Molecular characterization of two bacteriophages isolated from *Desulfovibrio vulgaris* NCIMB 8303 (Hildenborough)

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A preliminary endonuclease restriction map of a bacteriophage isolated from *Desulfovibrio vulgaris* has been established. *Bam*HI cleaved whole phage DNA into four fragments while *Hind*III cut the same DNA into seven fragments. Mapping studies succeeded in linking the four *Bam*HI fragments into two DNA segments; however, no linkage between the two segments was detected. These data imply that two phages were induced from cultures of *D. vulgaris* and that the two segments represented the DNA from these phages. Support for this hypothesis came from size approximation of restriction enzyme fragments, electron micrographs, and density gradients.

Introduction

Bacteriophages are natural vehicles of genetic transfer. However, relatively little is known about the bacteriophages found in sulphate-reducing bacteria (SRB). Handley *et al.* (1973) reported bacteriophage-like particles induced from cultures of *Desulfovibrio vulgaris* NCIMB 8303 by UV radiation or treatment with mitomycin C. The phages have a hexagonal head and a long contractile tail (Handley *et al.*, 1973) and were morphologically similar to Bradley's group A bacteriophages (Bradley, 1967). Recently bacteriophages were found in two other species of *Desulfovibrio*. Rapp & Wall (1987) reported that in *D. desulfuricans* ATCC 27774, genetic transfer of rifampicin resistance was mediated by a phage by a generalized transduction mechanism. This phage is not inducible with mitomycin C and is morphologically distinct from the *D. vulgaris* phage. The third *Desulfovibrio* phage was identified in a halophilic SRB, *D. salexigens* NCIMB 8308 by Kamimura & Araki (1989); it was morphologically similar to the phage induced from *D. vulgaris* with the exception that the tail is non-contractile.

Besides morphological characterizations of these phages, a paucity of information exists concerning molecular biological analyses of these viruses because of difficulties in propagating and isolating them. By optimizing the conditions for inducing and isolation of a *D. vulgaris* bacteriophage we have succeeded in producing microgram quantities of phage DNA sensitive to

restriction enzyme digestion. This showed the phage DNA to be double stranded (Seyedirashti *et al.*, 1991). This study reports the restriction endonuclease mapping of DNA from phage induced from *D. vulgaris*. The findings imply that there are two different prophages present in *D. vulgaris*.

Methods

Growth and maintenance of cultures. *Desulfovibrio vulgaris* NCIMB 8303 (Hildenborough) was maintained on Postgate's medium C (Postgate, 1984) and routinely checked for purity according to Postgate (1984). Ten millilitres of 24 h cultures were transferred to 200 ml medium C in 300 ml Florence flasks and incubated under a K₂CO₃/pyrogallol seal.

Induction of bacteriophages. A 120 ml volume of 8-h-old cultures was irradiated for 1 min with ultraviolet light (15 W General Electric Germicidal Lamp) in a 30 × 20.5 cm stainless steel pan with gentle agitation. The UV source dosage was 0.8 Jm² (40 cm). The culture was then incubated anaerobically for 12 d at 37 °C, after which time the cultures were harvested for bacteriophages.

Purification of bacteriophages and extraction of DNA. Concentration of phage particles and extraction of DNA were performed using a modified procedure of Maniatis *et al.* (1982). After 12 d incubation of the induced cultures, the culture medium was centrifuged at 5000 g for 30 min. The supernatant fraction was supplemented with RNAase A and DNAase I, to give a final concentration of 1 µg ml⁻¹ for each enzyme and incubated at 37 °C for 30 min. Polyethylene glycol and NaCl were added to final concentrations of 10% (w/v) and 1 M, respectively, and the phage particles were allowed to precipitate at 4 °C overnight. The precipitated phage particles were centrifuged at 10000 g for 20 min and suspended in 1 ml TM (10 mM-Tris/HCl buffer, pH 8.0, 10 mM-MgSO₄). Phage DNA was extracted by the addition of SDS and EDTA to give final concentrations of 1% (w/v) and 10 mM, respectively, followed by phenol/chloroform (1:1) extraction and

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dialysis against TE buffer (10 mM-Tris/HCl, pH 7.6, 1 mM-EDTA). The DNA concentration was determined by measuring A_{260} .

