Large-Scale Production of Lambda Bacteriophage and Purified Lambda Deoxyribonucleic Acid

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Large amounts of a heat-inducible phage lambda mutant (λC1857) may be obtained under standardized conditions. The phage is harvested by simple polylethylene glycol (C 6000) precipitation and purified by CsCl density gradient banding. Deoxyribonucleic acid (DNA) is extracted by cold phenol and purified by sucrose density gradient sedimentation to yield a homogenous population of unbroken λ DNA molecules.

Escherichia coli 3102 (λC1857), a heat-inducible lysogen, was first isolated by Sussman and Jacob (7). A virulent phage growth cycle was found to be induced at temperatures above 40 C due to thermal lability of the phage repressor. With the phage repressor functional at lower temperatures, the lysogenic E. coli cells can be grown at temperatures 30 to 35 C. When, however, the growing culture is exposed to a temperature near 45 C even for a brief period of time (e.g., 10 min), the repressor is inactivated, and phage development is induced. To obtain maximal yields of phage in the lysate, some authors (4) recommend that the temperature be lowered again after induction (to 35–37 C) and the growth be continued until a clear lysate is obtained. Different procedures were described to achieve the necessary temperature adjustments (4, 6).

Wherever large volumes of sterile media are used, the steps required for instantaneous temperature adjustments become increasingly difficult. We here describe a feasible process for large-scale phage production which would require a minimum of attention and handling when applied in the laboratory or fermentation plant.

We also describe a modification of phage purification and deoxyribonucleic acid (DNA) extraction which proved convenient for harvesting homogenous native λ DNA in quantity.

EXPERIMENTAL

Bacteria. E. coli 3102 (λC1857) was a gift from C. Richardson, Harvard University School of Medicine, Cambridge, Mass.

E. coli C-600 was used as indicator strain for plaque assays.

Media. Two types of liquid media were used for bacterial culture growth: (i) a modified Vogel-Bonner medium (8) and (ii) 1% tryptone broth.

Stock solutions of Vogel-Bonner medium (medium 1) were prepared as follows. Solution A consisted of: water, 670 ml; MgSO4·7H2O, 10 g; H3C6H5O7·H2O (citric acid), 100 g; KH2PO4, anhydrous, 500 g; and Na2HPO4·4H2O, 175 g. Solution B consisted of: water, 100 ml; CoSO4, 130 mg; H3BO3, 290 mg; (NH4)2MoO4, 750 mg; FeCl3, 480 mg; MnCl2, 280 mg; CuCl2, 270 mg; and ZnCl2, 2,000 mg. To 10 ml of solution A was added 5 ml of solution B, 100 ml of 25% glycerol in water, and 10 g of Casamino Acids, and water was added up to 1 liter. The medium was sterilized, and, after cooling, 1 ml of 0.5 M CaCl2 and 5 ml of 0.5 M MgCl2 were added under sterile conditions.

Tryptone-yeast extract broth (1% Difco Laboratories, Detroit, Mich.) was made to 0.5% in NaCl and 0.5% in MgSO4. This medium (medium 2) was used in a series of growth experiments as well as for lysate dilution in phage assays.

Phage assays. The technique described by Adams (1) was used for the determination of phage lambda titers in lysates and suspensions. Agar (1.1 and 0.7%) in Tryptone broth (medium 2) was used for the bottom and top layers in 9-cm petri dishes, respectively.

The indicator-strain bacterial culture was prepared as follows. An overnight culture (incubated at 37 C) of E. coli C-600 in medium 2 was centrifuged (5,000 rev/min.), and the sedimeted cells were suspended in 0.02 M MgSO4, to one-half of the original volume.

A cell suspension (0.3 ml) was incubated for 15 min at 36 C with 0.1 ml of lysate or phage suspension of the proper dilution. After addition of 2.5 ml of 0.7% agar, the mixture was poured over the bottom agar layer in a petri dish and incubated at 37 C.

Phage growth; preparation of inocula. (i) E. coli 3102 (λC1857) was passaged for several generations on suitable indicator strains, and the culture was adjusted to 108 and 109 cells per ml. The culture was centrifuged, and the cell suspension was used as the initial inoculum for the production steps. The cell density was checked by plating on indicator strains. E. coli C-600 was used as the indicator strain. (ii) E. coli C-600 lysates were prepared according to the following procedure:

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Phage growth; preparation of inocula. (i) E.
coli 3102 (ACI857) cells, from a slant culture kept in the cold, were suspended in liquid medium and streaked on bottom agar (1.1%). After overnight incubation at 32 °C, lysogenic colonies were easily identified by their transparent concentric rings and smooth edges. One such colony was suspended in tryptone broth (medium 2) and streaked again on agar. The next day, the colonies were harvested, re-suspended in liquid medium to a desired optical density at 550 nm (OD550) (see Table 1) according to the volume to be inoculated, and used for inoculation.

