APPLIED MICROBIOLOGY, Oct. 1968, p. 1483-1488

Vol. 16, No. 10
Printed in U.S.A.

Production of Bacteriophage T7

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Received for publication 3 June 1968

Bacteriophage T7 was grown with *Escherichia coli* B as the host organism in 3- and 20-liter vessels. Under the best growth conditions devised, the yields of T7 in the culture lysates averaged 1.33×10^{12} and 0.95×10^{12} plaque-forming units per ml, respectively, compared with the best previously reported yields of 10^{11} to 3×10^{11} plaque-forming units per ml in 1-liter batches grown in the presence of air, or double this in similar batches grown in the presence of oxygen. The bacteriophage was purified by a simple method which gave average yields of 143 mg/liter and 131 mg/liter from the 3- and 20-liter batches, respectively. The efficiency of plating of the final material ranged from 18 to 42%. The purified bacteriophage is a convenient source of monodisperse deoxyribonucleic acid, the molecular weight of which is about 25×10^6.

Bacteriophage T7, which is virulent for *Escherichia coli* B, was grown by Lunan and Sinsheimer (10) in 1-liter batches, in the glycerol-Casamino Acids medium of Frazer and Jerrel (8). Cultures of the host organism were grown to a density of 10^9/ml, treated with T7 at an input ratio of 1 bacteriophage to 10 bacteria, and aerated for a further 2 to 3 hr; foam was controlled with Dow-Corning antifoam A. The culture lysates so obtained had titers in the range 10^{11} to 3×10^{11} plaque-forming units (PFU)/ml, and the average value for 14 batches was 2.43×10^{11} PFU/ml. The crude product was purified by a series of centrifugation steps, and pure T7 was recovered in 41% overall yield, equivalent to 10^{11} PFU/ml of culture lysate. No estimate of the efficiency of plating of the pure product was given.

Lunan and Sinsheimer (10) used their purified bacteriophage suspension to prepare the monodisperse deoxyribonucleic acid (DNA). They recovered DNA equivalent to 4.9 mg/liter of culture and claimed a 77% recovery of the DNA present in the purified bacteriophage. If we accept the statement of Davison and Freifelder (3) that T7 contains 50% (by weight) of DNA, we may deduce that purified T7 equivalent to $(4.9 \times 100)/77 \times 100/50 = 12.7$ mg/liter of culture was obtained. We may go on to deduce the plating efficiency of the purified T7 suspension from the particle mass of 3.8×10^7 daltons given by Davison and Freifelder (3), Avogadro's number, 6.02×10^{23}, and the plaque count of 10^{14} PFU recovered per liter of culture. From these data, the mass of purified bacteriophage was $(10^{14} \times 3.8 \times 10^7 \times 10^8)/(6.02 \times 10^{23}) = 6.3$ mg/liter of culture. Therefore, the efficiency of plating was $6.3/12.7 \times 100 = 50\%$.

Davison and Freifelder (3) grew T7 in the same way and on the same scale. They reported that the bacteriophage yield was doubled when pure oxygen was bubbled through the culture in place of air. They devised a simpler method of purification in which the key step was the use of cesium chloride density gradient ultracentrifugation. They did not state the recovery of T7 in the purification process, but, if we assume that their extraction was as effective as that of the earlier workers, they would have obtained 25.4 mg of T7 per liter from cultures grown in the presence of pure oxygen. They did report, as a result of ultraviolet absorbance measurements at 260 nm, that, at most, 25% of the particles in their purified suspensions were infective.

The objective of the present work was to devise an improved method for the production and isolation of T7.

MATERIALS AND METHODS

Cultures. The host organism, *E. coli* B/R, and a sample of bacteriophage T7 were kindly provided by A. Loveless of the Chester Beatty Research Institute.

Bacteriophage seeds. The original sample of bacteriophage contained only 5.8×10^7 PFU. This was used as seed for a 3-liter culture, and the progeny so obtained were used as seed in all subsequent 3-liter cultures. The 20-liter experiments were all infected with bacteriophage T7 derived from one of these 3-liter cultures. By avoiding unnecessary serial passage, we sought to limit possible changes in the bacteriophage.

