Induction and partial purification of bacteriophages from *Desulfovibrio vulgaris* (Hildenborough) and *Desulfovibrio desulfuricans* ATCC 13541

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Bacteriophages were induced from cultures of *Desulfovibrio vulgaris* NCIMB 8303 and *Desulfovibrio desulfuricans* ATCC 13541 by UV light. The optimum time of UV exposure was 1 min and the maximum yield of phage was obtained 9–10 h after UV treatment. The two phage preparations were compared by restriction enzyme analysis and Southern blot hybridization. The nucleic acid from both phages was cut by restriction endonucleases specific for double-stranded DNA. The phage DNAs from *D. vulgaris* and *D. desulfuricans* showed different restriction enzyme cleavage patterns. No homology was observed between a 25 kb probe from the *D. vulgaris* phage DNA and the phage DNA from *D. desulfuricans*. Protein profiles of the phages from both sources were also studied; the *D. vulgaris* phage contained two major bands corresponding to M_r values of 37 000 and 56 000 while the *D. desulfuricans* phage contained only one major band, of M_r 38 000.

Introduction

The biochemistry of dissimilatory sulphate reduction by sulphate-reducing bacteria has been extensively investigated over the past three decades. Enzymes and electron-transport agents participating in the overall process of sulphate reduction to sulphide have been isolated and purified and the amino acid sequences of several of these proteins have been determined (Ambler *et al.*, 1971; Bruschi & LeGall, 1976; Bruschi *et al.*, 1979; Dubourdieu *et al.*, 1973; Travis *et al.*, 1971). In contrast, little information is available concerning the genetics of and genetic exchanges occurring among sulphate-reducing bacteria; this is due, in part to the difficulty in culturing these fastidious and unpredictable micro-organisms on solid media for performing genetic analyses. With advances in recombinant DNA technology, several molecular genetic approaches have been used to study the sulphate-reducing bacteria. However, due to the lack of characterized vectors, such as plasmids and bacteriophages, that can be used to mediate genetic transfer, molecular studies on these organisms have been limited to cloning and expression of bacterial genes, such as those coding for cytochrome c_3 and hydrogenase (Fons *et al.*, 1987; Li *et al.*, 1986; Menon *et al.*, 1987; Pollock *et al.*, 1991; van Rooijen *et al.*, 1989; Voordouw & Brenner, 1985, 1986; Voordouw *et al.*, 1985, 1989a, b).

Earlier work from this laboratory showed that phage-like particles were induced from cultures of *Desulfovibrio vulgaris* by mitomycin C or UV light treatment (Handley *et al.*, 1973). Postgate *et al.* (1984) reported the occurrence of plasmids in several species and strains of sulphate-reducing bacteria. More recently, Rapp & Wall (1987) described the isolation of phages from *Desulfovibrio desulfuricans* ATCC 27774 and demonstrated that these phages were involved in gene transfer by a generalized transduction process. These observations strongly suggest that genetic exchange among sulphate-reducing bacteria is possible, and open the way for significant advances in understanding the genetic make-up of these organisms. Thus, a detailed characterization of phages from sulphate-reducing bacteria, and studying their possible application as vectors for genetic transfer, would be valuable in understanding the molecular genetics of these bacteria.

As part of our overall programme to investigate the molecular biology and genetics of sulphate-reducing bacteria, we reinvestigated the induction of phage from *D. vulgaris* to (i) elucidate the parameters for obtaining high yields of phage particles, (ii) characterize the phage nucleic acid by restriction enzyme analysis, and (iii) obtain a protein profile of the phage. For comparative purposes we also succeeded in inducing a phage from *D. desulfuricans* ATCC 13541 ('*Vibro cholonicus*') (Postgate

& Campbell, 1966) and determined the differences in the DNA restriction enzyme pattern between the phages obtained from both organisms.

Methods

Growth of organisms. The organisms used in this study were *Desulfovibrio vulgaris* Hildenborough (NCIMB 8303) and *Desulfovibrio desulfuricans* (ATCC 13541), which was obtained from the American Type Culture Collection. Both strains were grown in medium C (Postgate, 1984) at 37 °C; *Escherichia coli* and *Salmonella typhimurium* were grown at 37 °C in Luria broth supplemented with 0.1% glucose and 10 mM-MgSO₄. All cultures were periodically checked for purity.

