Characterization of *Vibrio eltor* Typing Phages: Properties of the Eltor Phage e₄

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SUMMARY

Biophysical characteristics of *Vibrio eltor* phage e₄, a key phage in the *Vibrio cholerae* typing scheme were studied. This icosahedral phage was found to contain 12 structural polypeptides with mol. wt. ranging from 25 000 to 120 000. One of these polypeptides of mol. wt. 50 000 accounted for most of the structural proteins present and was probably the major phage capsid protein. The phage genome comprised a single linear, double-stranded DNA molecule, 69.2 kbp in length (45.6 × 10⁶ mol. wt.) as determined by electron microscopy and restriction fragment analyses. The G + C content was 34.6%. Electron microscopy data indicated that unlike the DNAs of other cholera phages, phage e₄ DNA is not circularly permuted. Adsorption under normal conditions was biphasic with rate constants of 1.02 × 10⁻⁹/ml/min up to 60% adsorption and 3 × 10⁻¹⁰/ml/min thereafter. Intracellular phage multiplication was characterized by a latent period of 27 min. The burst size was approximately 100 phage particles per infected cell.

*Vibrio cholerae*, the causative agent of cholera, comprises several biotypes. Of these, *eltor* and the classical biotypes were responsible for all the recent epidemics (Levine *et al.*, 1983). Although these biotypes differ in some biochemical properties (Finkelstein, 1973), they can be distinguished most conveniently on the basis of their susceptibility to different phages (Mukherjee, 1978). A number of phage typing schemes have been proposed (Mukherjee, 1978; Newman & Eisenstark, 1964; Basu & Mukherjee, 1968), the most recent one with 14 phages (Lee & Furniss, 1981). Cholera phages thus have received considerable attention but most of the studies have focused on serological properties and morphology on the basis of electron microscopy. Results have sometimes been conflicting and it was not always clear which phage was being examined (Ackermann *et al.*, 1984). This is due to the lack of information on the physicochemical and physiological properties of most of these phages, with the exception of a few phages infecting classical strains of *V. cholerae* (Chatterjee & Maity, 1984). Data on viral nucleic acid are available for only three phages: φ149 (Sengupta *et al.*, 1985), CP-T1 (Guidolin *et al.*, 1984) and φ138 (Chowdhury & Das, 1986). In our laboratory we have undertaken a programme to characterize physicochemically the different phages used in typing the *eltor* strains (Lee & Furniss, 1981). The results presented here describe some properties of the phage e₄ which specifically infects *eltor* strains.

This phage and its host, *V. eltor* Mak 757 were obtained from National Institute of Cholera and Enteric Diseases, Calcutta. To prepare high titre phage stock, host cells were grown in the medium described by Roy *et al.* (1982). Mid log phase cells (about 5 × 10⁸ cells/ml) were infected with e₄ at a m.o.i. of 0.1 and incubated with shaking until complete lysis occurred. A yield of 8 × 10⁹ to 2 × 10¹⁰ p.f.u./ml was obtained. The phage was concentrated by precipitation with polyethylene glycol 6000 as described by Yamamoto *et al.* (1970) and was subsequently purified...
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by centrifugation (35000 r.p.m. for 1 h; Beckman SW 50.1) in a CsCl density step gradient ranging from 1.3 to 1.7 g/ml. The recovered phage band was dialysed overnight against buffer (10 mM-Tris–HCl pH 8.0, 1 mM-EDTA) and stored at 4 °C.

Phage adsorption to host cells and the one-step growth parameters were studied as described by Adams (1959). Sixty % of phage particles were adsorbed with a rate constant of 1.02 × 10^{-9}/ml/min. Thereafter the process continued at a much lower rate with an adsorption constant of 3 × 10^{-10}/ml/min. No satisfactory explanation is readily available for the biphasic nature of adsorption for any phage studied so far (Feary et al., 1964; Kropinski & Warren, 1970; Schade & Adler, 1967). The possibility that the phage preparations contained a significant population of slowly adsorbing contaminant phage particles was ruled out by the fact that the phage recovered from the supernatant after the first rapid phase of adsorption or phage purified from a single plaque showed identical biphasic adsorption kinetics. This has been found for all cholera phages examined so far (Chatterjee & Maity, 1984). The intracellular development of phage e₄ was characterized by a latent period of 27 min and an eclipse period of 24 min i.e. about 90% of the latent period. In this respect this phage differed significantly from other cholera phages, where the eclipse period is about 60% of the latent period (Chatterjee & Maity, 1984).

The average burst size obtained was around 100 p.f.u.

Cholera phages PL 163/10 and e₄ have been classified as belonging to the family Podoviridae species I on the basis of their morphology (Ackermann et al., 1984). To see whether this similarity in morphology is a reflection of similar structural polypeptides of these phages, phage e₄ was purified by three methods (two successive runs to equilibrium in caesium chloride; step gradient followed by a reverse step gradient (Thomas & Davis, 1975); single step gradient), dissociated by heating in buffer (0.0625 M-Tris–HCl pH 6.8; 2% SDS; 5% 2-mercaptoethanol; 10% glycerol; 0.001% bromophenol blue) for 2 min in a water bath at 100 °C and then analysed by SDS-polyacrylamide gel (7.5%) electrophoresis according to Laemmli (1970). In each case, 12 structural polypeptides with mol. wt. 25000 to 120000 were found (Fig. 1). The major component had a mol. wt. of 50000. Under identical conditions PL 163/10 yielded only four polypeptide components (Chatterjee & Maity, 1984).