Assays. Glycerol was determined by the periodate oxidation method (9). Bacterial dry weights were determined by heating a sample of wet cells at 160 C to constant weight in an infrared oven as described by Ford (7). Carbon dioxide gas analysis was carried out by using either a type SB/H infrared gas analyzer (Infra Red Development Co. Ltd., Welwyn Garden City, Herts) as described by Telling, Elsworth, and East (12), or a Cambridge analyzer based on a katherometer power supply unit (Cambridge Instrument Co. Ltd., London, S.W.1). Bacteriophage counts were made by the agar layer-plaque-assay technique described by Adams (1). The soft agar contained: NaCl, 1.0%; agar no. 3 (Oxoid), 0.65%; tryptone L.42 (Oxoid), 1.0%; yeast extract (Oxoid), 0.5%. The firm agar contained a tryptic digest of beef. The host bacteria were grown in a medium having the same composition as the soft agar, but containing 0.1% glucose instead of agar.

Growth conditions. All cultures, including bacterial seeds, were grown at 37 C generally as described by Sargeant and Yeo (11). Particular attention was paid to following the methods given for avoiding the premature contamination of cultures with bacteriophage. Medium 2 was used exclusively in the 3-liter and 20-liter cultures. The medium for 3-liter cultures was sterilized separately in a 4-liter bottle and added, at room temperature, to the sterilized (free steam, 4 hr) vessel. The medium for 20-liter cultures was sterilized in the culture vessel (15 lb/in^2, 20 min). Antifoam was either a 25% solution, in demineralized water, of silicone MS antifoam emulsion RD (Hopkin and Williams Ltd., Chadwell Heath, Essex) sterilized in a 1-liter bottle or silicone MS antifoam A (Hopkin and Williams) added from a sterilized grease gun. In all cases, except where stated otherwise, "sterilized" means maintained in thermal equilibrium with steam at 20 lb/in^2 for 30 min.

The 3-liter culture vessel was previously described (11); the 20-liter vessel was used with a modified impeller having the following dimensions (original values in parentheses): A, 4 inches (3 inches); B, 1-3/16 inches (1 inch); C, 5/8 inch (5/8 inch); D, 1/8 inch (0 inch). The flow rate of sterile air used in each vessel was one culture volume per min. This gave a sulfite oxidation rate in the 3-liter vessel, as measured by the method of Elsworth, Williams, and Harris-Smith (6), of 220 mmoles of oxygen per liter per hr. The sulfite oxidation rate attained in the modified 20-liter system was not measured, but was probably just over 220 mmoles of oxygen per liter per hr.

For each vessel, a preliminary culture of the host organism was grown, and the carbon dioxide concentration in the culture effluent gas was monitored continuously and later correlated with the viable bacteria counts determined by plating. Thus, in cultures which were to be infected with bacteriophage, carbon dioxide gas effluent analysis was used as a guide to the bacterial cell density in the culture.

After bacteriophage infection, cultural conditions were maintained for 3 hr and antifoam was added as necessary.

Centrifugation. Centrifugation of culture lysates, to give clarified supernatant fluids by the removal of bacterial debris, was done at *p*H 7.0 (adjusted with 2 N NaOH) for 30 min at 13,000 $\times$ *g* in the 6- by 250-ml rotor of an MSE 18 refrigerated centrifuge (Measuring & Scientific Equipment, Ltd., London) at 4 C, or in batches of 20 liters at a flow rate of 300 ml/min in a deLaval model 3000 centrifuge.

No special effort was made to cool the 20-liter batches of culture lysate rapidly. The clarified supernatant fluid was stored in a cold room held at 4 C, and subsequent operations were conducted at this temperature. No appreciable fall in plaque count occurred during 7 days of storage at 4 C.

Isolation of bacteriophage T7 was by centrifugation for 30 min at 105,000 $\times$ *g* in the no. 30 rotor of a Spinco model L ultracentrifuge or at 109,000 $\times$ *g* in the 8 $\times$ 50 ml rotor of an MSE 65 centrifuge. In either case, the same set of tubes was repeatedly filled and centrifuged until 1- to 2.4-liter lots of clarified supernatant fluid had been processed.

