BACTERIOPHAGE GROWTH IN PROTOPLASTS OF *BACILLUS MEGATERIUM**

by

SYDNEY BRENNER** AND GUNTHER S. STENT

Virus Laboratory, University of California, Berkeley, Calif. (U.S.A.)

Treatment with lysozyme of suspensions of *Bacillus megaterium* in 0.2 *M* sucrose solution converts the bacterial cells into spherical protoplasts. These protoplasts are bounded by a membrane which, no longer supported by the rigid cell wall, is readily disrupted by any reduction in the osmotic pressure of the suspending medium. The protoplasts constitute 80% of the cell mass and preserve some of the physiological properties of intact bacteria, but they are incapable of colony formation[1]. It is the purpose of this report to show that such protoplasts are capable of supporting bacteriophage growth. SALTON AND MCQUILLEN have independently also demonstrated bacteriophage formation in protoplasts and, by mutual agreement, the results of the two investigations have been submitted simultaneously.

MATERIALS AND METHODS

Bacterium and phage. Strain KM of *Bacillus megaterium* and the *megaterium* bacteriophage, strain C, active on KM were obtained from the Department of Bacteriology, University of California, Berkeley.

Asparagine-tris buffer medium. 1 g L-asparagine; 10 ml 1.0 *M* tris (tris-hydroxymethylaminomethane) buffer, pH 7.2; 10 ml salts (a solution of 54 g NaCl, 30 g KCl, 0.1 m*M* $CaCl_2$, 1.0 m*M* $MgCl_2$ in 1000 ml of distilled water); 2.2 mg KH_2PO_4; 2.3 mg Na_2SO_4; 80 ml distilled water.

Sucrose-buffer. 0.2 *M* sucrose in 0.03 *M* phosphate buffer, pH 7.0.

Sucrose-buffer-peptone. 2 g bacto-peptone DIFCO in 100 ml sucrose-buffer.

Bacteriophage assay. The double layer plating method[2] was used; 2.5 ml of 5% peptone, 0.7% agar containing the phage and bacteria was poured on the surface of plates containing 5% peptone, 2% agar.

EXPERIMENTS AND RESULTS

It was reported by WEIBULL[1] that protoplasts of *B. megaterium* do not adsorb a bacteriophage active on the intact cells, indicating that the lysozyme-sensitive cell wall is necessary for phage fixation. This is complemented by the observation that such bacteriophage *is* readily adsorbed to isolated *B. megaterium* cell walls[3]. In order to examine the possibility of bacteriophage growth in protoplasts, therefore, it is necessary first to infect the intact bacteria and then convert the infected cells into protoplasts by lysozyme treatment in sucrose. Phage development can then be followed in a one-step growth experiment, such as described below.

* This investigation was supported by grants from the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service, Lederle Laboratories, and the Rockefeller Foundation.

** Fellow of the Carnegie Corporation of New York. Present address: Department of Physiology, University of the Witwatersrand Medical School, Johannesburg, South Africa.

References p. 475.

A culture of *B. megaterium*, strain KM, was grown in asparagine-tris buffer medium to a density of $5 \cdot 10^7$ cells per ml, centrifuged and resuspended in half of its original volume of 5% peptone water. The culture was then infected with about $2 \cdot 10^6$ phages of strain C per ml. After incubation for 5 minutes at 37° C, more than 90% of the phages were adsorbed. The culture was centrifuged, washed and resuspended in sucrose-buffer. One aliquot of this suspension was digested at room temperature with 10 μg per ml lysozyme. Microscopic examination indicated protoplast formation to be complete after 30 minutes. A second aliquot of the suspension of infected cells remained untreated. At time $t = 0$, both aliquots were diluted one-thousand fold into sucrose-buffer-peptone maintained at 25° C, and sampled and assayed periodically for infective centres.

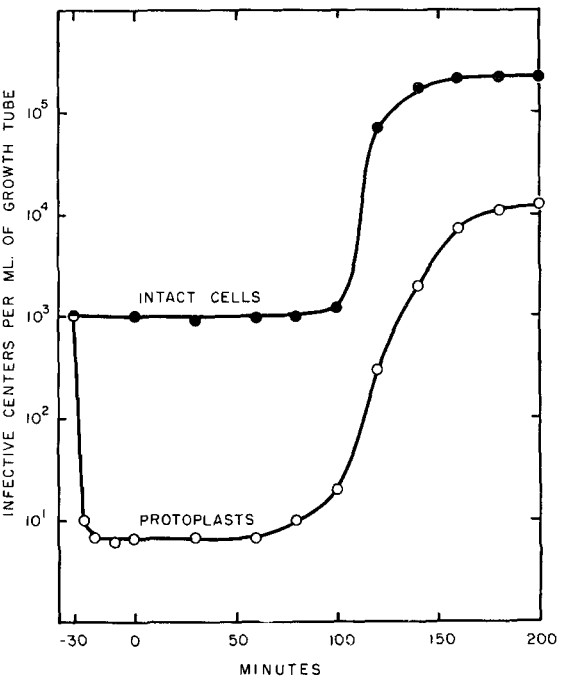

Fig. 1. One-step growth curves of intact cells of *B. megaterium* and of protoplasts infected with phage C at 25° C. Lysozyme treatment for protoplast formation commenced at $t = -30$ min. Phage development was initiated at $t = 0$ min.

