) cells of the parent culture. In other terms:

100 R cells → 2 S
2 S → 20 S before lysis occurs.
20 S × 50 (burst size) = 1,000 virus particles.

Thus, the bacteriophage/bacteria ratio of 10/1 is obtained by dividing the total bacteriophage released by the total cells of the culture.

The conclusion arrived at, then, is that a culture of *B. megaterium* growing under the conditions here used has a reversion rate to sensitivity, to its lysogenic virus. This rate of one cell in 50 or 2 per cent is very high, if indeed one is here dealing with a genetic change which leads to susceptibility, and it may be that the "induction" theory of Lwoff and Gutmann is more tenable. Admittedly these computations are based on few experimental data. Yet, it seems conclusive that the free bacteriophage in this culture is the result of lysis of certain cells. With regard to this point, our experiments confirm those of Lwoff and Gutmann (1949*a*,*b*,*c*; 1950*a*,*b*). Our calculated burst size is based on few observations, but it agrees reasonably well with that of 74 obtained by the preceding authors. Our calculation of the number of progeny which the original susceptible cell can form before lysis of all ensues, may be based on a false assumption—if one exists. It may well be that one susceptible may continue to divide throughout the growth cycle of the culture. The generation time of the bacterial cell may be slightly less than that of the virus, so that lysis does not overtake all susceptibles until the growth of the culture slows down. When this occurs, all susceptibles would be lysed and only resistant cells would remain to sporulate, incorporating the virus into the spore. Our data, however, indicate that such is not the case since lysis of all susceptible cells always ensued before very long, leaving a sterile microdrop.

Since the majority of cells in *B. megaterium* carry bacteriophage or its precursor and liberate none into the medium, we suggest that the term "lysophoric" be applied to them, reserving the usual term "lysogenic" to the culture as a whole or to those cells which do give rise to free virus, and in so doing, perish.

SUMMARY

The growth of the lysogenic *Bacillus megaterium*, strain 899, in a trypticase-yeast extract broth is paralleled by an increase in free bacteriophage throughout the logarithmic growth phase of the organism.

The bacteriophage/bacteria ratio in a culture growing under these conditions is approximately 13/1.

Bacteriophage release by the lysogenic *B. megaterium* is correlated with the lysis of certain cells in the culture.

The release of bacteriophage is not continuous but occurs in "bursts." Those cells which undergo lysis are designated susceptibles, and evidence is presented which indicates that they arise at a regular rate under the conditions employed here.

The results of Lwoff and Gutmann, with regard to bacteriophage release by this culture, are confirmed.

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