Halobacterium halobium phage ϕ H

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Phage ϕ H, a novel virus of the archaebacterium *Halobacterium halobium*, resembles in size and morphology two other *Halobacterium* phages. One-step growth curves show a 5.5 h eclipse, a latent period of 7 h, and an apparent burst size of 170. Phage ϕ H contains linear, double-stranded DNA which has a molecular weight of 39×10^6 and a GC content of 65%. A packaging model accounting for the partial circular permutation and terminal redundancy of ϕ H DNA is suggested. Partial homology of ϕ H DNA with the DNA of *H. halobium*, predominantly with the AT-rich satellite DNA, was observed. The presence of minor restriction fragments of ϕ H DNA which could be removed by purification of phage from single plaques suggests the existence of phage variants with rearranged DNA. A strain of *H. halobium* containing ϕ H DNA was isolated which is resistant to infection by phage ϕ H.

Key words: archaebacteria/virus/genome organization/DNA packaging

Introduction

The genus *Halobacterium* belongs to a group of organisms now known as archaebacteria which differ in many respects from both eucaryotes and eubacteria (Fox *et al.*, 1980). Two phages of *Halobacterium* have been described (Torsvik and Dundas, 1974 and 1980; Wais *et al.*, 1975), but nothing is known about the organization of their DNA.

This paper describes the growth and general properties of a new *Halobacterium* phage. In addition, it presents the first analysis of the DNA of an archaebacterial phage.

Results

Phage growth and purification

Optimal growth of phage was obtained by the procedure given in **Materials and methods** which resulted in a yield of between 5×10^9 and 3×10^{10} plaque forming units (p.f.u.)/ml. A high multiplicity of infection of undiluted cultures did not give reproducible results. Phage purification followed conventional procedures except that sufficiently high salt concentrations had to be maintained to prevent disintegration of the phage.

Latent period and eclipse

The one-step growth curve of ϕ H is shown in Figure 1. The number of infected cells was determined from the difference between total p.f.u. (infected cells plus free phage) and chloroform-resistant p.f.u. (complete phage) during the first hours following infection. After an eclipse of 5.5 h, mature

phage began to appear inside the cells, whereas lysis started after a latent period of 7 h. The apparent burst size was 170.

Phage morphology

An electron micrograph of negatively stained ϕ H (Figure 2) shows a polyhedral head (ϕ 64 nm) and a tail 170 nm long and 18 nm thick, similar to other halophilic phages (Torsvik and Dundas, 1974; Wais *et al.*, 1975).

Salt requirement

ϕ H viability is maintained only in solutions with high concentration of salt (Figure 3). In the presence of Mg^{2+} , lower salt concentrations are tolerated than in its absence.

Fig. 1. One-step growth curve of phage ϕ H on *H. halobium* R₁: O, total p.f.u./ml, representing the sum of infected cells and free phages; ● p.f.u./ml after chloroform treatment of aliquots, representing complete phage particles (free and inside the cells).

Fig. 2. Electron micrograph of phage ϕ H negatively stained with uranyl acetate. The bar represents 0.1 μ m.

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Fig. 3. Survival of phage ϕ H after storage for 12 h at various salt concentrations: ○, NaCl; ●, NaCl + 0.1 M Mg^{2+} ; △, KCl; ▲, KCl + 0.1 M Mg^{2+} .

Fig. 4. SDS polyacrylamide gel electrophoresis of phage ϕ H proteins.

Plaque formation

Phage ϕ H formed plaques on *Halobacterium halobium* strain R₁ which is deficient in vacuole formation. Plating efficiency was reduced by a factor of 10^3 – 10^4 if the wild-type was used as a host, but phage stocks which have been grown on the wild-type strain grow with normal efficiency on both hosts, suggesting the possible existence of a restriction and modification system (S. Yeats, personal communication).

Protein composition of ϕ H

Analysis of the proteins of purified phage by SDS polyacrylamide gel electrophoresis (Figure 4) shows three major proteins with molecular weights (mol. wts.) of 80 000, 53 000, and 22 000, and many minor components.