(ii) An overnight culture of E. coli 3102 (ACI857) was grown at 32 °C from a single colony. After spinning down, the cells were twice suspended and sedimented from 0.9% aqueous NaCl (isotonic saline). Finally, the cells were suspended in 50% glycerol, diluted to proper OD100, distributed to samples of several milliliters, and kept at −15 °C. These samples, when used as inocula, yield reproducible results even after prolonged storage. Table 2 shows the cell survival after storage for periods up to four months.

Cell growth and lysis. Cell growth and lysis in a laboratory scale were as follows. At the time of inoculation, the medium (10 liters in a 20-liter glass bottle) was prewarmed to 40 ± 0.5 °C and was kept at this temperature throughout the experiment with vigorous aeration (approximately 15 liters of air per 1 min per 10 liters of medium via sintered glass spargers). After lysis, detected by a drop in OD100 (Fig. 1), 0.1% chloroform was added to the lysate, and aeration was continued for another 10 min in an ice bath. Viable cell count was usually in the order of 10^7 to 10^8 cells/ml which is a good indication of complete lysis (Fig. 2).

Cell growth and lysis in a fermentation plant (10-liter fermentors) were as follows. It was found that increased or decreased aeration affected the rate of culture growth but did not show any substantial effect on the phage yields in the lysate. This is interpreted to mean that, even with aeration on laboratory scale (i.e., lower than in fermentors), the medium is saturated with oxygen.

To eliminate heavy foam formation, Antifoam (Antifoam A. Spray, Dow Corning Co.) was always added to the growing culture.

Lysate processing. During the last 10 min of aeration (after 1 ml of CHCl3 was added per liter of lysate), crystalline NaCl was added to make the lysate

| Days of storage | Bacterial colonies per ml | Per cent survival | PFU/ml |
|----------------|--------------------------|-------------------|--------|
| 0              | 3 x 10^4                 | 100               | 3 x 10^4 |
| 60             | 1.7 x 10^4               | 57                | 4 x 10^4 |
| 120            | 6 x 10^3                 | 20                | 1.2 x 10^4 |

* The decrease in viable cell count is at least partly due to spontaneous lysis as manifested by the increasing plaque-forming unit (PFU) count with time.

**Table 1. Effect of inoculum size on phage yield**

| Inoculum (OD138) | Growth (hr) | Max OD138 | OD100 of lysate | Lysate (PFU/ml) |
|------------------|-------------| ---------|----------------|-----------------|
| 0.50             | 15          | 0.225    | 0.000          | 11              |
| 1.00             | 14          | 0.145    | 0.005          | 1               |
| 1.50             | 15          | 0.148    | 0.004          | 1               |
| 3.60             | 13          | 0.115    | 0.014          | 4               |
| 4.65             | 12          | 0.105    | 0.023          | 0.3             |
| 5.45             | 13          | 0.083    | 0.000          | 3               |
| 6.5              | 11          | 0.087    | 0.037          | 0.4             |
| 7.5              | 12          | 0.041    | 0.003          | 1.2             |
| 8.2              | 11          | 0.105    | 0.000          | 3.7             |

* Yields of phage from 10-liter lysates prepared in fermentors. Escherichia coli 3102 (ACI857) cells were employed at differing optical densities for use in 1.0-ml amounts as inocula. Except for very small inocula, the phage yields do not show any apparent dependence on the number of bacteria used for inoculation.

* Plaque-forming units.
0.5 M in NaCl. When NaCl was completely dissolved, 120 g of Polyethylene Glycol 6000 (Fisher Scientific Co.) was added per liter of lysate. After 2 hr in the cold, the precipitate was pelleted by sedimentation at 10,000 rev/min for 20 min, with almost 100% recovery of the phage. After gentle but thorough suspension of the pellet in 1:100 of the original lysate volume of tris(hydroxymethyl)aminomethane (Tris) buffer (per liter of buffer: 4 M NaCl, 4.3 M; 1% gelatin, 10 ml; 1 M Tris, 10 ml, pH 7.4) most of the cell debris was removed by centrifugation at 5,000 rev/min for 10 min. The phage suspension was then adjusted to density 1.5 by adding crystalline CsCl (biochemical grade; Harshaw Chemical Co.). It was found easy and precise to adjust the refractive index of the suspension to the value of 1.381 with an Abbe refractometer. The phage was banded at 40,000 rev/min for 24 hr at 4 C (Spinco type 60Ti rotor). After collection of the phage band, it was rebanded once more in CsCl. The second banding is very important because only in this way were phage lambda preparations of high purity obtained in 50 to 85% yields (Table 3).