Induction of phages. Portions (120 ml) of 8 h old cultures were irradiated for 1 min with UV light in a 30 × 20.5 cm pan with gentle agitation. The UV source was a 15 W General Electric Germicidal lamp. The dosage at a distance of 40 cm was 0.8 J m⁻² s⁻¹. After exposure to UV, the cultures were incubated anaerobically for various lengths of time (0–96 h).

Purification of phage particles and extraction of DNA. Concentration of phages and extraction of DNA were performed by a modification of the procedure of Maniatis *et al.* (1982). Briefly, induced cultures were centrifuged at 5000 g for 30 min. The supernatant fraction was treated with RNAase A and DNAase I, each at a final concentration of 1 µg ml⁻¹, and incubated at 37 °C for 30 min. Polyethylene glycol and NaCl were added at final concentrations of 10% (w/v) and 1 M, respectively, and the mixture was stored at 4 °C overnight. The precipitated phage particles were sedimented at 10000 g for 20 min and suspended in 1 ml TM buffer (10 mM-Tris/HCl buffer, pH 8.0, containing 10 mM-MgSO₄). For quantification of phage DNA yield, phage DNA was extracted by the addition of SDS and EDTA at final concentrations of 1% (w/v) and 10 mM, respectively, followed by extraction with phenol/chloroform (1:1, v/v) and dialysis against TE buffer (10 mM-Tris/HCl buffer, pH 7.6, containing 1 mM-EDTA). Absorbance was measured at 260 and 280 nm with a Beckman DU-40 spectrophotometer.

For large-scale preparations, phages were purified using a three-step CsCl gradient (1.3, 1.5, 1.7 g ml⁻¹) prior to extraction of DNA. The gradient was centrifuged at 37000 r.p.m. in a Beckman SW-41 rotor for 1.5 h at 18 °C in a Beckman L2-65B ultracentrifuge. The phage band was collected, dialysed against TM buffer, and stored at 4 °C. Phage DNA was extracted as described above and was purified by centrifugation (45000 r.p.m., Beckman 50Ti rotor, L2-65B ultracentrifuge) for 36 h at 18 °C in CsCl (1.5 g ml⁻¹) solution containing 125 µg ethidium bromide 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 overnight and stored at 4 °C.

Restriction endonuclease analyses. Phage DNA was digested with restriction endonucleases purchased from New England Biolabs and used as recommended by the manufacturer. Digestions were performed for 2 h at 37 °C using 3 units of enzyme per µg of DNA. Restriction enzyme patterns on gels were recorded using Polaroid type 667 film.

Molecular cloning. Phage DNA from *D. vulgaris* was digested with HindIII and DNA fragments were ligated to the HindIII site of the cosmid vector PJB8 (Ish-Horowicz & Burke 1981). The resulting recombinant cosmids were packaged *in vitro* according to Ish-Horowicz & Burke (1981) and used to infect *E. coli* 803. Infected cells were plated on LB agar plates containing 100 µg ampicillin ml⁻¹ (Maniatis *et al.*, 1982).

Southern blot hybridization. Phage DNAs, either uncut or cut with restriction enzymes, were subjected to electrophoresis in 0.7% agarose gels. Phage DNAs were depurinated by immersion of the gel in 0.1 M-HCl for 15 min at room temperature followed by immersion in 0.5 M-NaOH, containing 1.5 M-NaCl, at the same temperature for 15 min. The gel was neutralized with 0.5 M-Tris/HCl buffer, pH 7.5, containing 3 M-NaCl. The DNA was subsequently transferred to nitrocellulose filter paper (Schleicher and Schuell) by capillary blotting for 16 h in 20 × SSC (Southern, 1975), followed by baking in a vacuum oven for 2 h at 80 °C. The nitrocellulose paper was soaked at 42 °C in a solution consisting of 50% (v/v) deionized formamide, 5 × SSC, 0.1% BSA, 0.1% polyvinylpyrrolidone, 0.1% Ficoll, 1% (w/v) glycine, 50 mM-sodium phosphate buffer, pH 6.5, and 250 µg denatured salmon sperm DNA ml⁻¹. The probe DNA was labelled with [α -³²P]dATP; 5 × 10⁶ c.p.m. were used for hybridization studies. These were performed at 42 °C for 16 h with the phage DNA on nitrocellulose paper immersed in a solution containing 50% (v/v) deionized formamide, 5 × SSC, 0.02% BSA, 0.2% polyvinylpyrrolidone, 0.02% Ficoll, 20 mM-sodium phosphate buffer, pH 6.5, 10% dextran sulphate, and 50 µg denatured salmon sperm DNA ml⁻¹. The filter paper was washed successively in 2 × SSC containing 0.1% SDS, and 0.2% SSC containing 0.1% SDS, for 15 min each at 42 °C. Autoradiography was performed at -70 °C on Dupont Cronex film using a Lightning Plus intensifying screen (Dupont).