For large-scale preparations of phage DNA, the phage particles were purified using a three-step CsCl gradient (1.3, 1.5 and 1.7 g ml⁻¹). The gradient was centrifuged at 37000 r.p.m. in a Beckman SW-41 rotor for 1.5 h at 18 °C. The phage band was collected, dialysed against TM, and stored at 4 °C. Phage DNA was extracted as described above and further purified by centrifugation at 45000 r.p.m. (Beckman 50 Ti rotor) for 36 h at 18 °C in CsCl solution (1.5 g ml⁻¹) containing ethidium bromide (125 µg ml⁻¹). The DNA band was collected with a syringe and needle and the ethidium bromide was extracted with isoamyl alcohol saturated with CsCl. The DNA preparation was dialysed against TE buffer.

Cloning and analyses of bacteriophage DNA. Manipulations of nucleic acids such as restriction enzyme treatment, Southern blot hybridization and subcloning of DNA segments were performed using standard methods described by Maniatis *et al.* (1982). The cosmid vector pJB8, used for subcloning of the phage DNA segments was kindly provided by Dr Ish-Horowicz (Ish-Horowicz & Burke, 1981).

Electron microscopy. For visualization of phage DNA, the non-aqueous (formamide) procedure was used (Garon, 1986; Westmoreland *et al.*, 1969). A stock spreading solution (700 µl deionized formamide; 100 µl Tris/HCl pH 8.0; 50 µl 5 M-NaCl; 20 µl 0.5 M-EDTA and 130 µl deionized water) was prepared. Nucleic acid (8 µl containing 1–5 µg ml⁻¹) and cytochrome *c* (1 µl of a 1 mg ml⁻¹ solution) were added to 40 µl stock spreading solution. A drop of this preparation was pipetted down a clean glass slide onto the surface of a hypophase (20%, v/v, formamide; 10 mM-Tris HCl, pH 8.5; 1 mM-EDTA) that completely filled a plastic Petri dish. The resulting monolayer was immediately transferred to a parlodion-coated grid by gently touching it to the hypophase surface. The grid was stained with uranyl acetate for 30 s and destained with 90% (v/v) ethanol for 10 s. After the grid was air-dried, it was rotary shadowed with platinum–palladium (80:20) at an angle of 6–10°. The grid was examined with a Philips EM 300 electron microscope.

Results and Discussion

Restriction analyses of *D. vulgaris* NCIMB 8303 bacteriophage

The restriction enzyme pattern of the purified phage DNA digested with *Bam*HI is shown in Fig. 1 (a), lane 2. The sizes of the four bands were estimated to be > 25, 21.9, 18.8 and 6.6 kb, and designated as Ba, Bb, Bc and Bd, respectively. No additional fragments were detected even when phage DNA was radioactively labelled to enhance the detection of small fragments. Digestion with *Hind*III appeared to produce seven fragments (Fig. 1b, lane 2, and Fig. 1c, lane 2) estimated to be 25, 13.5, 5.8, 3.7, 3.3, 2.1 and 1.8 kb in size, and designated as Ha, Hb, Hc, Hd, He, Hf and Hg, respectively. The top two bands, 25 and 13.5 kb, are much more intense than the smaller bands. In order for the smaller bands to show up clearly in the gel, more DNA had to be used for analysis. Comparing Fig. 1 (b), lane 2 and Fig. 1 (c), lane 2, about three times as much DNA was used in Fig. 1 (c). The 13.5 kb band appears to be diffuse; it is possible that

Fig. 1. *Hind*III digestion pattern of *Bam*HI fragments of phage DNA from *D. vulgaris*. The *Bam*HI digestion pattern of phage DNA is shown in (a), lane 2. The *Hind*III digestion pattern of total phage DNA is shown in lane 2 of (b) and (c). The *Bam*HI fragments of phage DNA were recovered by electroelution and then digested with *Hind*III. (b) Lane 3 is the *Hind*III restriction of the Ba fragment. (c) Lanes 3, 4 and 5 are *Hind*III restrictions of Bb, Bc and Bd, respectively. *Hind*III-digested λ phage DNA is shown in lane 1 of each panel.

there may be a protein or carbohydrate tightly bound to this DNA fragment which may result in the fuzzy appearance in the gel. The sum of the *Hind*III fragments was calculated to be approximately 55 kb, while the size of the phage genome estimated by *Bam*HI digestion was over 72 kb.