To save the rather expensive high-purity CsCl, and time as well, we modified the first purification step in the following ways. Polycarbonate screw-cap tubes [1 by 3.5 inch (2.54 by 8.89 cm), Spinco type 30 rotor], were almost filled with phage suspension (~33 ml) in Tris buffer. An amount (5 ml) of saturated solution of CsCl in Tris buffer (density approximately 1.94) was carefully pipetted to the bottom to form an underlayer. The tubes were placed in the rotor and spun for 1 hr at 23,000 rev/min. This was found to be sufficient to obtain a distinct phage band in the CsCl solution where, by diffusion, the density equilibrated to 1.5. The phage bands from all tubes were easily picked up by a Pasteur pipette. After pooling and minor readjustment of the density to 1.5 (controlled by refractive index reading), the phage suspension was banded for the second time as described above. (For data on recoveries see Table 3.) In this way, a phage suspension was obtained, the purity of which was checked in the electron microscope.

We found that for prolonged storage it was best to keep the phage in CsCl solution (density ~1.5) where it showed better survival (Fig. 3) than in 0.014 M NaCl, 0.01 M Tris, and 0.01% gelatine, pH 7.4.$

**DNA extraction.** For the extraction of DNA from phage λ, we used the standard cold-phenol method. To approximately 5 ml of phage in 0.017 M NaCl, 0.01 M Tris, and 0.01% gelatin (pH 7.4, OD_{260} between 8.0 and 10.0) was added an equal volume of freshly distilled phenol. To reduce the possibility of oxidative attack possibly resulting in DNA breakage, the phenol was distilled in the presence of Zn powder and in a N\textsubscript{2} atmosphere and was collected under 1 M Tris, pH 7.4, prior to use.

The phage suspension and phenol layers were rotated at approximately 30 rev/min at room temperature (no difference was found between room temperature and 4 C) for 1 hr. The 13-ml conical, glass test tube in which the mixture was rotated was then put in a clinical centrifuge and spun at 3,000 rev/min for 10 min to facilitate separation of the two liquid phases. The phenolic (lower) phase was then carefully removed with a Pasteur pipette and discarded.

**Table 3.** Yields of highly purified phage in 10 representative experiments

| Lot | PFU/ml (× 10^{11}) | Lystrate | Total PFU | Total 2nd CsCl banding | Phage yield* |
|-----|----------------|----------|-----------|------------------------|--------------|
| 1   | 1.6           | 2.5      | 4 × 10^{11} | 54.6                   | 72           |
| 2   | 1.5           | 3.0      | 4.5 × 10^{11} | 53.6                   | 66           |
| 3   | 2.6           | 4.0      | 1.04 × 10^{14} | 158.9                  | 84           |
| 4   | 3.3           | 2.8      | 9.24 × 10^{11} | 118                    | 70           |
| 5   | 1.4           | 3.6      | 5.04 × 10^{13} | 47.4                   | 51           |
| 6   | 1.7           | 3.4      | 5.78 × 10^{13} | 80.6                   | 76           |
| 7   | 2.9           | 3.3      | 9.6 × 10^{11}  | 124                    | 71           |
| 8   | 1.8           | 3.5      | 6.3 × 10^{11}  | 73                     | 63           |
| 9   | 2.7           | 3.0      | 8.1 × 10^{11}  | 78                     | 53           |
| 10  | 2.1           | 4.0      | 8.4 × 10^{11}  | 110                    | 72           |

* Plaque-forming units.

* Phage yield expressed as percent recovery based on total phage in the crude lysate.
A fresh portion of phenol was then added. This extraction step was repeated three times with rotation times reduced to 20 min the second and third time.

After 36 hr of dialysis against three exchanges of 2-liter batches of 1.0 M NaCl, 0.01 M Tris, and 10^{-4} M ethylenediaminetetraacetic acid (EDTA), pH 7.4, (to remove phenol), the DNA was retrieved from the dialysis bag with a wide-bore Pasteur pipette (to avoid shear breakage of the DNA) usually with a \( \sim 50\% \) yield of the original OD_{260} of the phage suspension.

**DNA purification.** To remove fragmented DNA of unknown provenance, the specimen obtained by phenol extraction had to be purified by sucrose density gradient sedimentation.

