His1 and His2 are distantly related, spindle-shaped haloviruses belonging to the novel virus group, *Salterprovirus*

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Abstract

Spindle-shaped viruses are a dominant morphotype in hypersaline waters but their molecular characteristics and their relationship to other archaeal viruses have not been determined. Here, we describe the isolation, characteristics and genome sequence of His2, a spindle-shaped halovirus, and compare it to the previously reported halovirus His1. Their particle dimensions, host-ranges and buoyant densities were found to be similar but they differed in their stabilities to raised temperature, low salinity and chloroform. The genomes of both viruses were linear dsDNA, of similar size (His1, 14,464 bp; His2, 16,067 bp) and mol% G + C (∼40%), with long, inverted terminal repeat sequences. The genomic termini of both viruses are likely to possess bound proteins. They shared little nucleotide similarity and, except for their putative DNA polymerase ORFs, no significant similarity at the predicted protein level. A few of the 35 predicted ORFs of both viruses showed significant matches to sequences in GenBank, and these were always to proteins of haloarchaea. Their DNA polymerases showed 42% aa identity, and belonged to the type B group of replicases that use protein-priming. Purified His2 particles were composed of four main proteins (62, 36, 28 and 21 kDa) and the gene for the major capsid protein was identified. Hypothetical proteins similar to His2 VP1 are present in four haloarchaeal genomes but are not part of complete prophages. This, and other evidence, suggests a high frequency of recombination between haloviruses and their hosts. His1 and His2 are unlike fuselloviruses and have been placed in a new virus group, *Salterprovirus*.

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Introduction

At the extreme salinities found in salt lakes, the majority of cells are prokaryotes belonging to the archaeal family Halobacteriaceae (Grant et al., 2001). As in marine waters, the concentration of virus-like particles (VLPs) in salt lakes greatly exceeds that of the cells (Wommack and Colwell, 2000) and viruses appear to significantly limit the microbial population (Guixa-Boixareu et al., 1996). Direct electron microscopy of hypersaline waters has shown that spindle-shaped and round VLPs are the predominant morphotypes. Head-tail particles are less common (Guixa-Boixareu et al., 1996; Oren et al., 1997) but represent the majority of reported halovirus isolates (Dyall-Smith et al., 2003; Witte et al., 1997; Zillig et al., 1988). The first spindle-shaped halovirus, His1, was reported in 1998 (Bath and Dyall-Smith, 1998) and the first round halovirus, SH1, was described in 2005 (Porter et al., 2005). However, several years before the His1 report, spindle-shaped viruses of *Sulfolobus* (a thermophilic crenarchaeon) had been discovered and subsequently studied in elegant detail (Palm et al., 1991; Stedman et al., 2003; Wiedenheft et al., 2004; Zillig et al., 1996). These crenarchaeal viruses were classified in a novel family of archaeal viruses, the Fusellovirusidae, with the type species being SSV1 (for *Sulfolobus* spindle-shaped virus 1).

Since the isolation of SSV1, other spindle-shaped archaeal viruses (and VLPs) with similar properties have been described. These include several viruses of *Sulfolobus* that are similar to SSV1 (Prangishvili and Garrett, 2005; Stedman et al., 2003; Wiedenheft et al., 2004); PAV1, a *Pyrococcus* VLP (Geslin et al., 2003) that resembles SSV1 in genome
size and structure (18 kb, circular, dsDNA); and a VLP observed in cultures of *Methanococcus voltae* A3 (Wood et al., 1989) that has a circular, dsDNA genome (23 kb) and can integrate into the host chromosome (as does SSV1). All of these examples can replicate without obvious lysis of host cultures.

The resemblance of His1 to SSV1 in morphology and genome size led us to propose that His1 be included in the Fuselloviridae family (Bath and Dyall-Smith, 1998) but it was not known at that time if His1 shared a similar replication strategy or any sequence identity with members of this family. Here, we present the isolation and characteristics of a second spindle-shaped halovirus, His2, and show that His1 and His2 are distantly related to each other but are not related to members of the Fuselloviridae or to other spindle-shaped viruses. We also show that their genomes are linear and, probably possess terminal-bound proteins, and are likely to replicate via a protein-primed DNA polymerase. In addition, the major capsid protein of His2 has homologues in three of the five completed haloarchaeal genome sequences.

Results

Isolation, host range and plaque morphology of His2

The Pink Lakes (36° 24.221′ S, 141° 57.556′ E) are a group of relatively small (1.3 km² or less), closely spaced salt lakes in a protected national park in the remote, semi-arid northwest of Victoria, Australia. In 1995, a water sample collected from these lakes was plated on overlay lawns of *Haloarcula hispanica*, *Haloferax volcanii* and *Haloferax lucentense*. After incubation (2 days, 37 °C), a single plaque formed on the *Har. hispanica* lawn, and from this a halovirus, designated His2, was isolated that produced clear plaques of about 2 mm diameter, with an undefined hazy edge. The plaques were quite distinct from those of His1 (Bath and Dyall-Smith, 1998), a halovirus of *Har. hispanica* that was isolated in southern Victoria (Fig. 1). The optimum plaquing temperature for His1 was 30 °C, while His2 plaqued best at 37 °C. The plaque morphology of His2 was the same at both temperatures, but plaques took longer to appear when plates were incubated at 30 °C.

