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

II. The Growth of Bacillus megaterium in Synthetic Medium

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It has been reported by Wahl (1946) that the bacteriophages of the lysogenic *Bacillus megaterium*, strain 899, could not lyse a sensitive *B. megaterium* in the absence of Ca^{++}. He stated, however, that the lysogenic *B. megaterium*, grown in a Ca^{++}-free synthetic medium containing sodium citrate as a carbon source, still released bacteriophage during its growth. The conclusions drawn were that bacteriophage multiplication and cell lysis were two separate phenomena, and that the bacteriophage found in lysogenic *B. megaterium* cultures is released by a method other than lysis.

Wahl (1946) did not, however, conduct adsorption experiments in a Ca^{++}-free medium, and it was felt that Ca^{++} might be an adsorption cofactor for 899 bacteriophage. If this were the case, it would follow that plaques would not be formed on the indicator strain in the absence of Ca^{++}. The lysogenic *B. megaterium* apparently transmits its bacteriophage internally at division (Lwoff and Gutmann, 1949*c;* Clarke and Cowles, 1952), therefore, precluding the necessity of adsorption as part of the virus multiplication cycle. To test the hypothesis that Ca^{++} was an adsorption cofactor, experiments with *B. megaterium*, strain PR3E, and 899 bacteriophage were conducted. Preliminary experiments confirmed Wahl's findings (1946) that 899 bacteriophage would not form plaques in the absence of Ca^{++}.

MATERIALS AND METHODS

All materials and methods are identical with those described in a previous paper (Clarke and Cowles, 1952). The synthetic medium of Wahl (1946) consisted of 3 g sodium citrate, 2 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, and 1,000 ml distilled water. The pH was adjusted to 7.3 to 7.4 with NaOH.

I. *The effects of Ca^{++} on adsorption of B. megaterium bacteriophage. B. megaterium*, strain PR3E, cells were removed from an 18-hour trypticase-yeast extract slant and washed 3 times in cold saline. Sufficient cells were then added to tubes containing 5.0 ml of the test medium to give a final titer of 2×10^7 cells per ml. One-tenth ml of broth containing 1,300 virus particles was then added to each tube. Control tubes contained only the test medium and virus. Adsorp-

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tion was allowed to proceed for 30 minutes at 5 C. The tubes were then centrifuged in the cold for 10 minutes and the supernate titered for bacteriophage. The results are presented in table 1.

These results demonstrated the finding that Ca^{++} was not an adsorption cofactor and that the Ca^{++}-free media tested did not inactivate the bacteriophage of *B. megaterium*. It became necessary, therefore, to seek another explanation for the failure of this bacteriophage to multiply, in the absence of Ca^{++}, on the sensitive *B. megaterium* strains.

II. *The growth of B. megaterium in* Ca^{++}*-free synthetic medium. B. megaterium* has been shown to release bacteriophage by the lysis of certain cells in the culture (Lwoff and Gutmann, 1950*a;* Clarke and Cowles, 1952), and also to release virus in a synthetic Ca^{++}-free medium (Wahl, 1946). Ca^{++}, then, cannot be required for cell lysis or adsorption and is postulated to be required for the penetration of the virus into the host cell.

It was believed that if the lysogenic *B. megaterium* was grown in a Ca^{++}-free medium the bacteriophage would eventually be eliminated from the culture. It has been shown (Lwoff and Gutmann, 1950*a;* Clarke and Cowles, 1952) that a susceptible *B. megaterium*, strain 899, cell was capable of division although infected by bacteriophage. Experiments in this laboratory have shown it to be possible to obtain nonlysogenic sensitive cells from rapidly multiplying lysogenic *B. megaterium* cultures by random picking of well isolated colonies. In isolating such sensitive variants it proved necessary to eliminate as much as possible of the free bacteriophage in the culture by centrifugation and washing. It may be noted here that the reversion of a lysogenic cell to a nonlysogenic sensitive cell must occur at a fairly high rate in rapidly multiplying cultures, otherwise it would be difficult to demonstrate such cells by plating techniques. Such a reversion is probably a two step process; a *lysogenic* resistant cell reverts to a *nonlysogenic* resistant cell, which then changes to a nonlysogenic *sensitive* cell. In this respect the phenomenon of lysogenicity is analogous to the phenomenon described by Preer (1948) for *kappa*. This worker has demonstrated that rapid division of *kappa*-containing paramecium results in the host paramecium losing *kappa* and becoming sensitive to paramecin.

