LYSOGENESIS OF BACILLUS MEGATHERIUM[1]

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It has been questioned for a long time whether a virus-infected cell is capable of multiplying with that virus present internally; or, in other words, whether the infected cell is capable of carrying on its own metabolic functions simultaneously with those required for virus synthesis. Such a state is called lysogenesis and has been doubted by many workers. For instance, Demerec and Fano (1945) and Delbrück (1946) thought that lysogenesis in bacteria-bacterial-virus systems is more apparent than real. In reality, they thought that a lysogenic culture was one in which most cells were virus-resistant mutants. However, a few mutants were constantly changing back to virus-susceptible cells. Any phage carried along externally would infect these cells and multiply.

We have found that cells of *Bacillus megatherium* can multiply with virus present within them, and that such infected cells are resistant to lysis by external virus. We were able also to show that, by adding glucose to a medium relatively rich in nitrogen, we could rid the infected cell of its internal virus and thus return it to its susceptible state.

MATERIALS AND METHODS

In this work *B. megatherium* strain 1 (University of Toronto, Canada) and megatherium phage φ 16 B (University of Toronto, Canada) were used. Vegetative, lysogenic cultures were developed from spores experimentally infected by essentially the methods of Den Dooren de Jong (1931) and Cowles (1931). Infected spores were heat-shocked at 85 C and germinated in tryptone beef extract broth. The germinate was plated on nutrient agar, and a mottled colony, assumed to be lysogenic by the criteria of De Jong (1931), was isolated and cultured on an agar medium of tryptone and beef extract. Such a culture has been carried along in stock and used for subsequent experiments.

All media used in these experiments contained 3 g Difco beef extract and 5 g Difco tryptone in 1,000 ml distilled water. Glucose and agar were added in the required amounts when needed.

To show that our lysogenic strain produced virus, equal amounts (0.1 or 0.2 ml) of a serially diluted suspension of a 24-hour lysogenic culture were put on the surface of each nutrient agar plate and spread by rotating the plate. These plates were incubated at 30 C. Virus titrations were made of the inoculum and

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of the growth on the nutrient agar plates by the method of Hershey (1943). To assay the plates for external virus, the growth of each was suspended in 5 ml sterile broth; the cells were then removed by centrifugation, and the supernatant was titered.

Since some virus-infected cells have been shown to stain more basophilically than uninfected cells, we wished to show cytochemically that the lysogenic cells contained internal virus by adapting for this purpose the nuclear staining technique of Robinow (1942) as modified by Beumer and Quersin (1947). A lysogenic culture on plain tryptone was suspended in distilled water and autoclaved for 20 minutes at 15 pounds pressure. One-half ml of the autoclaved culture was treated with one-half of a solution of crystalline ribonuclease (Armour) in a water bath at 45 C for 1 hour to remove the gram-positive layer from the cell surface (Bartholomew and Umbreit, 1944). The enzyme solution contained 0.1 mg enzyme per ml. Three loopfuls of this digest were put on a slide, air-dried, and fixed in the vapors of 2 per cent osmic acid for 2 minutes. After thorough washing under tap water, the preparation was treated in 4 N HCl at 56 C for 20 minutes and washed under running tap water for at least 5 minutes. Then the preparation was stained for 1 hour with Giemsa solution (Eimer and Amend) and diluted to 2.5 per cent with distilled water. The slides were subsequently washed under tap water, dried, and examined under oil immersion. In all experiments a control of an uninfected culture of *B. megatherium* was run simultaneously with the lysogenic culture. This technique has been shown to be specific for desoxyribonucleic acid (DRNA) by Vendrely and Lipardy (1946) and Tulasne and Vendrely (1947).

Cohen (1948) has shown that bacterial cells infected with virus are richer in DRNA than are uninfected cells. Consequently we attempted to show chemically that virus was present internally in the lysogenic cells by performing a DRNA analysis. A lysogenic culture and an uninfected culture on nutrient agar were harvested separately in distilled water at about 14 hours. The suspensions were autoclaved for 20 minutes at 15 pounds pressure to inactivate all enzymes and coagulate the proteins. The cultures were then washed three times in distilled water and lyophilized. After the dry weight of the lyophilized cultures had been determined, they were suspended in 5 per cent trichloroacetic acid to make a 0.5 per cent suspension. To extract the DRNA, the suspensions were then heated to 90 C for 15 minutes, allowing 1 minute for coming up to temperature. Dilutions of standard DRNA (Nutritional Biochemicals Company) in 5 per cent trichloroacetic acid were simultaneously heated to 90 C for 15 minutes. The suspensions were then spun down and known volumes of supernatant analyzed for DRNA by the Dische method (1930). The percentage of DRNA in the dry weight of the bacterial cells was calculated from the formula:

$$\frac{\text{mg per ml of DRNA in supernatant}}{\text{5 mg per ml bacterial cells in suspension}} \times 100$$

The DRNA content in the standard DRNA solution was determined from micro-Kjeldahl nitrogen. According to the nitrogen value given by Cohen (1948),

16.9 mg of nitrogen are contained in 100 mg DRNA, the DRNA concentration in the standard solution being calculated from this value.

To show the effect of glucose on the lysogenic strain, cultures were inoculated onto nutrient agar containing 1 per cent glucose and analyzed after 14 hours by the cytochemical and chemical techniques just described. Plain nutrient agar slants inoculated with the lysogenic strain were analyzed simultaneously with the others.

RESULTS

The lysogenic culture, which we isolated, produced virus during its growth, as shown in table 1. No increase in external virus occurred after the second day.