Mapping of the phage genome

To map the *Hind*III sites on each *Bam*HI fragment, phage DNA was digested with *Bam*HI and each of the four *Bam*HI fragments was extracted from a preparative agarose gel after electrophoresis. Each individual fragment was isolated and digested with *Hind*III, and the patterns were compared to the pattern of the whole phage DNA digested with the same enzyme. The results are shown in Fig. 1 (b) and (c). For the Ba fragment (Fig. 1b, lane 3), digestion with *Hind*III gave three bands of 18.5, 13.5 and 1.8 kb. The faint bands above the 18.5 kb fragment are probably due to partial digestion of the DNA. The migration of the 13.5 and 1.8 kb fragments on agarose gel was identical to the Hb (13.5 kb) and the Hg (1.8 kb) bands resulting from the digestion of the phage DNA by *Hind*III (Fig. 1b, lane 2). To confirm that they were the same fragments, the Ba fragment was radio-labelled and hybridized to phage DNA digested with *Hind*III. Three different fragments hybridized with labelled Ba fragment. The Hb (13.5 kb) and Hg (1.8 kb) fragments hybridized as expected, but in addition, the

Fig. 2. Proposed arrangement of *Hind*III and *Bam*HI fragments of phage DNA from *D. vulgaris*. Restriction enzymes are abbreviated as *Bam*HI (B) and *Hind*III (H). (a) Schematic representation of the Ha1 fragment cloned in pJBB. (b) Linkage map of Ba and Bd fragments. (c) Assumed linkage map of Bb and Bc fragments.

Ha (approximately 25 kb) fragment hybridized with the Ba fragment. This implied that the 18.5 kb fragment was part of a larger phage *Hind*III fragment (Ha) that contained a *Bam*HI site. The map of the Ba fragment is shown in Fig. 2(b). Similarly the other *Bam*HI fragments (Bb, Bc, Bd) of the phage DNA were digested with *Hind*III (Fig. 1c) and confirmed by hybridization as described for the Ba fragment. Interestingly, all four Ba fragments hybridized to the 25 kb Ha band, implying that there may be more than one fragment in the 25 kb band. The mapping of the *Hind*III sites on the four *Bam*HI fragments is shown in Fig. 2.

For the Ba fragment, it is not clear whether the *Hind*III site is located exactly at the end of the Ba fragment (Fig. 2b). However, the existence of this *Hind*III site was confirmed by subcloning the Hb fragment directly into the *Hind*III site of a plasmid. It is possible that a very small oligonucleotide fragment, which we did not detect, may indeed exist 5' to the *Hind*III site at the 5' end of the Ba fragment.

To establish the orders of the *Hind*III sites within the *Bam*HI fragments, two approaches were used. First, each isolated *Bam*HI fragment was end-labelled and then cut with *Hind*III, which enabled us to identify the end fragments. For example, when the 18.8 kb (Bc) fragment was labelled and cut with *Hind*III (Fig. 2), the 11.5 kb and a small 0.3 kb (Hx) fragments were labelled whilst the 3.3 (He) and 3.7 kb (Hd) fragments were not, indicating that the latter two fragments (Hd and He) were internal. Similar end-labelling experiments were performed on the other *Bam*HI fragments and the orders of most of the *Hind*III sites within the *Bam*HI fragments were determined (Fig. 2b, c). To confirm these results, total phage DNA was partially digested with *Hind*III and then hybridized with various cloned fragments in