SDS-PAGE of phage proteins. Structural proteins from both phages, together with low molecular weight standards (Bio-Rad) were separated by electrophoresis on 12.5% (w/v) denaturing SDS-polyacrylamide gels. Samples to be run were boiled in 50 µl Laemmli sample buffer with 2-mercaptoethanol according to Laemmli (1970). After cooling, the samples were loaded on the gel. After the run, the gel was fixed and stained with 0.125% Coomassie Brilliant Blue R-250.

Chemicals. Mitomycin C, Coomassie Brilliant Blue R-250, ethidium bromide, Ficoll, polyvinylpyrrolidone, salmon sperm DNA and [α -³²P]dATP were purchased from Sigma. Restriction enzymes were obtained from Pharmacia Fine Chemicals and New England Biolabs.

Results

UV induction studies

Handley *et al.* (1973) reported that phage-like particles could be induced from *D. vulgaris* by mitomycin C or UV treatment. In our hands the yield of phage induced by mitomycin C treatment (2.5 µg ml⁻¹ for 15 h) according to Handley *et al.* (1973) was consistently lower than that observed for UV irradiation. Since it was important that the maximum yield of phage was obtained, we chose the latter for phage induction in this study. The UV exposure time for maximum phage production, as indicated by the amount of DNA recovered, was determined to be 60–90 s. Phage DNA was quantified by absorbance measurements at 260 nm. To determine the optimal incubation time after induction, cultures were UV irradiated for 1 min and harvested at various times (Fig. 1). In both uninduced and induced cultures we observed phage liberation in small quantities (7 µg per 240 ml of culture) for up to 6 h after UV irradiation. This indicated that some spontaneous lysis of cells was occurring. However,

Fig. 1. Effect of time on *D. vulgaris* phage yield. Cultures 8 h old were exposed to UV for 1 min and harvested at the indicated times. Phage DNA was extracted and quantified as described in the text.

a sudden increase in phage yield was observed (25 µg per 240 ml culture) 9–10 h after irradiation and the phage titre then remained constant for up to 96 h.

Preliminary characterization of the phage nucleic acids from D. vulgaris and D. desulfuricans

The nucleic acid from both phages was easily cut by double-stranded-DNA-specific restriction endonu-

cleases, indicating that the genomes consisted of double-stranded DNA. Fig. 2(a) shows the comparative restriction enzyme patterns of phage DNA from *D. vulgaris* and *D. desulfuricans*. The various restriction enzymes tested cut DNA from both phages, but their patterns were significantly different. This indicated that the genomes of these phages share no gross homology. In the case of *D. vulgaris*, a large *Hind*III fragment is seen (lane D) above the 23 kb marker resulting from λ DNA digested with *Hind*III (lanes A and N). This fragment was cloned into a cosmid and was estimated to be approximately 25 kb in size (data not shown). In addition to this fragment, bands of 13.5 and 1.8 kb in size are seen. Based on the various restriction patterns the *D. vulgaris* phage genome was estimated to be at least 40.3 kb. *D. desulfuricans* phage DNA was also cut by *Hind*III into several fragments ranging from 13.1 to 2.9 kb and its genome was estimated to be approximately 45 kb in size. *Eco*RI and *Bam*HI also digested *D. desulfuricans* phage DNA extensively; however, these enzymes only partially digested *D. vulgaris* phage DNA, as some uncut DNA can be seen. Other enzymes such as *Cla*I cut the *D. vulgaris* phage DNA but did not appear to cut the DNA from the *D. desulfuricans* phage.

Fig. 2. Restriction endonuclease patterns of phage DNA from *D. vulgaris* NCIMB 8303 and *D. desulfuricans* ATCC 13541. (a) Lanes A and N, λ phage DNA digested with *Hind*III. Uncut DNAs from *D. vulgaris* and *D. desulfuricans* are shown in lanes B and C, respectively. The patterns for *D. vulgaris* phage DNA using *Hind*III, *Eco*RI, *Bam*HI, *Cla*I and *Ava*I are shown in lanes D, F, H, J and L. The patterns for the phage from *D. desulfuricans* using the same enzymes are shown in lanes E, G, I, K and M. (b) Autoradiogram of a Southern blot of the DNA in (a) using a 32 P-labelled 25 kb probe from *D. vulgaris* phage DNA.