The host range of His2 was investigated by spotting a high titred virus lysate (10¹⁰ plaque-forming units (PFU) per ml) onto plate overlays containing one of 17 members of the family Halobacteriaceae (see Experimental procedures), including members of six genera and three additional species of *Haloarcula* (*Har. marismortui*, *Har. sinaiensis* and *Har. vallismortis*). Plaques were only observed on lawns of *Har. hispanica*.

His2-infected liquid cultures of *Har. hispanica* produced high virus titres (≥10¹⁰ PFU/ml). The culture optical density increased over the first 24 h incubation then decreased to reach a minimum at 2–3 days. Cultures generally remained turbid but, in about 10% of cases, complete lysis would occur in small volume cultures (10 ml). Lysis did not produce higher virus titres. The clear plaques and occasional lysis of liquid cultures indicated that His2 was lytic in nature. Attempts to isolate lysogens from infected cultures were unsuccessful, although uninfected virus-resistant strains, and carrier-state (persistently infected) strains, could be readily obtained. Cells of the latter strains did not contain virus DNA in a prophage state, either chromosomally integrated or as a plasmid (data not shown).

Stability of His2 virions

The stability of His2 and His1 under various conditions was compared and the results are summarised in Fig. 2. In high salt conditions, both viruses maintained their initial titres for at least 3 weeks when incubated at temperatures between 4 and 37 °C (data not shown). At temperatures above 50 °C, His2 lost infectivity rapidly, while His1 was slightly more resistant, losing infectivity above 60 °C (Fig. 2A). A large difference was observed in the stability of His1 and His2 to low salt: His2 lost infectivity immediately upon dilution while His1 only dropped about twofold in titre over 1 h (Fig. 2B). Both viruses were relatively stable over a wide pH range (Fig. 2C) and were sensitive to chloroform (Fig. 2D).
Prior to examining the single-step growth characteristics of the two viruses, a series of preliminary experiments were performed to determine the conditions that would maximise the proportion of infected cells. The multiplicity (3–60 PFU/cell), adsorption time (0–60 min) and incubation temperature (4–37 °C) were varied and the percentage of infected cells determined (data not shown). For His2, infection of 100% of cells was achieved by a 1 h adsorption time at 37 °C, and a multiplicity of 30. These conditions were also the best for infection of cells by His1, although only 25% of cells could be infected. Increasing the adsorption time to 3 h did not improve this percentage.

Single-step growth experiments were performed using early exponential phase cells infected with His1 or His2 under the conditions described above. Typical results are shown in Fig. 3. For His2, experiments performed in parallel, using identical conditions and virus, produced different growth curves and titres at 24 h after infection (Fig. 3A, 4 × 10^{11} PFU/ml; Fig. 3B, 5 × 10^{10} PFU/ml). In the example in Fig. 3A, a steep rise in titre was observed between 2 and 3 h after infection and the calculated ‘burst size’ at this time was 315 PFU/ml. In contrast, Fig. 3B shows a delayed and small initial rise at 4–5 h after infection (calculated ‘burst size’ of 10 PFU/ml at 5 h after infection), followed by a gradual increase over the next 5–24 h. For His1 (Fig. 3C), a slow initial rise in virus titre occurred, followed by a gradual increase over the next 10–24 h. Infected cultures grew more slowly than uninfected cultures grown under identical conditions (Fig. 3, open circles). The latent periods for both viruses were similar (4 h for His1 and 2–4 h for His2). For both viruses, the growth curves did not fit the classical pattern of lytic bacteriophages, such as T4. There was no phase of rapid cell lysis and the OD_{550} of the cultures rose steadily over 24 h.

**His2 purification**

Large-scale liquid cultures (500 ml) of His1- or His2-infected cells reached maximum titre at 2 days after infection and consistently produced 10^{11} to 10^{12} PFU/ml. His2 was purified using methods based on those used for the lipid-containing viruses PM2 (Kivelä et al., 1999) and SH1 (Porter et al., 2005). A typical His2 purification (Supplementary Table 1) gave a final virus titre of 7 × 10^{12} PFU/ml (6.6 × 10^{10} PFU A_{260}). The density
of His2 particles was 1.30 g/ml, which is similar to the density determined for His1 (1.28 g/ml) (Bath and Dyall-Smith, 1998). When examined by transmission electron microscopy (TEM) using a standard negative-stain method, His2 particles (purified or unpurified) were often rounded, distorted or completely disrupted (Fig. 4A). Glutaraldehyde fixation did not improve the preservation of particle morphology (data not shown). The least distorted virus particles were spindle-shaped (44 × 67 nm; average of 15 particles) and, occasionally, a short tail structure was visible at one end (Figs. 4B, C). These dimensions are similar to those of His1 (44 × 77 nm) (Bath and Dyall-Smith, 1998). The same purification method was not sufficient to fully purify His1, as it bound strongly to cellular material and flagella (data not shown).