TABLE 1

Adsorption of 899 bacteriophage to Bacillus megaterium

TESTING MEDIUM	PER CENT BACTERIOPHAGE ADSORBED	PER CENT SURVIVAL BACTERIOPHAGE (CONTROL TUBES)
Trypticase-yeast extract broth	95	100
Wahl's citrate medium	100	100
Trypticase-yeast extract broth + 1 per cent sodium citrate	95	100

In a Ca^{++}-free medium the nonlysogenic sensitive cells would probably be protected from lysis by the free bacteriophage present, since Ca^{++} is postulated to be necessary for the penetration of the virus into the host cell.

If the rate of formation of such cells is as high as it appears to be, the passage of the lysogenic *B. megaterium* in a Ca^{++}-free medium should result in a culture which shows a slow, progressive drop in free virus, since the nonlysogenic cells would gradually replace the lysogenic cells. As Delbrück (1946) has pointed out, the resistant cells in a lysogenic culture may persist only in the presence of the selective action of the bacteriophage.

A preliminary experiment was set up to test this hypothesis. The lysogenic *B. megaterium* from a broth culture was inoculated into 10 ml of Wahl's (1946) Ca^{++}-free synthetic medium. The culture was continually aerated and transfers were made every 48 hours, using 0.2 ml for each transfer. The cultures were checked on the 5th, 15th, and 25th passage for lysogenicity and were still producing bacteriophage. They were not checked again until the 61st passage at which time no demonstrable virus could be found in the culture filtrate. The 61st passage material was plated onto trypticase-yeast extract agar and 5 of the resulting colonies selected at random. All 5 colonies were nonlysogenic and sensitive to all *B. megaterium* phages tested against them.

The experiment was repeated using the same procedures. Titrations of the bacteriophage were made at intervals during the passage of the culture. Before titration, each culture was adjusted to a reading of 180 ± 10 on a Klett-Summerson photometer. The results of this experiment showed that a slow progressive drop in free bacteriophage occurred as the culture was passed in synthetic Ca^{++}-free medium. The initial bacteriophage titer was 9×10^5 per ml, on the 40th passage the titer had fallen to 8×10^4 per ml, and on the 71st passage the titer of free bacteriophage was 4×10^2 per ml, a reduction of about 99.95 per cent.

Lwoff (1951) has recently repeated this experiment and confirmed the findings. In his laboratory, passage of *B. megaterium*, strain 899, in a synthetic medium containing M/125 oxalic acid resulted in a loss of lysogenicity on the 34th passage. The nonlysogenic strain thus obtained proved sensitive to the parent strain's bacteriophage.

DISCUSSION

The results obtained in these studies confirm the findings of Lwoff and Gutmann (1949*a*,*b*,*c*; 1950*a*,*b*), that the free bacteriophage found in cultures of *B. megaterium* arises from the lysis of a limited number of cells of the culture. There can be little doubt that the bacteriophage is passed from cell to cell at division, and in all probability multiplies along with the cell. Our own work does not eliminate the possibility that during cell division there is a "dilution" of internal bacteriophage, so that after sufficient cell divisions the internal virus is lost from a cell, and reinfection by free virus liberated from other cells becomes possible. However, Lwoff and Gutmann (1950*b*) have followed one cell through 19 divisions, never observing lysis or demonstrating free bacteriophage. The cells of the 19th generation were all lysogenic. By simple calculation they point out that it would have required over 500,000 bacteriophage units to be present in the original cell if each daughter cell were to receive only one unit at division and still remain lysogenic. On a volume basis they calculate that a single *B. megaterium* cell could contain only 66,000 bacteriophage units. The internal virus,

called *probacteriophage* by Lwoff and Gutmann (1950*b*), must multiply during cell division.