Fig. 3. SDS-PAGE analysis of phage protein profiles. Lane A, M_r standards; lane B, proteins from *D. vulgaris* phage; lane C, proteins from *D. desulfuricans* phage.

Hybridization of *D. vulgaris* and *D. desulfuricans* phage DNA

Phage DNA from *D. vulgaris* was digested with *Hind*III and the fragments were subcloned into the *E. coli* cosmid vector PJB8. One subclone contained a 25 kb phage insert and this was used to check for cross-hybridization between the phage DNAs from *D. vulgaris* and *D. desulfuricans*. The autoradiogram of the Southern blot is shown in Fig. 2(b). The 25 kb nick-translated probe from *D. vulgaris* phage DNA hybridized with itself as expected, but did not hybridize with the phage DNA from *D. desulfuricans*. These results clearly indicated that the phages from the two organisms were different and shared no DNA sequence homology.

Protein profile of the phage particles

Large quantities of the phages from both organisms were harvested and intact particles were obtained by caesium chloride gradient centrifugation. The protein profiles of the phages were analysed by SDS-PAGE (Fig. 3). Two major and six minor bands were obtained from the phage particles from *D. vulgaris*. The minor bands were very faint. The M_r values of the minor species were calculated to be approximately 38000, 40000, 44000, 48000 and 50000, with one being above 97000; the major bands had M_r values of 37000 and 56000. In the phage of *D. desulfuricans*, only one major and eight minor bands were detected. The major band was calculated to have an M_r of approximately 38000, while those of the minor bands ranged from 42000 to over 97000.

Discussion

Both *D. vulgaris* NCIMB 8303 and *D. desulfuricans* ATCC 13541 are lysogenic. However, the phages that were induced from these two organisms were quite distinct from each other as judged by restriction endonuclease patterns. Furthermore, the lack of hybridization between the *D. vulgaris* and *D. desulfuricans* phages, using a labelled *D. vulgaris* phage DNA fragment as probe (Fig. 2b), implies that the two phages may not have common ancestries.

In our restriction analyses of the phage DNA, a complete digest was not obtained, especially with some enzymes such as *Ava*I and *Bgl*II. This incomplete digestion may imply some unique properties of the phage DNA, such as the presence of unusual bases and/or strong association with protein or polysaccharide that could not be dissociated under standard conditions of nucleic acid purification, or a low frequency of the specific sequences recognized by these enzymes.

The protein profiles of the phage particles showed conspicuous differences in number and size of their major bands. The two major bands from the structural proteins of the *D. vulgaris* phage, of M_r 37000 and 56000, corresponded to the two major structural protein bands observed in a bacteriophage lytic for *Desulfovibrio salexigens*, a halophilic sulphate-reducing bacterium (Kamimura & Araki, 1989). If the structural proteins from *D. vulgaris* phage are similar to those reported for *D. salexigens* phage, the M_r 37000 protein in this study should correspond to the head while the M_r 56000 protein band represents the tail protein. This would be in agreement with our finding that the phage from *D. desulfuricans* showed only one major protein band, of M_r 38000. This protein presumably corresponds to the head protein of the phage; no major tail protein band was observed in the M_r region of 56000, probably because this phage has a short tail (unpublished observations).

The major difficulty in studying the properties of these phages is the lack of a suitable indicator strain. The *D. vulgaris* NCIMB 8303 phage was not able to grow on *E. coli* or *Salmonella typhimurium*, as determined by plaque formation, or, as determined by phage DNA detection, on *D. desulfuricans* strains ATCC 13541 and NCIMB 8310. Compounding this problem is the difficulty of culturing these micro-organisms on solid media, especially to establish a lawn of organisms to detect plaques. The technique used by Kamimura & Araki (1989) to form a lawn of these organisms may be the answer to this problem. We are currently concentrating on the phage from *D. vulgaris* because much of the biochemistry and physiology of this organism is already known. Although the phage from *D. vulgaris* was induced from a pure culture, we have not eliminated the possibility that more

than one type of phage was induced since it was not possible to purify the phage by the plaque assay method. We have initiated the subcloning of several restriction enzyme fragments of *D. vulgaris* phage DNA into *E. coli* using cosmid vectors, and are in the process of elucidating the physical map of this phage DNA.

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