The cytochemical test showed that the lysogenic culture contained DRNA distributed throughout the cell, but the uninfected cell contained DRNA only in a more central region. The lysogenic cultures stained an even purple, whereas the uninfected cells showed a purple-staining, oval, central body surrounded by

TABLE 1

Virus production of lysogenic culture on solid medium

DAY OF OBSERVATION	VIRUS TITER PER ML OF SUSPENSION AFTER WASHING OFF CULTURE*
Initial	4×10^5
1	2.4×10^5
2	2.1×10^7
3	6.8×10^6
4	5.1×10^6
5	8.3×10^6

* In a constant volume (5 ml) of suspension.

pink-staining material. Electron micrographs of 14-hour-old autoclaved cells showed that the pink-staining area was not a capsule but an integral part of the cell structure, since this material contained distinct cross walls. Lysogenic cells were not distinguishable from uninfected cells in the electron micrographs.

Chemically it was possible to show that the lysogenic cells contained about two times as much DRNA as the uninfected cells (table 2). That a higher than normal DRNA content is indicative of virus synthesis in the bacterial cell can be inferred from the work of Cohen (1948).

If the infected cells were exposed to the usual nutrient agar medium that contained 1 per cent anhydrous glucose (Mallinckrodt), staining after 14 hours of exposure showed a complete loss of internal virus. The cells showed the same cytological picture as the uninfected cells. Suitable controls were run in these experiments.

Chemical analyses of lysogenic cultures exposed to 1 per cent glucose for 14 hours gave similar results. A loss of DRNA to about one-half the initial value was obtained. The results are listed in table 2.

We were able to show that glucose does not act selectively for mutants of

susceptible cells in an otherwise resistant culture. By plating a lysogenic culture by the spread technique on ordinary nutrient medium and nutrient medium containing 1 per cent glucose, the same number of colonies was obtained in both cases (table 3). A far smaller number of colonies should have been obtained on the glucose medium if it had selective action.

Glucose does not inhibit lysis of uninfected cells by its virus. If such cells, previously exposed to 1 per cent glucose in two successive transfers, are exposed

TABLE 2

Percentage of DRNA in dry uninfected cells, lysogenic cells, and recovered cells of B. megatherium

EXPERIMENT	PERCENTAGE OF DRNA IN DRIED CELLS		
	Uninfected cells	Infected cells	Recovered cells
A	1.5	3.0	—
B	1.1	2.3	—
C*	—	2.9	1.4
D†	—	2.4	1.7

* Cells 15 hours old at time of harvest.
† Cells 17 hours old at time of harvest.

TABLE 3

Test for the selective effect of glucose on the lysogenic culture

EXPERIMENT	DILUTION	NO. OF COLONIES ON 1.0 PER CENT GLUCOSE AGAR	NO. OF COLONIES ON PLAIN NUTRIENT AGAR
A	0.2×10^{-3}	110	130
B	0.1×10^{-3}	536	258

TABLE 4

Effect of glucose on lysis of a normal culture of B. megatherium by phage

EXPERIMENT	VIRUS TITER ON PLAIN NUTRIENT MEDIUM	VIRUS TITER ON NUTRIENT MEDIUM WITH 1.0 PER CENT GLUCOSE*
A	1.7×10^7	1.3×10^7
B	1.3×10^7	1.3×10^7

* Susceptible cells previously exposed to 1.0 per cent glucose; glucose added to all media used in the titration.

to virus in the presence of glucose and titered by the Hershey method, approximately the same number of plaques are obtained as without glucose (table 4).

A lysogenic culture of *B. megatherium* can be distinguished from an uninfected culture on nutrient medium by its ground-glass appearance and iridescence in transmitted light. The uninfected culture is opaque under these conditions. The lysogenic cells are arranged in parallel in a colony, but the uninfected cells are arranged in criss-cross fashion. Glucose in the medium changes the ground-glass

appearance of the lysogenic culture into opaque growth. If a lysogenic culture is exposed to glucose, the resultant culture when mixed with stock virus will again lyse.

DISCUSSION

These experiments indicate that cells of *B. megatherium* can actually multiply with phage present within them and that such infected cells are resistant. This observation makes it possible to interpret differently the findings of other workers, who explained lysogenesis on a genetic basis.

It was possible to demonstrate that lysogenic cells when grown on a nutrient medium containing glucose would lose their internal virus. These conclusions were drawn from analyses of the DRNA content of uninfected and infected as well as lysogenic cells exposed to glucose. The DRNA content of lysogenic cells before exposure to glucose was decidedly higher than that of uninfected cells or that of lysogenic cells after exposure to glucose. The return of susceptibility was concurrent with a disappearance of external virus. In other words, no more virus was synthesized by the susceptible cells.

The effect of glucose may be a metabolic one since there was a loss of internal virus within 15 hours. If the pH of the medium during growth is the key factor, the lysogenic cells must be very sensitive to slight pH changes on the acid side. The pH of the glucose medium falls to about 6.2 in 1 to 2 days. It should also be mentioned that a glucose concentration of 0.1 per cent does not eliminate the lysogenic state. Further work is being done in an attempt to elucidate this problem.

The resistance of lysogenic cells to lysis by external phage can be explained by the interference phenomenon, discovered for bacteriophages by Delbrück and Luria (1942).

SUMMARY

Evidence for true lysogenesis in *Bacillus megatherium*, strain 1, with megatherium phage φ 16 B has been accumulated. Cytochemical and chemical data were obtained to clarify this point.

Glucose, added to the normal medium, was shown to render virus-resistant, lysogenic cells susceptible to the virus with which they were originally infected. Such cells showed a decrease in desoxyribonucleic acid after exposure to glucose. Glucose does not prevent lysis of susceptible cells exposed to virus.

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