ϕ H DNA, physical properties

ϕ H contains linear, double-stranded DNA (Figure 5). By contour length measurements, the mol. wt. was determined as $38.5 \times 10^6 \pm 4.8\%$ when compared with pBR322 and as $39 \times 10^6 \pm 2.5\%$ when compared with phage P2 DNA as reference. The melting temperature (T_m) of ϕ H DNA in 0.1 x

Fig. 5. Electron micrograph of ϕ H DNA. pBR322 (mol. wt. 2.88×10^6 , Sutcliffe, 1978) and phage P2 DNA (mol. wt. 22×10^6 , Inman and Bertani, 1969) were used as references in separate experiments. The bar represents 1 μ m.

Fig. 6. Cleavage of ϕ H DNA (a–c) and ϕ H 1 DNA (from single plaque purified phage, d–f) by several restriction enzymes. The arrows indicate minor bands in ϕ H DNA which are not present in ϕ H 1 DNA.

SSC was 80.8°C , corresponding to a GC content of 66%. This value agrees with the buoyant density of ϕ H DNA of 1.723 ± 0.002 , determined by CsCl equilibrium centrifugation, from which a GC content of $64 \pm 2\%$ was calculated.

Cleavage by restriction enzymes

The fragment patterns of ϕ H DNA obtained by cleavage with the restriction enzymes ClaI, BglI, and BglII are shown in Figure 6 a–c. Substoichiometric fragments (arrowed) could be removed by several single plaque purifications yielding the pattern of ϕ H 1 DNA shown in Figure 6 d–f. This observation led to the suggestion that the original phage is a population of at least two different phage variants. To investigate whether the minor fragments are additional DNA which is absent from the DNA of purified phage ϕ H 1, labelled ϕ H 1 DNA was prepared by nick translation and hybridized to a BglI digest of ϕ H DNA containing the minor fragments (Figure 7). The result, hybridization of ϕ H 1 DNA

Fig. 7. Hybridization of nick-translated ϕ H 1 DNA to the DNAs of ϕ H and ϕ H 1 digested with BglI. **a,c:** restriction patterns; **b,d:** hybridization patterns.

Fig. 8. Restriction patterns of ϕ H DNA (**a,c,e,g,i**) and corresponding autoradiographies of ϕ H DNA ^{32}P -labelled at the 5' ends (**b,d,f,h,j**).

with at least one minor fragment in ϕ H DNA, clearly indicates that there is sequence homology between these additional fragments and the DNA which yields the main bands, suggesting that the minor fragment may have originated from rearrangement of ϕ H DNA in phage variants which account for $\sim 10\%$ of the phage population (estimated from the intensity of ethidium bromide fluorescence of these minor bands).

Structure of ϕ H DNA

Only one labelled fragment can be detected in agarose gels when ϕ H DNA ^{32}P -labelled at the 5' ends is digested with several restriction enzymes (Figure 8), although two end fragments would be expected from a linear molecule. Only cleavage with PstI generates two labelled bands, the larger one appearing heterogeneous in length both in the gel and in the autoradiography. The other end-labelled PstI fragment, 5, has a defined length but is present in less than stoichiometric amounts as shown by densitometer tracing of a gel photograph (Figure 9). Another feature of ϕ H DNA is the existence of minor restriction fragments which, in contrast to those discussed above, cannot be removed by single plaque purification, e.g., PstI fragment 2 (Figure 9).

Fig. 9. Densitometer tracing of the PstI pattern of ϕ H 1 DNA. Arrows indicate end-labelled fragments.

Fig. 10. Electron micrograph of a circular duplex obtained after denaturation and reannealing of ϕ H 1 DNA. Arrows point to single-stranded (ss) tails. The contour length of this double-stranded circle corresponds to 37.5×10^6 , and the distance between the two ss tails is 5% of the genome. Length measurements were relative to phage P2 DNA. The bar represents 1 μm .