Proteins of purified His2

The proteins of purified virus preparations of His2 were separated by SDS-PAGE and stained using Brilliant Blue G. Four proteins were detected (designated VP1–VP4); a major band at 62 kDa and three smaller, less intensely stained bands of 36, 28 and 21 kDa, respectively (data not shown). No difference was seen when proteins were analysed under nonreducing conditions (data not shown). Tryptic peptides of VP1 and VP2 were analysed by MALDI-TOF. VP1 gave a clear result, with six peptides closely matching in MW to those predicted from ORF 29 (Supplementary Fig. 1; and see below). In addition, the sequence QWEAKALAYDYAAR was predicted from collision-induced dissociation data of one of these peptides (1655.78 Da), and this matched an internal tryptic peptide of ORF 29. Curiously, VP1 tryptic peptides that contained Asn-X-Ser/Thr motifs and were expected to be detected by MS were absent from the spectra. The full protein sequence of VP1 (derived from the His2 genome sequence, see below) is predicted to contain a signal sequence, several potential N-glycosylation sites and a membrane anchor region, suggesting that VP1 may be transported to the external surface of the host cell and glycosylated. However, when separated on an SDS-PAGE gel, VP1 did not show a reaction with a conventional periodic acid-schiff (PAS) stain (data not shown). VP2 did not give an interpretable result from MALDI-TOF.

Nature of the His2 genome

Nucleic acids were isolated from purified His2 virus preparations (with protease treatment), digested with restriction
enzymes and separated by agarose gel electrophoresis. Uncut DNA ran as a single, well-resolved band at around 16 kb. The DNA could be digested to discrete fragments by several type II restriction enzymes, e.g. *Ase*1, *Dra*I and *Hind*III. Examples of *Sac*II and *Tsp*RI digests are shown in Fig. 5. The fragments of each digest added up to the size of the undigested DNA and there were no bands that were under-represented or diffuse, indicating that the genome ends were not heterogeneous. The genome could be digested inwards from the termini by exonuclease Bal-31, and this was used in conjunction with restriction enzymes to construct a restriction map of the His2 genome (data not shown). The map was distinct from that of His1 (Bath and Dyall-Smith, 1998).

Together, the results of gel electrophoresis and enzyme treatments indicated that the His2 genome was linear, double-stranded DNA and slightly larger than the His1 genome (Bath and Dyall-Smith, 1998).

**Terminal proteins**

The genome sequences of His1 and His2 (see below) contained inverted repeat sequences (ITRs) at their termini. In addition, their putative DNA polymerases were similar to those of viruses and plasmids that replicate via protein priming. These features suggested that His1 and His2 may possess covalently bound terminal proteins (Grahn et al., 1994). To test this, the gel mobilities of His2 restriction fragments prepared from DNA that had been protease treated or not treated were compared. Mobility changes could not be detected in terminal fragments greater than 1 kb (data not shown) but small shifts were observed for both His1 (data not detected in terminal fragments greater than 1 kb (data not shown) and His2 in terminal fragments below this size. Fig. 5 shows His2 DNA restricted so as to give left-terminal (*Sac*II) and right-terminal (*Tsp*RI) fragments of 837 and 948 nt, respectively. These fragments (arrowed in Fig. 5) electrophoresed more slowly if they came from DNA that was not protease-treated.

![Fig. 5. Detection of terminal proteins of His2. DNA was extracted from purified virus particles either with protease (+) or without protease treatment (−). Terminal fragments of the expected size are indicated by white arrowheads. MW, DNA size standard (sizes in kb are given at the left).](image)

**Sequences of His1 and His2 genomes**

The linear, dsDNA genomes of both His1 and His2 were completely sequenced using a combination of cloned fragments and primer-walking on virus DNA (see Experimental procedures). The His1 genome was 14,464 bp and His2 was 16,067 bp (Genbank accessions AF191796 and AF191797, respectively). The palindromic CTAG was absent from both genomes, and the palindrome GATC was absent from His2. In the His1 genome, the GATC palindrome displayed a strikingly uneven distribution, with the left half (1–7575) having no sites, and the right half having 16, evenly spread sites (Fig. 6). The related tetramers CGAG and GCTC were strongly under-represented in both genomes. The termini of His1 and His2 possessed ITR sequences; His1 having an imperfect repeat of approximately 105 nt, and His2 having a perfect ITR of 525 nt. There was no nucleotide similarity between His1 and His2 ITRs, and only one region of very weak nucleotide similarity between the entire genomes; that encoding the predicted DNA polymerases (see below).