Cellulose-nitrate tubes (38.5 ml each; Spinco SW 27 rotor) were filled with 38 ml of 5 to 20% (Biochemical grade) sucrose density gradient in 1 M NaCl, 0.01 M Tris, and 10^{-4} M EDTA, pH 7.4. On top of the \(-4\) C cold gradient, 0.5 ml of heated and rapidly cooled DNA solution of not more than 0.6 OD_{260}/ml was carefully overlaid. The heating and rapid cooling disintegrates linear aggregates formed by hydrogen bonding between the complementary single-stranded "sticky" ends of \( \lambda \) DNA molecules.

After centrifugation at 18,000 rev/min for 18 hr at 4 C, the content of the test tube was collected (1 drop/second) in 1-ml fractions from the top of the test tube through a 1-mm inner diameter tubing with an Isco density fractionator equipped with a ultraviolet (UV) analyzer (Instrument Specialties Company, Inc., Lincoln, Nebr.). A characteristic UV absorption curve is shown in Fig. 4. The non-fragmented \( \lambda \) DNA was found usually in a sharp band containing 20 to 30\% of the original OD input on top of the gradient. The effect of the sucrose density gradient purification step became obvious when DNA samples before and after purification were examined in the electron microscope (Fig. 5) by the surface film technique (3).

The sucrose was removed by dialysis against an excess amount of 0.01 M NaCl, 10^{-4} M Tris, and 10^{-5} M EDTA to allow 100-fold concentration of the very dilute DNA solution obtained from the sucrose density gradient band after dialysis. In this way, solutions of final concentration from 3 to 5 OD_{260}/ml could be obtained. They contained homologous monodisperse population of unbroken \( \lambda \) DNA molecules.

**DISCUSSION**

The induction of virulent phage growth of *E. coli* 3102 (\( \lambda \)Cl857) requires temperatures in the range of 40 C to inactivate a heat-labile repressor.

Normally, this is done by growing bacteria at a permissive temperature to reach a desired cell population density and by a subsequent shift to high temperature where complete lysis ensues.

Since it is difficult to achieve the required temperature changes on a scale larger than normal in the laboratory, we were gratified to note that operation at a constant temperature—intermediate between normal growth and induction—gave rise to reproducibly high phage yields.

It is possible to interpret this observation in the following manner. There is a spontaneous and constant release of phage particles from a small proportion of cells in lysogenic culture even at permissive temperatures (5). As the temperature is raised, this proportion increases by heat inactivation of an increasing fraction of repressors (7). At the same time, growth of the surviving lysogenic bacteria becomes more vigorous, and certain equilibrium is reached.

Evidently, at 40 C, which is below the inducing temperature for massive lysis (45 C), the accumulation of lysogenic bacteria and free phage particles as a function of time permits

**FIG. 3.** Purified phage lambda survival in CsCl (density 1.5 in Tris buffer, pH 7.4) and Tris buffer (0.01 M Tris, 0.014 M NaCl, and 0.01\% gelatin).

**FIG. 4.** UV (260 nm) tracing of sucrose density gradient containing \( \lambda \) DNA molecules. Duration, 18 hr; speed, 18,000 rev/min; temperature, 4 C; rotor, Spinco SW27. 1, meniscus; 2, fragmented DNA; 3, linear \( \lambda \) DNA; 4, circular \( \lambda \) DNA.
an appropriate coexistence of the two processes so that large phage yields can be obtained.

After a certain period of time, a ratio of free phage particles to bacterial cells is reached where superinfection becomes predominant, resulting in the lysis of practically all the cells.

The immunity breakdown due to superinfection (2) delayed until considerable bacterial growth has occurred contributes to the large phage yield in the terminal lysates.

In our hands, the method described above yielded good results with sufficient reproducibility. In conclusion, we would like to point out a few technical details which, according to our experiences, proved to be important: (i) because of small inocula and long growth periods, absolute sterility is the basic requirement. (ii) Inocula should be periodically tested for viable cell counts to inoculate the medium always with the same amount of cells per liter of medium. (iii) Nonlysogens represent a contaminant whose presence would decrease the phage yields because of possible phage adsorption to the cells. Their colonies can be detected in the inocula by their characteristic appearance. Inocula containing nonlysogens should be discarded. (iv) The temperature fluctuations in the growing culture should not exceed 1°C. In this procedure, lysates with phage titers favorably comparable to other methods can be obtained with little attendance and within a predictable period of time. Conveniently, most of the growth can take place overnight.

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