These results, which should not be obtained from a population of linear, identical molecules, suggested that ϕ H DNA consists of partially circularly permuted, terminally redundant DNA molecules like those of phage P22 (Jackson *et al.*, 1978). This was confirmed by electron microscopy of denatured and renatured ϕ H DNA molecules. As expected, circular duplexes were observed (Figure 10), the circumference of which is a measure of the unit genome length, and corresponds to $37.9 \times 10^6 \pm 2.1\%$. The analysis of the circular homoduplexes indicates that the permutation is limited to $\sim 10\%$ of the genome and that the terminal repetition, estimated from the difference between linear and circular ϕ H DNA, comprises $\sim 3\%$ of the genome length.

Sequence homology between the DNAs of ϕ H and *H. halobium*

Radioactively labelled ϕ H DNA hybridizes with high specificity to certain bands in restriction digests of total *H. halobium* DNA (Figure 11). There is no difference between the hybridization patterns of total DNA from wild-type or strain R₁, and almost the same picture is also obtained when

Fig. 11. Hybridization of nick-translated ϕ H DNA to several DNAs from *H. halobium* digested with PstI. (a,c,e,g,i: restriction patterns; b,d,f,h,j: hybridization patterns) a,b: total DNA from *H. halobium* wild-type; c,d: total DNA from *H. halobium* strain R₁; e,f: plasmid; g,h: chromosomal DNA; i,j: satellite DNA, all from wild-type.

ϕ H DNA is hybridized to plasmid DNA from *H. halobium* wild-type purified by CsCl-ethidium bromide centrifugation. To determine whether the observed homology is between ϕ H DNA and plasmid only or also with chromosomal DNA, AT-rich satellite DNA, which contains the plasmid (Weidinger *et al.*, 1979), was completely separated from the chromosomal DNA by CsCl centrifugation, independent of intact supercoils, taking advantage of the 8% lower GC content of the satellite DNA (Moore and McCarthy, 1969). The purity of the chromosomal DNA was checked by hybridization with nick-translated plasmid; this showed that the chromosomal DNA contained no plasmid, because the restriction pattern of the complete plasmid genome was not seen in the autoradiography. There were, however, many other bands which could be seen indicating extensive sequence homology between the chromosomal DNA and the plasmid (not shown). The comparison between chromosomal and satellite DNA shows that ϕ H DNA hybridizes much more strongly to satellite DNA, which contains the plasmid, but it becomes apparent that the same restriction fragments with sequence homology to ϕ H DNA are also present in chromosomal DNA. Figure 12 shows the patterns obtained by hybridizing labelled plasmid to transferred restriction digests of ϕ H DNA.

ϕ H resistant strain of *H. halobium* R₁

All of 50 single colonies derived from surviving cells from phage lysates were phage-sensitive. However, some resistant clones could be obtained from concentrated lysates. Even high concentrations of phage did not yield plaques on these resistant clones. The absence of phage particles from cultures of resistant cells was checked by mixing them with sensitive bacteria; one resistant culture forming no plaques on sensitive cells was used for further analysis.

Colonies from this resistant strain were shown by colony hybridization to contain much more ϕ H-homologous DNA than sensitive colonies. The presence of ϕ H DNA was shown by hybridization of labelled ϕ H DNA to restriction fragmented DNA from this resistant strain (Figure 13). Many of the heavily labelled bands are identical in size to restriction fragments of ϕ H DNA. Therefore, this resistant strain con-

Fig. 12. Hybridization of nick-translated plasmid from *H. halobium* wild-type to ϕ H DNA digested with several restriction enzymes. (a,c,e,g: restriction patterns; b,d,f,h: hybridization patterns).

Fig. 13. Hybridization of nick-translated ϕ H DNA to DNA from a phage-resistant strain. R₁: control with DNA from phage-sensitive *H. halobium*; R₁ ϕ H^r: DNA from a phage-resistant strain derived from R₁; ϕ H: restriction pattern of ϕ H DNA digested with the same restriction enzyme.

tains most or all of ϕ H DNA in a partially rearranged way.