Using a number of genome annotation tools (see Experimental procedures), the putative ORFs and sequence features of the two genomes were examined, and the results are summarised in Fig. 6 and Supplementary Tables 1 and 2. Each genome was predicted to contain 35 ORFs, most of them very closely spaced and many of them overlapping (16/35 for His1; 11/35 for His2). The majority of predicted proteins had pI values less than 7 (22/35 for His1; 25/35 for His2), as would be expected for halohyphal protein (Ng et al., 2000). Cumulative AT- and GC-skew plots of His1 and His2 supported the strand assignments of the predicted ORFs, with major inflections (circled in Fig. 6) occurring at points where transcription would be expected to switch from one strand to the other, as found previously with the halovirus HF2 genome (Tang et al., 2002). The predicted ORFs of His2 formed a simple pattern, being directed outwards towards the termini from a central region containing several short repeats. The His1 ORFs were similarly arranged except for an apparent inversion of a segment that included the DNA polymerase gene (Fig. 6). Many of the predicted ORFs contained Cys-X-X-Cys motifs that may indicate the presence of zinc finger DNA binding domains (Iuchi, 2001).

A comparison of the predicted proteins of both viruses found that only the DNA polymerases showed significant similarity (see below). When all the ORFs were compared to those in the sequence databases, only a small number of significant matches were found (4 for His1, 5 for His2), and most of these were to hypothetical proteins of unknown function. Just three ORFs from the two viruses could be assigned putative functions: the two virus DNA polymerases (by sequence similarity) and the major virus capsid protein (VP1) of His2 (by analysis of purified virus proteins, see below). While there was no significant sequence similarity between His2 VP1 and any of the His1 ORFs, the characteristics of His1 ORF27 protein strongly resemble those of VP1, including its predicted size, pI, presence of a signal sequence, glycosylation sites, a carboxy-terminal
membrane anchor region (Supplementary Tables 2 and 3) and its genomic position relative to the DNA polymerase (Fig. 6).

The mol% G + C of both genomes was 39–40 while that of its host, Har. hispanica, is 62.7 (Juez et al., 1986). The 20% difference in mol% G + C between the viral genomes and that of their host was analysed further. The full genome sequence of Har. marismortui (a close relative of Har. hispanica, and with a similar mol% G + C) was used to calculate the Codon Adaptation Index (CAI) for His1 and His2 when growing in these cells. The CAI measures how well the codon usage of each virus matches that of their host (Sharp and Li, 1987). Both His1 and His2 had low CAI values (0.16 and 0.19, respectively), indicating that they may not be well adapted to replicate in Haloarcula. No tRNA genes could be detected in the virus genomes using the program tRNAscan-SE (Lowe and Eddy, 1997).

The predicted DNA polymerase proteins of these viruses showed an overall amino acid identity of 42% but the best alignments required several insertions/deletions of between 1 and 38 residues (Fig. 7). Comparison to the sequence databases showed that they were most similar to the type B DNA polymerases found in plant and fungal mitochondria, and certain viruses. This subset of polymerases is unusual in that it uses proteins attached to the 5′ termini of linear dsDNA genomes to prime replication. His1 polymerase was most similar to the mitochondrial polymerase of Daucus carota (E = 10−5), while the His2 polymerase was most similar to those of tectiviruses, such as Bacillus thuringiensis phage GIL16c (E = 10−3) (Verheust et al., 2005). The predicted virus proteins contained all the characteristic sequence motifs found in this family of polymerases (Braithwaite and Ito, 1993), including the highly conserved motif, Kx3NSxYG, also known as motif B (found in Region 2A), that has been shown to be involved in both template/primer binding and dNTP selection (Saturno et al., 1997). VP1 of His2 (see Supplementary Table 3, ORF 29) was found to have related sequences in four haloarchaea, three of which have been completely sequenced. Har. marismortui, Hfx. volcanii and Natronomonas pharaonis each encode two VP1-like proteins, and the plasmid pHK2 of Hfx. lucentense encodes one. On closer inspection, the ORFs encoding VP1-like proteins

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**Fig. 6.** Diagram of the His1 and His2 genomes. The major features are shown, including the predicted ORFs (yellow and green arrows), putative DNA polymerases (blue arrows), VP1 and its His1 homologue (red arrows), terminal inverted repeat sequences (purple arrowheads) and regions of nucleotide sequence repeats (grey boxes). Scale bars, in bp, are shown above each genome diagram. ORFs are numbered (left to right), and some have been named (pol, DNA Polymerase; VP1, major capsid protein) or the accession numbers of closely related sequences given. The template strand for each ORF is indicated by the direction of arrows and their position above or below the black line. The vertical arrows above the His1 genome scale bar show positions of the sequence GATC (see text for details). Plots of cumulative GC-skew (solid black line) and AT-skew (dashed blue line) are shown below each genome diagram, with inflection points indicated by circles.
in *H. marismortui* and *Hfx. volcanii* form parts of longer regions of similarity to His2 that include three downstream ORFs, corresponding to His2 ORFs 30, 31 and 33 (Fig. 8). Beyond these tight clusters of His2-like ORFs, the similarity to His2 disappears.