The molecular size of e₄ DNA was determined by electron microscopy and restriction fragment analyses. Phage DNA was prepared essentially according to the method described earlier (Ghosh & Guhathakurta, 1983). For electron microscopy, DNA was spread using the formamide method as described by Davis & Hyman (1971). Briefly, the hyperphase contained 50 ng phage DNA, 25 ng of double-stranded φX174 DNA, 5 μg cytochrome c (type V; Sigma) in 100 mM-Na₂EDTA and 44% formamide in a total volume of 50 μl (Kinchington et al., 1984). The DNA was spread at room temperature in a perspex hood on to the hypophase containing 10 mM-Tris–HCl pH 8.5, 1 mM-Na₂EDTA and 14% formamide. The cytochrome film was picked up on a collodium-coated grid (Kleinschmidt, 1968), stained with uranyl acetate in 90% alcohol, rinsed with 90% alcohol, dried in 2-methylbutane, shadowed with gold (40%) and platinum (60%) on a rotating stage, and coated with carbon. The grids were photographed in a Philips 310 electron microscope. Molecules were measured using a Bruhl digitizer interfaced with a DEC 20 computer. The native phage DNA was linear and double-stranded (Fig. 2); by contour length measurement, its size was determined as 69279 ± 839 bp. Double-stranded φX174 DNA, which is known to contain 5386 bp, was used as the internal standard. Restriction

Fig. 1. SDS-polyacrylamide gel electrophoresis of the structural polypeptides of phage e₄. Lysozyme, ovalbumin, bovine serum albumin, phosphorylase B and β-galactosidase were used as mol. wt. markers; sizes are indicated × 10^{-3}. 

120 108 89 78 76 62 50 45 42 37 32.5 25.8
enzyme analyses (see below) yielded a value of 68.9 kbp for e₄ DNA which is in good agreement with the electron microscopy data.

Analysis of DNA restriction fragments is a powerful method for comparing the genomes of different bacteriophages. One to 2 μg of the DNA from phage e₄ was digested with various restriction enzymes under conditions specified by the supplier (New England Biolabs). After incubation, the reaction mixture was heated at 65 °C for 10 min and the restriction fragments were separated by electrophoresis on vertical 1% agarose gel slabs as described by Ghosh et al.
(1985). Bands were visualized by staining with ethidium bromide (1 μg/ml). The gels were photographed with a Fotodyne DNA photography system using a 254 nm wavelength transilluminator. The sizes of the restriction fragments were determined by comparing their mobilities on the gel relative to that of fragments of phage λ DNA digested with HindIII. Digestion of the phage DNA with HaeIII resulted in 15 bands, one of them (band 5 from the top, 3.9 kb) appearing double (Fig. 3a). XbaI yielded 20 bands of which bands 6 (3.6 kb), 7 (2.9 kb) and 8 (2.6 kb), from the top, appeared as doublets (Fig. 3b). However, when a small amount of phage DNA was digested with XbaI and end-labelled according to Downing et al. (1979) before electrophoresis for a longer time followed by autoradiography, two doublet bands of around 3.6 kb in size were evident at the position of band 6 (Fig. 3c). The molecular size of e4 DNA obtained from the sizes of restriction fragments was 68.9 and 69.2 kb for HaeIII and XbaI digests respectively. This size is intermediate between that of φ149 (104 kb) (Sengupta et al. 1985) and those of CP-T1 (43.5 kb) (Guidolin et al., 1984) and phage φ138 (45 kb) (Chowdhury & Das, 1986). The e4 DNA was resistant to PstI, BamHI, BglI and BgII whereas the DNAs of the other three phages were digested by PstI and BamHI.

A thermal transition profile of e4 DNA was obtained using a Zeiss spectrophotometer (VSU 2-P) equipped with a thermospacer cell holder and a MLW type U1 thermostatic water bath.
The cuvette was heated at approximately 1 °C/min to a final temperature of 95 °C. The $T_m$ of the phage DNA was found to be 83.5 °C in 0.15 M-NaCl, 0.015 M-sodium citrate, which corresponds to a G + C content of 34.6 ± 0.1% according to the formula of Marmur & Doty (1962).

The DNAs from three cholera phages, namely CP-T1 (Guidolin et al., 1984), φ149 (Sengupta et al., 1984) and φ138 (Chowdhury & Das, 1986) have been mapped with restriction enzymes. All of these phages contain linear double-stranded DNAs which are circularly permuted and terminally redundant. Such DNA molecules yield faint bands besides the major discrete ones and fail to yield discrete terminal ends upon restriction enzyme digestion. Moreover, when denatured and renatured, double-stranded circular molecules with single-stranded tails are formed. To ascertain whether phage $e_4$ DNA was also circularly permuted, phage DNA was examined under the electron microscope after denaturation and renaturing, essentially according to the method described by Davis et al. (1971). No circular molecules were visible under the above conditions. Moreover, digestion of $e_4$ DNA with various restriction enzymes always gave discrete bands only, and both the terminal fragments could be clearly identified (data not shown). These results clearly indicated that, unlike the other three phages mentioned above, $e_4$ DNA contained only unique sequences.

At present phage typing is still the most powerful method for distinguishing the different biotypes of V. cholerae but it seems that there is some confusion in identifying cholera phages (Ackermann et al., 1984). Because there is little information on their physiological and physicochemical properties, it was recommended that individual phages be properly characterized prior to use in typing (Ackermann et al., 1983). In this paper we have attempted an initial characterization of the phage $e_4$, a key phage in one of the most recommended V. cholerae typing schemes available at present.

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REFERENCES


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