Subclones of a single resistant colony show variations in the pattern of ϕ H-homologous restriction fragments. This indicates that changes in the physical structure can happen with high frequency when the phage DNA is in this state.

Discussion

ϕ H is the first archaeobacterial phage whose molecular properties have been investigated. Its morphology is similar to that of two other *Halobacterium* phages which have been described (Torsvik and Dundas, 1974; Wais *et al.*, 1975) and also to that of some *Escherichia coli* phages, e.g., lambda. Its growth cycle on *H. halobium* R₁ is completed ~15 h after infection, which is several times longer than the 5 h generation time of the host.

Phage ϕ H has a complex protein composition and contains linear, double-stranded DNA. The existence of substoichiometric restriction fragments in ϕ H DNA which can be removed by purification of phage from single plaques (ϕ H 1) suggests that there are phage variants with similar, but not com-

pletely identical, DNA. These variants could have arisen by recombinational events involving insertion, deletion, or inversion, leading to the simultaneous reduction in the amount of one or two major fragments by $\sim 10\%$, which would not be visible in the gel. As the relative proportion of the minor bands remains constant after several growth cycles of the phage, it can be assumed that the variant is frequently formed from ϕ H 1 or that it grows as well as ϕ H 1. Therefore, attempts are currently under way to isolate the variant from single plaques.

Figure 14 shows a putative packaging model which was developed in analogy to phage P22 (Jackson *et al.*, 1978) and accounts for the partially permuted structure of ϕ H DNA. Packaging of mature DNA molecules from concatemeric precursors would begin with the introduction of a sequence-specific cut at the "pac" site and proceed by filling of phage heads; this would lead to packaging of slightly more than one genome length and to inexact cutting of the DNA. The two end-proximal PstI sites would yield the end fragments 3 and 5 (compare Figure 9) and, if molecular ends fall past the right PstI site, also the intact fragment 2. The single end-labelled fragment seen in all other digests must originate from the "pac" end of the mature molecules. The other ends are heterogeneous in size and therefore, the bands in the gel will be spread over a wide range which results in concentrations of label too low to be detected.

The hybridization of ϕ H DNA to only a few restriction fragments of relatively small size in restriction digests of *H. halobium* DNA clearly indicates that neither wild-type nor strain R_1 is generally lysogenic for phage ϕ H. ϕ H DNA hybridizes predominantly, but not exclusively, to the plasmid of *H. halobium*. The fact that the size of the ϕ H-homologous restriction fragments is the same in plasmid and chromosomal DNA suggests that they are cut from larger sequences which are present in both DNAs. There are several possible explanations for the observed sequence homology.

- During the evolution of ϕ H, host sequences were incorporated into its DNA which are now present as an integral part of the phage genome.
- ϕ H DNA could be integrated into the plasmid at one or very few specific sites. By inexact excision, transducing phages would be formed which carry pieces of host DNA attached to their own. This would mean that ϕ H DNA can integrate into the plasmid.
- It is known that the plasmid of *H. halobium* is subject to an extraordinary degree of structural alteration by insertion and deletion (Pfeifer *et al.*, 1981b). The possibility exists that such insertions could also occur in the phage and that the homology is due to the simultaneous presence of the same inserted sequence in plasmid or chromosome and ϕ H DNA.

The fact that plasmid DNA hybridizes to several bands in restriction digests of ϕ H DNA, some of which cannot be seen in the gels after staining (compare Figure 12), suggests that several minor DNA species in ϕ H DNA carrying plasmid-homologous sequences must exist. This could point towards possibility (c) because these minor DNAs might contain multiple insertions homologous to the plasmid, causing labelling by hybridization of bands which are not visible in the gels.