**Discussion**

Spindle-shaped viruses have so far only been found in Archaea. These viruses are commonly observed in environments rich in thermophilic or extremely halophilic Archaea and, in the former organisms, are represented by numerous isolates (Prangishvili and Garrett, 2005; Wiedenheft et al., 2004). The results of the present study show that the spindle morphotype does not represent a homogenous virus lineage, but instead encompasses viruses with radically different replication strategies and phylogenies. Members of the thermophilic Fuselloviridae are temperate viruses with circular dsDNA genomes that do not encode a DNA polymerase. In contrast, the group represented by His1 and His2 are lytic viruses with linear, dsDNA genomes that contain ITR sequences and terminal proteins, and encode their own DNA polymerase. These haloviruses probably replicate via protein-priming, a mode that has not been reported previously in archaeal viruses (or indeed, in Archaea). In addition to the gel-shift results, the presence and significance of the terminal proteins have been demonstrated by nuclease protection, the specific binding of terminal restriction fragments to silica, and the ability of viral DNA to transfect host cells (manuscript in preparation). Protein-primed replication is widespread although not common in nature and includes some mammalian viruses (e.g. adenovirus), bacteriophages (e.g. Φ29, PRD1, Cp-1), linear Streptomyces plasmids and plant mitochondrial DNA (Salas, 1991). The round halovirus, SH1, may also use this strategy as its genome is linear dsDNA, with ITR sequences (Bamford et al., 2005) and terminal proteins attached (unpublished data).

Spindle-shaped viruses appear to be widespread, having been observed in hypersaline waters in Europe (Díez et al., 2000; Guixa-Boixareu et al., 1996) and the Middle East (Oren et al., 1997), and isolated in Australia (Bath and

![Alignment of the predicted DNA polymerase proteins of His1 and His2](image)

Fig. 7. Alignment of the predicted DNA polymerase proteins of His1 and His2. Identical amino acids are shown by white text on a dark background, and similar amino acids are indicated by black text with a surrounding box. Dots represent gaps introduced to maximise the sequence identity of the alignment. Conserved sequence motifs found in family B DNA polymerases are indicated (e.g. Region 1), and the most conserved residues of these motifs indicated by asterisks.
Fig. 8. Diagram of the right end of the His2 genome, aligned with one of the two regions in each of the *Har. marismortui* and the *Hfx. volcanii* genomes that show clear similarity to ORF 29 protein (encoding VP1) and nearby ORFs. Shaded regions between ORFs of the two genomes indicate similar predicted protein sequences, with the percentage amino acid identity (to the His2 proteins) indicated. The ORF numbers for His2 are the same as in Supplementary Table 3, and the ORFs for *Har. marismortui* are the GenBank accessions. The rulers above and below show scale and nucleotide positions. AAA, ATPase motif (CDD 25315) found in His2 ORF 33 and the corresponding ORFs in *Har. marismortui* (tm2294) and *Hfx. volcanii*. The *Hfx. volcanii* genome has not yet been annotated, and nt numbers refer to contig 446 of the draft genome (www.tigr.org). For reference, the predicted amino acid sequence of the VP1-like ORF of *Hfx. volcanii* that is shown in this figure is given in Supplementary Fig. 2.

Dyall-Smith, 1998) (this study). His1 was isolated from a coastal salt pan crystalliser and His2 from an inland salt lake some 174 km distant, yet the two haloviruses share a similar morphology, host range and genome structure. Their DNA polymerases are clearly related at the amino acid level and possess all the characteristic sequence features of family B DNA polymerases that use protein priming. However, the halovirus polymerases appear to be only distantly related to other known members of this family. The weak nucleotide similarity between His1 and His2 DNA polymerase genes, and the significant number of gaps required to align their predicted amino acid sequences suggest that these two isolates are members of a very diverse virus family, a proposition that is supported by the lack of any significant nucleotide (or predicted amino acid sequence) similarity outside of these genes. Metagenomic studies of hypersaline environments should provide a clearer picture of the significance and diversity of this and other halo virus groups.

The ability of His1 and His2 to exit cells without causing cell lysis is a feature that is shared with halovirus SH1 and members of the Fuselloviridae (Porter et al., 2005; Prangishvili et al., 2001; Xiang et al., 2005). The crenarchaeal spindle-shaped viruses, such as SSV1 and STSV1, are believed to exit through the membrane of their host, *Sulfolobus*, without lysis, although they do slow cell growth. However, His2 and His1, unlike fuselloviruses, show occasional lysis of infected-cell cultures, and the clear plaques and the inability to isolate lysogens indicate that they are virulent. The nature of their interaction with host cells requires further investigation, but it appears that they enter a kind of chronic infection that maximises virus production (by allowing prolonged cell survival while constantly producing virus) but slows cell growth and eventually leads to cell death and lysis.