The *probacteriophage* is apparently noninfective since Cowles (1950) has been unable to demonstrate it by rupture of lysogenic cells by a wide variety of methods. Yet, in certain cells in a lysogenic culture the *probacteriophage* literally "comes to life" and multiplies to form fully infective and active bacteriophage. Doermann (1948) has reported his inability to demonstrate active bacteriophage in *Escherichia coli* cells infected with T3 bacteriophage until about the mid-point of the normal latent period; and Cowles (1949) was unable to detect active bacteriophage by disrupting infected sensitive cells of *B. megaterium* by a wide variety of methods. However, after the mid-point of the latent period, lysozyme lysis freed increasing amounts of active virus. It may well be that the *probacteriophage* is in the "latent" state into which bacteriophage enters immediately after infecting a cell.

Cohen (1949) reported that during the first 7 minutes after infection of *E. coli* cells with bacteriophage only protein is synthesized. Seven minutes after infection desoxyribose nucleic acid synthesis begins and is four times as rapid as in normal cells. In comparing the rate of desoxyribose nucleic acid synthesis with intracellular bacteriophage synthesis, Cohen (1949) has demonstrated that desoxyribose nucleic acid synthesis occurs several minutes before the completion of intact virus, and therefore, the laying down of desoxyribose nucleic acid alone does not complete the virus. Presumably the *probacteriophage* may be a protein-desoxyribose nucleic acid complex or consist of protein alone. The report of Ehrlich and Watson (1949) states that lysogenic *B. megaterium* cells may be recognized by their high desoxyribose nucleic acid content, and this is presumably due to internal virus. The work of these authors should be repeated with *B. megaterium*, strain 899.

One of the most interesting aspects of the problem of lysogenicity is the question of what upsets the balance between the host cell and *probacteriophage*, and allows the *probacteriophage* to develop into active virus. Lwoff and Gutmann (1949*a,b,c;* 1950*a,b*) have demonstrated, as we have, that lysis of single cells in the lysogenic *B. megaterium* does not occur. Lysis has always been observed in groups of cells. Lwoff *et al.* (1950*c*) in a series of interesting experiments have demonstrated that it is possible to alter the percentage of cells releasing bacteriophage by altering the environment of the culture or by irradiation. On the basis of their experiments, an "activation" hypothesis has been developed to explain the conversion of *probacteriophage* to bacteriophage.

Our experiments indicate that the phenomenon of lysogenicity is the result of resistant cells reverting to sensitive cells at a fairly high rate, ca. 2 per cent (Clarke and Cowles, 1951). The factors involved in initiating this reversion are not brought out by our experiments. With such a high rate of reversion to sensitivity, the resistant cells could easily be outgrown by the sensitive forms, were the latter not selectively destroyed by bacteriophage. Thus, elimination of the bacteriophage should result in a completely sensitive culture after a short time. In the present report, elimination of the bacteriophage was successful and the

resulting culture proved to be sensitive to the original bacteriophage. It also seems significant that of the many variant colonies isolated from the lysogenic *B. megaterium* in this laboratory, none has proven to be *nonlysogenic* and *resistant.* A loss of lysogenicity always has resulted in the development of a colony of sensitive cells. Also significant seems the observation that certain cells of the culture do not liberate as much virus as most cells in the culture. For example one isolate, although liberating normal bacteriophage in broth cultures, yields less than 100 bacteriophage units per ml under optimum growth conditions. The lysogenic parent strain under the same conditions produces as much as 10^9 bacteriophage units per ml. Since the two bacteriophages are apparently identical, the differences must lie in the natural reversion rate to sensitivity of the two cultures. Wide differences in the free bacteriophage titers of other lysogenic strains have been reported (Cowles, 1950; Rountree, 1949).

The *probacteriophage,* which can apparently multiply, along with the cell, causing no damage, seems to represent an ideal state of parasitism. Indeed, if the lysogenic bacteria did not occasionally revert to sensitivity and allow the *probacteriophage* to form active virus, the culture would never be recognized, by present methods, as being infected by a virus. One would be dealing with a true saprophytic virus which would be indistinguishable from normal cell constituents.

SUMMARY

Passage of the lysogenic *B. megaterium,* strain 899, in a synthetic Ca^{++}-free medium results in a loss of lysogenicity.

A loss of lysogenicity results in the culture reverting to sensitivity to its original bacteriophage.

The phenomenon of lysogenicity is discussed.

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