Fig. 3. Southern blot hybridization of He (a) and Hd (b) fragments with partial *Hind*III-digested bacteriophage DNA. He and Hd fragments of phage DNA were cloned into pUC13, amplified, cut out of pUC13 with *Hind*III, electrophoresed on agarose gel, electroeluted, nick-translated and hybridized to partial *Hind*III-digested phage DNA. Bacteriophage DNA in both panels was digested with 1:3 serial dilutions of *Hind*III, starting with 12 units of enzyme, shown in lanes 1–5 (lane 5 contained 12 units).

order to elucidate the *Hind*III sites within the *Bam*HI fragments. An example of one of these experiments with the 18.8 kb (Bc) fragment is shown in Fig. 3. Total phage DNA was partially digested with increasing amounts of *Hind*III so that lower concentrations of enzyme digested the DNA partially and the higher concentrations of enzyme digested the DNA completely. The digested DNA was then hybridized to either the 3.3 (He) or 3.7 (Hd) fragment (Fig. 2c) that was subcloned into a pUC13 vector. Fig. 3(a) shows that the He probe hybridized to its 3.3 kb counterpart as expected. In addition, it also hybridized to two partially digested 6.9 and 7.2 kb bands. This implied that the 6.9 kb band consisted of Hd (3.7 kb) plus He (3.3 kb) while the 7.2 kb band contained three fragments, Hd, He and Hx and, furthermore, that the Hx band must be the 0.3 kb fragment which constituted the end of the Bc fragment. Since the results from the hybridization with the He (3.3 kb) fragment (Fig. 3a) did not provide evidence that Hx was connected to Hd or He, a hybridization experiment was done with partially digested phage DNA with Hd (3.7 kb) as the probe. The results (Fig. 3b) show that Hd hybridized with its counterpart band (3.7 kb), the 6.9 kb (He + Hd) and 7.2 kb (He + Hd + Hx) bands. In addi-

tion, it also hybridized with a 4 kb band that was not observed when the He probe was used (Fig. 3a). These data indicated that Hx (0.3 kb) is connected to the Hd fragment as shown in Fig. 2(c), which accounts for the 4 kb band that hybridized with the Hd probe. In this way the *Hind*III sites were mapped on the Bc fragment. Similar partial digestions and hybridizations were performed with all the other *Bam*HI fragments to establish the map shown in Fig. 2.

To link the four *Bam*HI fragments of the phage, a *Bam*HI-*Hind*III double digestion approach was used to identify all new fragments that were generated after double digestion, i.e. those that were not found in either digestion by *Bam*HI or *Hind*III alone. Connecting the *Bam*HI-*Hind*III fragments would allow linkage of the four *Bam*HI fragments. *Hind*III digestion of the *Bam*HI fragments, Ba-Bd, generated four new fragments of 18.5, 14, 11.5 and 6.2 kb (Fig. 1), that did not correspond to any *Hind*III-digested bands of total phage DNA. When each of these four *Bam*HI fragments was labelled and hybridized to a *Hind*III digest of phage DNA, all of the fragments hybridized to the 25 kb Ha fragment (data not shown). This implied that the 25 kb Ha band consisted of two fragments of approximately 25 kb in size that were not resolved during electrophoresis. This was confirmed when *Bam*HI was used to digest the Ha fragment purified from the *Hind*III-digested phage DNA; the four fragments, 18.5, 14, 11.5 and 6.2 kb, were released. Assuming that the Ha band was composed of two separate 25 kb fragments, the Ha fragment(s) was ligated into cosmid pJB8 and cloned into *Escherichia coli* HB101 (Fig. 2a). Digestion of a cosmid insert with *Bam*HI released the 18.5 and 6.2 kb fragments. The Ha resulting from this clone was designated Ha1 (24.7 kb). This result implied that the 18.5 and 6.2 kb fragments were linked (Fig. 2a, b). Because we concluded that there was the possibility that two different 25 kb fragments existed, the second fragment was designated Ha2 (25.5 kb), which was assumed to contain the 14 and 11.5 kb fragments. We attempted to subclone the Ha2 fragment into a cosmid vector but were not able to obtain a clone containing the fragment corresponding to Ha2. Since the restriction enzyme data clearly indicated the existence of a second *Hind*III fragment, we concluded that Ha2 must be present in the original Ha fragment. By this method we established the relationship between Ba and Bd as well as that between Bb and Bc.