The mol% G + C of previously sequenced halovirus genomes ranges from 56–70% (Dyall-Smith et al., 2003), values that are similar to their hosts. It was surprising then to find that the His1 and His2 genomes were around 40 (mol% G + C), or about 20% lower than that of *Har. hispanica*. The calculated CAUs suggested that His1 and His2 are poorly adapted to grow in *Haloarcula*, and no virus-encoded RNA genes were discovered that could have compensated for this difference. Perhaps these viruses have alternative hosts with genomes of lower GC content, but all current members of the family Halobacteriaceae have GC-rich DNA (Grant et al., 2001). However, our isolate of the square haloarchaeon of Walsby (Burns et al., 2004) is estimated to have a relatively low mol% G + C of 47 (J. Eisen, personal communication), and this organism has been observed to be commonly infected with lemon-shaped VLPs (Guixa-Boixareu et al., 1996; Oren et al., 1997).

Another interesting feature of the His1 and His2 genomes was the avoidance of certain tetrameric sequences, particularly CTAG and its reverse, GATC. On average, such sequences would be expected to occur about once every 256 nt, but these palindromes are absent in the genomes of haloviruses HF1, HF2 and SH1 (accessions NC_004927, NC_003345, NC_007217). They are present in halovirus *ΦCh1* (accession NC_004084). The elimination of palindromic target sequences in virus genomes is thought to be due to strong selection pressure by restriction–modification (R–M) systems operating in host cells (Bickle and Krüger, 1993), and there is evidence that CTAG and GATC sequences are used by haloarchaeal R–M systems. For example, CTAG sites are methylated in the *Hfx. volcanii* genome (Allers and Mevarech, 2005), while Dam-methylated DNA (i.e. containing GmATC sequences) entering these cells is restricted (Holmes et al., 1991). The extremely asymmetrical distribution of GATC sites in His1 is remarkable, and is probably the result of a recombination event between two parental viruses, one of which had many GATC sites, and the other none. Evidence for recombination between haloviruses has been described previously for HF1 and HF2 (Tang et al., 2004).

The major capsid protein of His2 is predicted to be exported and membrane-anchored. The presence of lipids in His2 (and His1) is supported by chloroform sensitivity results and the flexible nature of virus particles seen by EM, but direct chemical analyses are required to prove this. The detection of homologues of VP1 in haloarchaeal genomes is intriguing, particularly as three of these species (*Har. marismortui*, *Hfx. volcanii* and *Nmm. pharaonis*) each carry two copies. In the first two species, these homologues are part of longer segments of similarity (and conserved gene arrangement) to His2. For example, one of the *Har. marismortui* segments includes a VP1-like ORF as well as three downstream His2-like ORFs (Fig. 8). These segments are not part of His2-like prophage genomes as the sequences flanking these ORFs: (a) are not similar to His2
Bacto-agar (Difco Laboratories, Detroit, USA) was added to

Experimental procedures

Media

A concentrated salt stock solution (30% wt vol\(^{-1}\)) of artificial salt water (SW) was prepared as described in the online handbook, The HaloHandbook (http://www.microbiol.unimelb.edu.au/people/dyallsmith/HaloHandbook/) and contained per litre: 240 g NaCl, 30 g MgCl\(_2\)-6H\(_2\)O, 35 g MgSO\(_4\)-7H\(_2\)O, 7 g KCl, 0.5 g CaCl\(_2\)-2H\(_2\)O, adjusted to pH 7.5 using a small volume (about 2 ml) of 1 M Tris, 12, 18 or 23% (vol/vol) SW were prepared as previously described (Bamford et al., 2005). Bacto-agar (Difco Laboratories, Detroit, USA) was added to give solid (15 g/L) or top-layer (7 g/L) media.