The sediment was removed from the tubes with a spatula, transferred to a stoppered flask containing 60 to 70 ml of 0.5 M sodium acetate per 0.001 M magnesium chloride, and stirred mechanically for about 2 hr. Less severe methods of resuspending the sediment were tried, for example, allowing it to stand covered with solvent overnight. Presumably because of the large amount of material involved, no obvious improvement in the resuspension process was achieved. Mechanical stirring was continued until an homogeneous suspension was obtained. At this stage, loss of biological activity rarely exceeded 10%.

The suspension (80 g) was added to 60 g of cesium chloride, and the mixture was distributed between the three buckets of a Spinco SW 25 rotor. Centrifuging for 24 hr at 73,000 $\times$ *g* was sufficient to separate the T7 band cleanly near the middle of the tubes, but, after this short time, the wax-like pad which formed at the top of the tubes was fragile and not easy to remove. After centrifuging for 72 hr, this pad was much more compact and could be very easily removed with a spatula. The band of T7 was removed from the tubes by pipette, diluted, and dialyzed against several changes of the sodium acetate-magnesium chloride solvent.

Characterization of T7 suspensions. The total of bacteriophage T7 in the final solutions was estimated from absorption measurements at 260 nm. Corrections for scattered light were applied in the manner described by Davison and Freifelder (3). The correction amounted to about 10% at 260 nm. The number of phage particles present in final suspensions was calculated on the basis that unit absorbance at 260 nm was equivalent to 1.5 $\times$ 10^{12} phage/ml. The plating efficiencies were then calculated from the plaque counts.

In a few cases, the concentration of phage was also calculated from assays of the DNA content of the final suspensions. For these assays, phage suspensions were heated in 10% perchloric acid at 70 C for 20 min, and DNA was determined by the diphenylamine method as modified by Burton (2). The number of phage present was calculated taking 1 mg of DNA as equivalent to 3.2 $\times$ 10^{13} phage. The results obtained were in reason-

able agreement with those found by the absorption method.

The mass of bacteriophage in the final suspension was in each case calculated, taking 1 mg of phage as equivalent to 1.6×10^{13} particles. This factor and those used in the two DNA assays are based on the observations of Davison and Freifelder (3) that the molecular weight of the phage is 38×10^6, that it contains 50% DNA, and that for the phage suspensions the mean extinction coefficient per mole of phosphorus, $\sum (P) = 7{,}300$.

More recent estimates give the molecular weight of T7 DNA as 25×10^6 (13) instead of 19×10^6 used above. Taking this new value for the molecular weight would reduce the estimates of the number of phage particles per milliliter by 24%, and the plating efficiencies reported in the penultimate column of Table 3 would be increased.

RESULTS

Carbon dioxide evolution figures. The peak concentrations of CO_2 in the culture effluent gas from cultures of the host organism were in the range 3.5 to 4.5% for 3-liter cultures, and 4.0 to 5.0% for 20-liter cultures. The differences in the peak CO_2 concentrations observed in each group were at least partly due to the different amounts of antifoam required by different cultures. It is nevertheless clear that the use of the larger impeller improved the oxygen solution rate in the 20-liter vessel to a value slightly higher than that in the 3-liter vessel.

Growth of T7 in 3-liter vessels. The results are summarized in Table 1. The first culture, which was inoculated at the very low virus-cell input ratio of 4.7×10^{-6}:1 gave a lysate which was used as inoculum for the remaining 3-liter cultures. The inoculation was carried out at a cell density of 4.1×10^9/ml, when the CO_2 concentration in the culture effluent gas was 1.6%. The CO_2 concentration reached a peak value of 3.6% 1 hr later, and fell to 0.5% during the subsequent 0.5 hr.

Cultures 2 and 3 were also inoculated at relatively low cell densities, but with much higher phage-cell input ratios. In culture 2, the CO_2 evolution rate remained constant at 0.5% for 20 min after infection and fell rapidly thereafter. In culture 3, it rose from 0.7 to 1.1% during the 20 min following infection and thereafter fell rapidly.