Two populations of phage DNA detected in *D. vulgaris*

Although we were able to demonstrate the direct linking of Ba to Bd and Bb to Bc, we could not establish the linking of the Ba-Bd fragment to the Bb-Bc fragment. We observed that when a purified Ha fragment

Fig. 4. *Bam*HI restriction endonuclease pattern of phage DNA from *D. vulgaris* after fractionation on CsCl density gradient. Fractions containing DNA, as judged by A_{260} , were ethanol-precipitated, digested with *Bam*HI and electrophoresed on 0.6% agarose gel. Lanes 2-9, *Bam*HI-digested DNA in fractions with densities of 1.718, 1.709, 1.706, 1.704, 1.698, 1.693, 1.684 and 1.679, respectively. *Hind*III-digested λ phage DNA is shown in lane 1.

(Ha1 + Ha2) was digested with *Bam*HI, the resulting bands varied in intensity on the ethidium-bromide-stained agarose gel. Careful examination of the digestion pattern indicated that the 18.5 and 6.2 kb bands (Ha1) were always more intense than the 14 and 11.5 kb bands (Ha2). This intensity variation was also noticed in both the *Hind*III and *Bam*HI digestion bands of total phage DNA. This observation implied that the Ha1 fragment was present in larger amounts in the Ha (Ha1 + Ha2) band, and that the Ha1 and Ha2 arose from two different phage DNA molecules.

To determine if there were two types of phage DNA molecules present in our preparations, phage particles were further purified using a CsCl gradient. A bacteriophage DNA preparation was mixed in a CsCl solution at a final concentration of 1.72 g ml⁻¹ and centrifuged for 88 h in a Beckman 50 Ti rotor at 38000 r.p.m. The resulting gradient was fractionated (in 100 μ l fractions) and the A_{260} and density of each fraction measured. The results reproducibly showed two peaks in the gradient at densities 1.704 and 1.693 g ml⁻¹. The DNA from each fraction was precipitated with ethanol, digested with *Bam*HI, and separated by electrophoresis. Fig. 4, lane 7 clearly shows that Bb (21.9 kb) and Bc (18.8 kb) fragments, assumed earlier to be linked, were not present in the second peak. The first peak (corresponding to Fig. 4, lane 5), however, contained both DNA populations. Furthermore, the absence of the Bb and Bc fragments in

Fig. 4, lane 7, is accompanied by higher intensity bands of Ba and Bd fragments. This strongly supported the conclusion that there were two different DNA populations in cultures of *D. vulgaris* NCIMB 8303. These results were consistently reproduced with three different gradients with different concentrations of CsCl.

Electron micrograph of D. vulgaris phage DNA

Based on the size of the restriction enzyme fragments, the total size of each of the two phage DNA should be approximately 40 kb. It was also of interest to us to determine whether the bacteriophage DNA was linear or circular. Electron micrographs of the phage DNA showed that the DNA was linear and, using the cosmid pJB8 as a reference molecule (5.4 kb), the size of the phage DNA molecule was estimated to be 40–41 kb.

The infection of a bacterial cell by two unrelated bacteriophages is not unique to *D. vulgaris*. Superinfection by an unrelated phage has been documented in *E. coli* K12 in the case of T5 and λ phage (Adams, 1959; Weigle & Delbruck, 1951). The same phenomenon has been demonstrated with the unrelated temperate phages P1 and P8 in *Pseudomonas aeruginosa* 13 (Adams, 1959). In these studies, if the lysogenic strain 13 is induced with UV and then superinfected with P1, the majority of the bacteria liberate P1 and P8.

This study describes the first molecular characterization of a phage from a sulphate-reducing bacterium. The results strongly suggest that two different phages were induced from cultures of *D. vulgaris* NCIMB 8303. It is not clear whether these phages were released from the same cell and if so, what roles they play in infection, e.g. helper phage for each other. Further studies are necessary to address these questions.

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