Haloarchaeal and haloviral strains and growth conditions

The haloarchaeal strains used in this study include *Har. hispanica* ATCC 33960\(^T\) (Juez et al., 1986), *Har. marismortui* ATCC 43049\(^T\) (Oren et al., 1990), “Har. sinaiensis” ATCC 33800 (Toreblanca et al., 1986), *Har. vallismortis* ATCC 29715\(^T\) (Gonzalez et al., 1978), *Halobacterium salinarum* NCIMB 786, *Hbt. salinarum* NCIMB 763, *Hbt. salinarum* NCIMB 777, *Halobacterium* sp. GRB (kindly supplied by U. Rdest) (Soppa and Oesterhelt, 1989), *Haloferax gibbonsii* ATCC 33959 (Juez et al., 1986), *Hfx. lucentense* JCM 9276\(^T\) (Gutierrez et al., 2002), *H. mediterranei* ATCC 33500\(^T\), *Hfx. volcanii* NCIMB 2012 (Toreblanca et al., 1986), *Haloarcula coriense* ACM 3911\(^T\) (Kamekura and Dyall-Smith, 1995), *Haloarcula lacusprofundi* ATCC 49239\(^T\) (Frimanzan et al., 1988), *H. saccharovorum* NCIMB 2081 (Ventosa et al., 1999), Haloterrigena turkenimensis NCMB 784 (Ventosa et al., 1999) and Natrinema asiatica 172P1 JCM 9576\(^T\) (Kamekura and Dyall-Smith, 1995). Haloarchaeal cultures were grown in MGM containing a salt concentration appropriate for each strain and were incubated with shaking (except ‘Har. sinaiensis’, which was not shaken) at 37 °C.

The halovirus strains used in this study include His1 (Bath and Dyall-Smith, 1998) and the novel halovirus His2 (described in this study). Virus cultures were grown by infection (multiplicity of 0.1) of an early exponential *Har. hispanica* culture in 18% MGM. Incubation was at 30 °C (His1) or 37 °C (His2), with shaking for 1 to 4 days. For virus lysates, the cell debris was pelleted (5000 × g, 10 min, room temperature) and the supernatant filtered through a 0.45 μm then a 0.2 μm Millipore filter (Sartorius AG, Germany), and stored at 4 °C. Virus titres were determined by plaque assay on 18% MGM with an indicator lawn of *Har. hispanica*, using the method previously described (Nuttall and Dyall-Smith, 1993). Plates were incubated at 30 °C (His1) or 37 °C (His2) for 48 to 72 h for visible plaques.

His2 isolation

A water sample from the Pink Lakes, Victoria, Australia (36° 24.221′ S, 141° 57.556′ E) was centrifuged (5000 × g, 10 min, RT) and filtered (0.25 μm membrane filter) to remove cells and particulate material. This water was then screened directly for infectious viruses by plaque assay using indicator lawns of *Har. hispanica*, *Hfx. volcanii* or *Hfx. lucentense*. A single plaque was obtained from the *Har. hispanica* lawn on 18% MGM, incubated at 37 °C. After two rounds of plaque purification, this virus was designated His2.

His2 host range

Concentrated virus solutions (10\(^{10}\) PFU/ml) were spotted onto haloarchaeal lawns and incubated at both 30 °C and 37 °C until the lawn had adequately grown. A range of salt concentrations (12 to 23%) was examined in an attempt to stimulate plaquing (Daniels and Wais, 1990; Torsvik and Dundas, 1980).
His2 stability

After each treatment, samples were removed, diluted in HVD and virus titres determined by plaque assay. Temperature stability was tested by incubation of virus lysates at various temperatures for 1 h, after which samples were removed and cooled to 4 °C before plaque assay. Sensitivity to pH was tested by dilution (1:100) of the halovirus lysates in the appropriate pH buffer (HVD, buffered with Tris–Cl for pH 7–10, or potassium phosphate for pH 4–6). Virus was incubated for 30 min at room temperature with constant agitation then diluted in standard HVD (pH 7.5). Sensitivity to chloroform was tested by exposure of virus stocks to solvent, in a volume ratio of 1:4 (chloroform: virus). Incubation was at room temperature, with constant agitation. Stability in low salt was tested by diluting virus lysates (grown in 18% MGM) 1000-fold with pure water. Incubation was at room temperature.

His2 single-step growth curve

A 10 ml culture of early exponential phase Har. hispanica cells (23% MGM, 37 °C, shaken at 180 rpm) was centrifuged (5000 × g, 15 min, RT) and the pelleted cells resuspended in 10 ml of 18% MGM containing virus at an MOI of 30. After an adsorption period of 1 h at 37 °C, the cells were washed free of virus by centrifuging the cells (as above), discarding the supernatant and resuspending the cells in 10 ml of fresh medium (18% MGM). This was repeated two further times, after which the cell suspension centrifuged again, and the cell pellet resuspended in 100 ml of 18% MGM and incubated either at 30 °C (His1) or 37 °C (His2), with slow shaking (100 rpm). Samples were taken at hourly intervals and the OD550 and number of infective centres determined immediately.