Cultures 4 to 6 were aimed at "superinfecting"

TABLE 1. *Production of bacteriophage T7 in a 3-liter culture vessel*

Culture no.[a]	Viable bacteria at infection	Bacterial dry weight at infection	No. of infective particles per bacterium at infection	No. of infective particles in final culture	No. of infective particles in final culture supernatant fluid	No. of infective particles produced per bacterium
	per ml	*g/liter*		*per ml*	*per ml*	
1	4.1×10^9	0.9	4.7×10^{-6}		4.3×10^{11}	105
2	2.1×10^9	0.7	3.0	1.9×10^{11}	1.3×10^{11}	62
3	2.5×10^9	1.8	0.1	2.2×10^{11}	3.4×10^{11}	136
Avg. 1–3	2.9×10^9				3.0×10^{11}	103
4	9.2×10^9	3.8	13.0	5.8×10^{11}	6.3×10^{11}	68
5	10.5×10^9	—	7.8	6.6×10^{11}	8.3×10^{11}	79
6	13.3×10^9	5.0	10.7	4.0×10^{11}	4.4×10^{11}	33
Avg. 4–6	11.0×10^9				6.3×10^{11}	57
7	14.6×10^9	5.8	0.8	6.6×10^{11}	6.7×10^{11}	46
8	15.5×10^9	7.4	0.9	20.8×10^{11}	16.8×10^{11}	115
9	19.5×10^9	7.5	1.2	14.7×10^{11}	14.4×10^{11}	74
10	22.1×10^9	8.3	0.2	17.2×10^{11}	12.4×10^{11}	56
Avg. 7–10	17.9×10^9		0.8		12.6×10^{11}	70
11	26.9×10^9	8.4	0.4	9.0×10^{11}	9.8×10^{11}	36
12	29.6×10^9	10.8	0.6	15.3×10^{11}	18.2×10^{11}	61
13	30.8×10^9	10.1	0.6	13.5×10^{11}	14.9×10^{11}	48
Avg. 11–13	29.1×10^9		0.5		14.3×10^{11}	49
Avg. 7–13	22.7×10^9		0.7		13.3×10^{11}	59

[a] Cultures 1–3 were inoculated with phage when the cell density was low. For cultures 4–6, a high phage-cell input ratio was used. Cultures 7–10 were inoculated with phage when the cell density was high; no *p*H control was used. Cultures 11–13 were inoculated with phage when the cell density was high; *p*H control used.

TABLE 2. *Production of T7 in a 20-liter culture vessel*[a]

Culture no.	Viable bacteria at infection	Bacterial dry weight at infection	No. of infective particles per bacterium at infection	No. of infective particles in final culture	No. of infective particles in final culture supernatant fluid	No. of infective particles produced per bacterium
	per ml	*g/liter*		*per ml*	*per ml*	
1	25.1×10^9	8.2	0.5	8.2×10^{11}	8.8×10^{11}	35
2	26.9×10^9	9.4	0.7	10.8×10^{11}	13.5×10^{11}	50
3	27.7×10^9	10.6	1.0	11.0×10^{11}	9.0×10^{11}	33
4	35.4×10^9	8.1	0.5	6.2×10^{11}	6.7×10^{11}	19
Avg. 1–4	28.5×10^9		0.7		9.5×10^{11}	33

[a] Cultures were inoculated with phage when the cell density was high. No *p*H control was used.

the cells with bacteriophage. Culture 4 received the bacteriophage seed as a single addition, but cultures 5 and 6 were infected with bacteriophage on two separate occasions. One-sixth of the bacteriophage seed was added first and the remainder was added 10 min later. In all cases the first bacteriophage addition was made about 30 min before the anticipated peak CO_2 evolution rate. In all cases the CO_2 evolution rate was declining rapidly about 20 min after the first addition. The average final yield in these experiments was 57 PFU/cell or 6.3×10^{11} PFU/ml.

Cultures 7 to 10 were inoculated with a single addition of bacteriophage at an average input ratio of 0.8, when the CO_2 evolution rate was judged to have reached its peak value. In all cases the CO_2 evolution rate was declining rapidly about 30 min after inoculation. The average yield of bacteriophage in the culture lysate supernatant fluids was 70 PFU per cell or 12.6×10^{11} PFU/ml.