It was possible to isolate a resistant strain of *H. halobium* which contains ϕ H DNA and therefore could be immune. Its existence probably means that the phage can establish a stable lysogenic state. The resistance of cells containing ϕ H DNA to

Fig. 14. Model for packaging of ϕ H DNA. The inner circle represents the unit genome, the concatemeric precursor is illustrated by the spiral surrounding it. Only those two PstI sites closest to the molecular ends are shown. Numbers correspond to PstI fragments (see Fig. 9).

further infection suggests the synthesis of a phage-coded repressor.

An interesting observation which is currently under investigation in our laboratory is the fact that subclones of a resistant colony show variations in the pattern of ϕ H-homologous restriction fragments. Possibly, ϕ H DNA in these cells is subject to the same high frequency of rearrangement that has been observed for the plasmid (Pfeifer *et al.*, 1981b).

Materials and methods

Bacterial and phage strains

H. halobium NRL wild-type and *H. halobium* NRL R_1 (deficient in vacuole formation) were obtained from D. Oesterhelt. The phage described in this paper appeared spontaneously in cultures of *H. halobium* R_1 .

Culture medium and phage growth

The host bacterium (*H. halobium* R_1) was grown at 42°C in a medium similar to that described by Oesterhelt and Stoekenius (1974) which contained in 1000 ml: NaCl, 250 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20 g; trisodium citrate $\cdot 2\text{H}_2\text{O}$, 3 g; KCl, 2 g; yeast extract (Difco), 5 g; casamino acids (Difco), 5 g; L-Arg, 2 g; 1 M Tris-Cl, pH 7.4, 50 ml. Exponentially growing cultures at A_{660} between 0.5 and 0.8 were diluted 1:100, infected with 10^6 phages/ml, and incubated for three days.

Phage assay

Phage solutions were assayed by the soft agar layer method (Adams, 1959). Plating and soft agar were prepared from medium diluted with 20% water. Plaques usually formed within three days of incubation at 42°C .

One-step growth curve

An exponentially growing culture of *H. halobium* R_1 at a concentration of 2×10^8 cells/ml was infected with a multiplicity of infection (m.o.i.) of 3. After an adsorption period of 45 min, the cells were collected by centrifugation in order to remove unadsorbed phages and, after one wash, taken up in the same volume of warm medium. The sample was then diluted by a factor of 10^3 into warm medium and incubated at 42°C . The phage titre was determined from aliquots after different times with and without addition of chloroform.

Purification of ϕ H

After three days of phage growth the lysate was filtered through diatomaceous earth (Hyflo Super Cel, Serva), and the phages were precipitated by 8% polyethylene glycol 6000 at 4°C over night. The precipitate was collected by centrifugation and taken up in basal salts (4 M NaCl, 0.12 M MgSO_4 , 0.03 M KCl, 0.01 M trisodium citrate). The concentrated phages were purified by CsCl step centrifugation for 12 h at 23 000 r.p.m. in an SW 27 rotor. Since KCl disturbs the density measurement of CsCl solutions by the refractive index, the exact composition of the step gradient is given. Four steps were mixed from CsCl stock solution (200 g plus 150 ml 0.05 M Tris-Cl, pH 7), KCl saturated at 20°C , and 1 M MgCl_2 in the following volume ratios: 6:3.5:0.5; 5:4.5:0.5; 4:5.5:0.5; 3:6.5:0.5. The phages concentrate on the second layer. The collected phage band was centrifuged to equilibrium for 12 h at 60 000 r.p.m. in a Beckmann VTi 65 rotor.

Preparation of ϕ H DNA

Purified phages in CsCl/KCl solution were dialyzed against 0.02 M Tris-Cl, 0.001 M EDTA, pH 8, followed by extraction with phenol saturated with the above buffer, chloroform-octanol mixture (9:1), and finally chloroform. The resulting DNA solution was extensively dialyzed against 0.02 M Tris-Cl, 0.001 M EDTA, pH 8, and stored at 4°C.

Electron microscopy

Cytochrome c spreading, denaturation and reannealing of ϕ H DNA were carried out essentially as described by Davis *et al.* (1971). Intact phages were prepared for electron microscopy by negative staining.

