Properties of the Virulent Form of a Mitomycin C- or Temperature-induced Thermophilic Bacteriophage

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SUMMARY

A virulent bacteriophage was isolated from lysates of *Bacillus stearothermophilus* strain NU-10 induced either by treatment with mitomycin C or by shifts in temperature. Optimum conditions for induction, morphology and other properties of the virus are described. Chloroform treatment and shifts in temperature of producer cells released approximately similar amounts of phage as did mitomycin induction, suggesting an effect on release rather than on synthesis of the virus.

Conflicting reports have appeared concerning the morphology of the thermophilic bacteriophage *φμ*-4. We noted that lysates produced after *φμ*-4 infection of *Bacillus stearothermophilus* strain NU-10 contained phage with a morphology similar to that described by Rabussy *et al.* (1970) and different from that reported initially by Shafia & Thompson (1964). The latter observation stimulated us to study the possible presence of a temperate phage in the host bacterium and its potential for induction when *φμ*-4 infects that host.

*B. stearothermophilus* strain NU-10, which is the only known host for *φμ*-4, was grown at 56 °C with constant shaking at 132 rev/min in a broth medium composed of 2% tryptase supplemented with 0.1% yeast extract and 0.1% glucose. Mid-exponential phase cultures (*A*<sub>600</sub> = 0.2) were treated with various concentrations of mitomycin C (0.01, 0.05, 0.1, 0.5, 1 and 5 μg/ml final concentration) and incubation continued for 6 h or until lysis occurred. Cell lysis was measured as a decrease in absorbance at 600 nm and only concentrations of 0.05 and 0.1 μg/ml mitomycin C induced significant lysis. These data are similar to previous observations made on mitomycin C induction of *B. stearothermophilus* 1503-4R (Welker & Campbell, 1965).

Crude lysates contained mostly phage heads and plaques were not formed on any of the 26 thermophilic or eight mesophilic bacteria tested. However, after the lysate was concentrated 1000-fold, plaques did appear on *B. stearothermophilus* NU-10, the same strain from which the phage was obtained. Plaques were faint on initial observation, but following enrichment of the phage by passing it through NU-10, plaques were large, clear and identical to those produced by *φμ*-4 when plated under similar conditions. Optimum plaque production and morphology occurred after 10 to 18 h incubation at 56 °C in the presence of oxygen. The phage appears to be a virulent mutant of a temperate virus carried by *B. stearothermophilus* strain NU-10 (see below) and has been designated TP-10<sub>μμ</sub>. The only sensitive host obtained so far is strain NU-10 and titres of 10<sup>10</sup> to 10<sup>12</sup> p.f.u./ml have been obtained by several enrichment passages on this host.

Purification was achieved by the ammonium sulphate precipitation method of Shafia & Thompson (1964) for *φμ*-4 or by acid precipitation to the isoelectric point as described by Rabussy *et al.* (1970). Sucrose gradient (10 to 40%, w/v) centrifugation of the phage produced two distinct bands on the gradient column. More than 90% of the p.f.u. were recovered from the lower band, in the 30 to 35% region, after centrifuging at 86 860 g for 75 min. Centrifugation times beyond 75 min resulted in complete sedimentation of the phage.
Electron micrographs of the sucrose-purified phage revealed a morphology strikingly similar to $\phi u-4$ as reported by Rabussy et al. (1970). The head was hexagonal, having a face-to-face measurement of approx. 40 nm and a diam. across the vertices of 50 nm. The tails were long and flexible, measuring about 225 \times 10 \text{ nm}. The tail had cross-striations that occurred at 4-5 nm intervals and there was no evidence of a tail sheath or tail fibres.

The active fraction from sucrose gradient columns was homogeneous and yielded a single band when recycled on sucrose gradients of the same concentrations. However, sucrose-purified phage produced three bands when centrifuged in discontinuous CsCl gradients containing five densities (1.55, 1.46, 1.35, 1.23 and 1.13 g/ml). The lowest band contained approx. 99\% of the p.f.u. Electron micrographs of CsCl-purified TP-10 vi r indicated a loss of structural stability in the presence of CsCl. Heads appeared prolate without evidence of symmetry. Very few heads contained tails and numerous disrupted heads were observed. Free tails and tail-like structures that resembled the reassociated tails noted for Escherichia coli phage $\lambda$ (Bleviss & Easterbrook, 1971; Kuhl & Katsura, 1975) were seen. These structures measured about 20 nm wide and varied from 75 to as much as 1500 nm long.