The results of this experiment are presented in Fig. 1. It is seen, first of all, that the untreated infected bacteria exhibit a normal one-step growth curve, with latent period of 90 minutes and final burst size of 230. Lysozyme treatment of the infected cells, on the other hand, rapidly reduces the number of infective centres to less than 1% of its intitial level. (The surviving infective centres appear to be free phage, which had been either liberated by lysozyme treatment or carried over in the washings.) Sixty minutes after dilution of the infected protoplasts into the sucrose-buffer-peptone, however, a rapid rise in the number of infective centres commences until a level is reached fourteen times greater than the original number of infected cells. The initial decrease of the number of infective centres in the lysozyme-treated suspension must be due to the fact that the phage had entered the eclipse period[4] of intracellular virus development and that the instantaneous lysis of the protoplasts upon plating prevented any further maturation of the vegetative phage. The subsequent increase in infective centres is a reflection of the intracellular growth and maturation of the vegetative phage in the protoplasts maintained in sucrose-buffer-peptone.

A single burst experiment[5] was carried out in order to examine whether the phage ultimately produced in the lysozyme-treated suspension represented a small burst issuing from the majority or a very large burst issuing from a small minority of the infected protoplasts. In this experiment, an average of one infected protoplast suspended in sucrose-buffer-peptone was distributed into each of forty tubes and phage growth was allowed to take place for 4 hours at 25° C. The total contents of each tube were then

References p. 475.

assayed for infective centres. The result of this experiment is presented in Table I, where it may be seen that at least half of the infected protoplasts do produce phage and that the individual bursts are small.

The small burst size of the phage-infected protoplasts is probably due to an aggravation of the well-known fragility of *intact* bacteria at the conclusion of the eclipse period[4,6], thus terminating phage synthesis prematurely by spontaneous lysis shortly after the intracellular appearance of the first mature progeny phages. Microscopic examination reveals that *uninfected* protoplasts remain intact for up to six hours under the same experimental conditions.

TABLE I

SINGLE BURST EXPERIMENT ON INFECTED PROTOPLASTS

Number of plaques	*Number of tubes*
0	24
1	5
2	2
3	4
5	1
12	2
23	1
25	1

Fraction of tubes without burst: 24/40 = 0.60; Multiplicity of bursts per tube: $-\ln 0.60 = 0.51$; Multiplicity of infected protoplasts per tube: 1.0; Fraction of protoplasts yielding burst: 0.51/1.0 = 0.51.

B. megaterium protoplasts, therefore, preserve sufficient structural and functional integrity to be capable of carrying out all those reactions subsequent to invasion by the parental phage which are necessary for growth and maturation of infective progeny. There is presumptive evidence[7,8] that high molecular weight substances like enzymes, to which intact bacterial cells are impermeable, may be able to penetrate into protoplasts. The possibilities of influencing intracellular bacteriophage development through addition of specific macromolecular substances to protoplast suspensions are being explored.

SUMMARY

One-step growth experiments show that bacteriophage C can grow in protoplasts of *B. megaterium* KM if the cells are infected prior to treatment with lysozyme. The phage yield of infected protoplasts is smaller that that of intact cells, and a single burst experiment establishes that the burst size of individual protoplasts is small.

RÉSUMÉ

Des expériences de multiplication à cycle unique montrent que le bactériophage C peut croître dans les protoplastes de *B. megaterium* KM si les cellules sont infectées avant le traitement par le lysozyme. Le rendement en phage des protoplastes infectés est plus petit que celui des cellules intactes et une expérience de production individuelle établit que le rendement moyen des protoplastes individuels est faible.

ZUSAMMENFASSUNG

Fortpflanzungsversuche in einem einzigen Zyklus beweisen, dass sich der Bakteriophage C in Protoplasten von *B. megaterium* KM entwickeln kann, falls die Zellen vor der Lysozymbehandlung infiziert werden. Die Phagenausbeute aus infizierten Protoplasten ist geringer, als aus unversehrten Zellen, und ein individueller Produktionsversuch ergibt, dass die Durchschnittsausbeute der einzelnen Protoplasten gering ist.

REFERENCES

1 C. WEIBULL, *J. Bacteriol.*, 66 (1953) 688.
2 M. H. ADAMS, *Methods of Medical Research*, Vol. 2, The Year Book Publishers, Chicago, 1950.
3 M. R. J. SALTON AND G. S. STENT, unpublished results.
4 A. H. DOERMANN, *J. Gen. Physiol.*, 35 (1952) 645.
5 M. DELBRÜCK, *J. Bacteriol.*, 50 (1945) 131.
6 O. MAALØE, *Acta Path. Scand.*, 17 (1950) 680.
7 R. L. LESTER, *J. Am. Chem. Soc.*, 75 (1953) 5448.
8 M. BELJANSKI, *Biochim. Biophys. Acta*, 15 (1954) 425.

Received January 27th, 1955