The *p*H value of cultures 11 to 13 was controlled at 7.0 by the automatic addition of 2 N NaOH, so as to avoid the considerable drop in *p*H value which would otherwise have occurred in reaching very high cell densities. These cultures were inoculated from 30 to 60 min after the CO_2 evolution rate had reached its peak value, with a single addition of bacteriophage giving an average input ratio of 0.5. In all cases the CO_2 evolution rate declined rapidly from about 60 min after inoculation with bacteriophage. The average final yield in the culture lysate supernatant fluids was 49 PFU per cell or 14.3×10^{11} PFU/ml.

Growth of T7 in 20-liter vessels. The results are summarized in Table 2. Cultures 1 and 2 were infected with a single addition of T7 when the CO_2 evolution rate was judged to have just reached its peak value, and cultures 3 and 4 were similarly infected 0.5 hr after the observed peak. In no case was the *p*H controlled. The average input ratio was 0.7 PFU per cell. In cultures 1 and 2, the CO_2 evolution rate fell rapidly from about 30 min after bacteriophage was added; in cultures 3 and 4, the CO_2 evolution rate began to fall rapidly about 60 min after bacteriophage addition.

The average final yield in the culture lysate supernatant fluids was 33 PFU per cell or 9.5×10^{11} PFU/ml.

Isolation of T7. The results obtained with culture lysates derived from a number of 3-liter and 20-liter batches are summarized in Table 3. The average yield of pure T7 was 143 mg/liter from the 3-liter cultures and 131 mg/liter from the 20-liter cultures. The efficiency of plating of the final product was in the range of 18 to 42%.

DISCUSSION

The purpose of the 3-liter cultures was to discover how to produce culture lysates with the maximal T7 concentration by using a typical deep culture vessel. We sought to achieve this by growing bacterial cultures to a high cell density and infecting them with T7 in the hope that the greater the bacterial concentration at infection the greater would be the amount of progeny T7 released. We expected that this would happen only up to a certain cell density, as was the case in the production of bacteriophage $\mu 2$ (11). In that case, for 3-liter cultures infected at cell densities up to about 10^{10}/ml, 3,000 PFU per cell were produced. For cultures infected at higher cell densities, the yield was very much less, probably because some essential nutrient or nutrients became exhausted before complete lysis could occur.

The time from infection to complete lysis in the present system is much less than for the $\mu 2$ system under comparable cultural conditions, and we found that infection of cultures may be delayed until the bacterial density is much higher and satisfactory lysis will still occur. For 3-liter cultures infected with T7 at cell densities up to 3×10^{10}/ml, cell lysis takes place, and the slight decrease in yield of T7 per cell at the higher densities is more than outweighed by the increase in the number of cells present.

There is no evidence that a low multiplicity of initial T7 infection has much influence on the

TABLE 3. *Purification of T7 in culture supernatant fluids*

Experiment no.	No. of infective particles in culture supernatant fluid	Volume of culture supernatant fluid used	No. of infective particles used	No. of infective particles in final product	Percentage of infective particles recovered[a]	Mass of T7 recovered (E_{260nm})	Plating efficiency of final product[b]	Mass of T7 recovered per liter of culture[c]
	per ml	*liters*			%	*mg*	%	*mg*
3-Liter batches								
7	6.7×10^{11}	1.13	7.6×10^{14}	3.4	45	92	37	83
8	16.8×10^{11}	2.0	33.6×10^{14}	20.0	60	300	42	150
9	14.4×10^{11}	2.4	34.6×10^{14}	23.2	67	470	29	190
Avg. of 7–9								143
20-Liter batches								
1	8.8×10^{11}	1.0	8.8×10^{14}			211		211
2	13.5×10^{11}	1.0	13.5×10^{14}	4.4	34	152	18	152
3	9.0×10^{11}	1.0	9.0×10^{14}	2.7	30	89	19	89
4	6.7×10^{11}	1.0	6.7×10^{14}	2.0	30	72	18	72
Avg. of 1–4								131
Earlier work								
Lunan and Sinsheimer (10)	2.43	14.0	3.4	1.4	41	178[d]	50[d]	12.7[d]
Davison and Freifelder (3)	4.86 (O_2)						<25	25.4[d]

[a] Average of the seven experiments was 44%.
[b] Average of the seven experiments was 27%.
[c] Average of the seven experiments was 136.
[d] Estimated.

yield of T7 per cell. Thus, culture 1 (Table 1), which was infected with a very low phage-cell input ratio, gave 105 PFU per cell. Presumably several lytic cycles occurred before all the susceptible bacteria were infected, and during these the uninfected bacteria would have been growing normally. The true yield of T7 per bacterial cell available for infection was less than 105 PFU and was probably near to the average of 59 PFU per cell in cultures 7 to 13, which received an average phage-cell input ratio of 0.7:1.