Other properties exhibited by this phage were relative instability at $75 \degree C$ (98-5\% inactivation in 30 min), stability over a pH range of 4 to 12 and resistance to 100 \mu g/ml trypsin, DNase and RNase. Divalent cations were required for maximum plaque production as shown by significant reductions in p.f.u. titres (30 and 70\% respectively) when 0-5 and 1\% sodium citrate was present in soft agar overlays. Bacteriophage TP-10 vi r contains DNA with a density of 1.706 g/ml in CsCl. Heating the DNA to $100 \degree C$ in neutral CsCl increased the density to 1.713 g/ml but did not separate it into two strands.

A comparison of physical and biological properties of $\phi u-4$ and TP-10 vi r indicate that the two phages are similar but not identical. The most prominent shared characteristics include host range, burst size, morphology, neutralization with phage-specific antisera, sedimentation patterns in sucrose and CsCl gradients, and requirement for divalent cations for plaque formation. In contrast, bacteriophage $\phi u-4$ was stable at $75 \degree C$ for 1 h, was susceptible to trypsin activity as reported by Shafia & Thompson (1964) and contained double-stranded DNA with a density of 1.719 g/ml as previously reported (Rabussy et al., 1970).

During recent phage isolation studies it was noted that broth cultures of B. stearothermophilus strain NU-10 exhibited total or partial lysis and phage release following temperature drops from normal incubation conditions or when shaken with small volumes of chloroform. The rate and extent of lysis varied considerably from one culture to the next and attempts to standardize induction were unsuccessful. Parallel cultures grown from single colony isolates produced similar results during induction, chloroform treatment and infection experiments. Chloroform-mediated lysates of strain NU-10 gave phage titres approaching those from mitomycin C induction ($1 \times 10^3$ versus $1 \times 10^4$ p.f.u./ml) and a 1000- to 10000-fold increase over spontaneous clarified supernatants (35 p.f.u./ml). These titres represent the virulent mutant phage present in the lysates. However, electron microscopy studies of each lysate indicated a much larger phage population. Burst sizes could not be directly determined, although it was evident that a large cell population underwent lysis without phage release.

A closer examination of mitomycin C induction revealed a temperature-sensitive lysis and phage release (Fig. 1). At a drug concentration of 0-1 \mu g/ml total lysis occurred after 90 min incubation, whereas 0-05 \mu g/ml allowed an extended phase of cell growth and lytic resistance. When aliquots were removed to cuvettes at room temperature, a complete drop in turbidity was observed in 10 min giving a temperature at half lysis of approximately 47 \degree C. Removal to $56 \degree C$, without vigorous aeration, caused little change in absorbance during the same time interval, although chloroform addition readily facilitated lysis. Surprisingly, phage titres did not differ significantly under the two mitomycin C concentrations employed indicating that prolonged cell growth is not accompanied by any detectable continuation of phage synthesis.
Short communications

Fig. 1. Effect of temperature on mitomycin C induction of *B. stearothermophilus* strain NU-10. Cultures were grown to an $A_{600}$ of 0.2 at 56 °C at which time 0.05 or 0.1 μg/ml mitomycin C was added. Incubation was continued at 56 °C with aeration (inset graph) or removed to cuvettes at room temperatures (expanded graph) and absorbance monitored. Dotted lines on inset graph indicate time at which samples were removed to room temperature and subsequent lysis noted. O O, 0.05 μg/ml mitomycin C; A A, 0.1 μg/ml mitomycin C.

Microscopic observation of the cells during mitomycin C induction showed a definite filamentous cell formation before the onset of lysis. This is analogous to the findings of Welker & Campbell (1965) in their induction studies with *B. stearothermophilus* strain 1503-4R. Yamagami & Endo (1969) have shown that filamentous *E. coli* produced from treatment with a variety of agents including mitomycin C does not result in a decreased cell wall stability (as judged by resistance to EDTA and lysozyme) but rather appears to enhance membrane fragility or permeability.

It is suggested here that mitomycin C may not be inducing phage synthesis to any large extent but rather providing a means of overcoming a physical barrier by allowing phage release via lysozyme penetration of a weakened cell envelope. It is important to note that mitomycin C at concentrations producing maximum phage yields also resulted in maximum cell lysis. Release of this phage also accompanies a decrease in incubation temperature and could be associated with a conformational change in the membrane. This concept is supported by the observed effect of chloroform which is known to disrupt membranes to the extent that endolysin can gain access to the cell wall. It is also possible that this phage is 'pseudolysogenic' as are several *B. subtilis* phages, such as SP-10 (Bott & Strauss, 1965), but
is defective in lysis or the lysin has an optimum temperature much lower than normal growth temperatures for *B. stearothermophilus*. A lytic enzyme produced by *B. stearothermophilus* NU-10 which has optimum activity at 25 °C was reported by Cole (1973). We have isolated and partially characterized the enzyme, but it is not clear at present whether it is coded by phage genes or by host cell genes.

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**REFERENCES**


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