SDS polyacrylamide gel electrophoresis

Gel electrophoresis was performed according to Laemmli (1970), but in 5–25% exponential polyacrylamide gradients. The mol. wts. were estimated by comparison of the electrophoretic mobilities with those of reference proteins.

Cleavage of DNA with restriction enzymes

1–3 μ g of DNA were used for each digest, which was performed as specified by the supplier of the restriction enzymes (Boehringer, Mannheim). The restriction fragments were separated by agarose gel electrophoresis, which was carried out in Loening's buffer (36 mM Tris, 30 mM NaH_2PO_4 , 1 mM EDTA, pH 7.8; Loening, 1969), usually at 3 V/cm over night. After electrophoresis, bands were visualized by staining with 1 μ g of ethidium bromide and photographed under u.v. light.

Terminal labelling of ϕ H DNA with ^{32}P

Dephosphorylation of ϕ H DNA was carried out with 0.05 U/ μ g DNA of alkaline phosphatase in 70 mM Tris-Cl, 10 mM MgCl_2 , 5 mM β -mercaptoethanol, pH 8 for 30 min at 65°C. After extraction with phenol and chloroform, the DNA was precipitated with ethanol. 50 μ g of DNA were end-labelled in 100 μ l of the above buffer containing 100 μ Ci [γ - ^{32}P]ATP (3000 Ci/mmol, Amersham) and 10 U of polynucleotide kinase (Boehringer) for 30 min at 37°C. After phenol and chloroform extraction, the reaction mixture was sized on Sepharose 4B followed by ethanol precipitation of the DNA-containing fractions. After gel electrophoresis of labelled DNA, gels were dried and subjected to autoradiography.

Transfer of DNA fragments to DBM-paper

Preparation of DBM-paper, pretreatment of agarose gels, and transfer of DNA fragments were carried out as described by Alwine *et al.* (1977) and Wahl *et al.* (1979).

Hybridization

For prehybridization, the DBM-paper was sealed into a plastic bag containing preincubation buffer (50% formamide, 5 x SSC, 0.1% SDS, 1% glycine, 10 x Denhardt's, 250 μ g/ml denatured herring sperm DNA, 40 mM NaH_2PO_4 , pH 6.5) immediately after transfer and incubated for 3–12 h at 37°C. Denhardt's was 0.02% each of gelatine, polyvinyl pyrrolidone, and ficoll 400 (Denhardt, 1966). Hybridization was performed in 50% formamide, 5 x SSC, 0.1% SDS, 10 x Denhardt's for 24–48 h with 2×10^6 c.p.m. of nick-translated DNA (5×10^7 – 1×10^8 c.p.m./ μ g)/gel track. Nick-translation was carried out according to Maniatis *et al.* (1975). After hybridization, filters were washed three times with 1 x SSC, 0.1% SDS and three or more times with 0.1 x SSC, 0.01% SDS at 60°C followed by autoradiography.

Isolation of plasmid DNA from *H. halobium*

This was performed according to Pfeifer *et al.* (1981a).

Purification of chromosomal DNA

Cells which had grown to the stationary phase were collected by centrifugation, taken up in a small volume of basal salts (see above), lysed with sodium deoxycholate solution, and diluted 3–4-fold with 0.1 M Tris, 0.01 M EDTA, pH 8. The lysate was extracted with phenol, chloroform-octanol, and chloroform, and dialyzed against 0.02 M Tris, 0.001 M EDTA, pH 8. The resulting solution was diluted to an A_{260} of 10, CsCl was added to a density of 1.7 g/ml, and ethidium bromide to a final concentration of 0.5 μ g/ml. After centrifugation to equilibrium, two bands were clearly seen under u.v. light. The upper band, containing less material, corresponded to the AT-rich satellite DNA, the lower band to the plasmid-free chromosomal DNA. Both bands were collected and rebanded twice.

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