A high input ratio of T7 per cell does not lead to a significant change in the yield of progeny T7. This is surprising, because the plating assay system used in this work gives plaques which exhibit the phenomenon of lysis inhibition, i.e., there is a clear central zone surrounded by a halo of incomplete lysis. It is believed that halos of this kind are areas in which infected cells, about to lyse, are prevented from doing so by the adherence of extracellular phage to the cell walls. Such cells remain intact beyond the stage of normal lysis and continue to synthesize phage. Doermann (5) obtained enhanced yields of bacteriophages T2, 4, and 6 by the "superinfection" of *E. coli* grown in 25-ml broth cultures.

The 20-liter cultures were grown under conditions intended to simulate the best devised on the 3-liter scale and to give high yields of T7. To this end, the stirrer was fitted with the new impeller, larger than that used for $\mu 2$ production (11), so as to boost the aeration capacity to a value near to that of the 3-liter vessel. In fact, the 20-liter vessel had a slightly greater aeration efficiency than the 3-liter vessel and, as a result, the *p*H value of culture grown in it remained above 6.5 without the addition of NaOH, even when grown to completion.

The average yield of 9.5×10^{11} PFU/ml obtained in the 20-liter culture lysates is not regarded as indicating a significantly different final T7 concentration from that obtained in the 3-liter culture lysates (average of cultures 7 to 13 = 13.3×10^{11} PFU/ml). This conclusion is borne out by the similarity in mass recoveries of T7 from lysates from the two sources.

We conclude that the optimum cell density for infection with T7, at an input ratio close to 1 PFU/cell, is 1.5×10^{10} to 3.0×10^{10} cells per ml. In practical terms this means that bacteriophage infection should be carried out just as the carbon dioxide evolution rate reaches its peak value. Cultures 7 to 10 (Table 1) and 1 to 2 (Table 2) were grown under such conditions. The control

of *p*H value did not confer any significant advantage. These conclusions apply to cultures grown in vessels having sulfite oxidation rates of about 220 mmoles of O_2 per liter per hr.

The number of infective particles obtained per cell in this work was considerably lower than that obtained by Lunan and Sinsheimer (10). At least part of the difference can be accounted for by the low input ratio of 1 PFU per 10 cells used by the earlier workers compared with our input ratio of about 1 PFU per cell. Those cells not infected at first would multiply and provide a higher effective cell density for phage production. Even so, the high overall yields of infective particles reported here were the result of lysing very dense bacterial cultures rather than of obtaining large numbers of bacteriophage per cell.

The method of T7 isolation adopted is not well suited to the manipulation of large volumes but provides a simple and reliable method for preparing a few hundred milligrams of T7. The overall average yield of 136 mg/liter of culture compares favorably with the 12.7 mg/liter which we calculate was obtained by Lunan and Sinsheimer (10), and the 25.4 mg/liter which Davison and Freifelder (3) are presumed to have obtained by using cultures grown in the presence of pure oxygen rather than air.

The 44% average overall recovery of infective particles in the purification was close to the 41% reported by Lunan and Sinsheimer (10), who used a more tedious method for purification. The 27% average plating efficiency of the final product compared favorably with the 50% which we deduced for the product of Lunan and Sinsheimer (10) and the 25% reported by Davison and Freifelder (3).

The final product was a suitable source of monodisperse native DNA. Denaturation of the DNA showed, in agreement with Davison, Freifelder, and Holloway (4), that only about half of the single DNA chains were intact.

The bacteriophage and the DNA isolated from it have been used for studies on the action of mono- and bifunctional alkylating agents.

ACKNOWLEDGMENTS

We thank our colleagues J. W. S. Ford, D. Kitching, and D. Laughton for carrying out chemical and bacteriological laboratory work in support of this project.

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