Virus purification

To produce large cultures of His1 and His2, 500-mL cultures of early exponential Har. hispanica in 18% MGM were infected at a multiplicity of 0.1. The infected cultures were incubated aerobically at 30 °C (His1) or 37 °C (His2), with shaking (100 rpm) for 3–7 days. Although the cultures did not clear, purification proceeded if the titre was determined to be >1010 PFU/ml. Cell debris was removed by low speed centrifugation (Sorvall GSA; 6000 rpm, 30 min, 10 °C). For His2, virus was pelleted from the supernatant by centrifugation through a cushion of 30% (wt vol−1) sucrose (in HVD) (Beckman SW28; 20,000 rpm, 10 h, 10 °C). The pellet was resuspended in HVD, loaded onto a linear 30–70% sucrose gradient and centrifuged for 2 h (Beckman SW28; 23,000 rpm, 10 °C). The virus band was harvested and re-centrifuged to equilibrium in a 1.3 g/ml CsCl (in HVD) solution (Beckman 70Ti; 60,000 rpm, 20 h, 10 °C). The resulting virus band was harvested, diluted in HVD and virus pelleted (Beckman SW55; 35,000 rpm, 75 min, 10 °C). The virus pellet was resuspended in HVD and stored at 4 °C.

For His1, the cell debris was removed as above, then virus pelleted (without a sucrose cushion) (Beckman SW28; 20,000 rpm, 10 h, 10 °C). The pellet was resuspended in HVD, loaded onto a linear 30–70% sucrose gradient and centrifuged (Beckman SW28; 23,000 rpm, 2 h, 10 °C). The resulting virus band was harvested, diluted in HVD and pelleted (Beckman SW55; 35,000 rpm, 75 min, 10 °C). The virus pellet was resuspended in HVD and stored at 4 °C.

His2 negative-stain transmission electron microscopy

Halovirus preparations were examined by negative-stain TEM. Formvar (polyvinylformaldehyde) coated 400-mesh copper grids (ProSciTech, Australia) were placed (plastic side down) onto a 20 μl drop of sample and the virus particles were allowed to absorb for 7 min. Grids were then placed on a 20 μl drop of 2% (w/v) uranyl acetate (May and Baker Ltd., England) for 30 s. Alternatively, virus was diluted 1:10 in MES-buffered HVD and fixed in 2.5% glutaraldehyde for 30 min prior to staining as above with uranyl acetate. Excess stain was removed with filter paper and the grid was allowed to air dry for 5 min. Grids were examined on a Philips CM120 BioTwin transmission electron microscope, operating at 120 kV.

Preparation and analysis of virus DNA

For viral DNA extraction, two methods were used. In the first, samples of purified virus preparations were diluted one in 10 in water, and DNA was extracted using a conventional SDS-proteinase K method (Ausubel et al., 1994). After the SDS/proteinase K treatment, protein was removed by a phenol–chloroform extraction, and the DNA precipitated with ethanol. The final DNA pellet was dissolved in water. A second method was used to obtain DNA that still retained the terminal proteins. The diluted virus preparations were incubated with 0.1% SDS only (i.e. without protease) at 37 °C for 1 h, then the soluble proteins removed by phenol–chloroform extraction, and the DNA ethanol precipitated and dissolved in water or TE buffer (10 mM Tris–Cl, 0.1 mM EDTA, pH 8.0). Genomic DNA and restriction fragments were separated on 0.8–1.0% agarose gels in Tris–acetate–EDTA electrophoresis buffer (Ausubel et al., 1994).

Restriction endonucleases and Bal-31 nuclease were purchased from New England Biolabs Inc., Ipswich, MA, USA. All reagents were used according to the manufacturer’s instructions.

Protein profiles and mass spectroscopy

His2 structural proteins were analysed by SDS-PAGE using NuPAGE Novex Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) and stained with Brilliant Blue G. Protein sizes were estimated by comparison to Broad Range (2–212 kDa) Protein Markers (New England Biolabs, Inc., Ipswich, MA, USA). For MS analysis, protein bands of interest were excised from the gel and sent to the Australian Proteomics Analysis Facility (Macquarie University, NSW) for trypsin digestion and analysis by MALDI-
TOF MS using an Applied Biosystems 4700 Proteomics Analyser. The sample was irradiated with a Nd:YAG laser (355 nm), and spectra were acquired in reflectron mode with a mass range 750 to 3500 Th.

**Sequencing and bioinformatics analyses**

Viral DNA was sequenced on both strands using a combination of clones and primer walking (directly on viral DNA). Automated dsDNA sequencing PCR reactions were performed using the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit or the ABI PRISM BigDye Terminator Mix v2 or v3 according to the manufacturer’s instructions (Applied Biosystems, USA). Sequencing reactions were separated on Applied Biosystems DNA sequencing machines at the following facilities: the Applied Genetic Diagnostics Sequencing Service, Department Pathology, University of Melbourne; the Department of Microbiology and Immunology, University of Melbourne; and the Australian Genome Research Facility DNA Sequencing Laboratory, Melbourne.

DNA sequences were edited and aligned with Sequencer (Gene Codes Corporation, USA). Analysis and annotation of the His1 and His2 genome sequences, including ORFs and ITR sequences, were carried out using a number of programs, including Glimmer2 (http://www.tigr.org/software/) (Delcher et al., 1999), Sequin (http://www.ncbi.nlm.nih.gov/Sequin) and DNA Strider 1.2 (CEA, France). Protein pf, molecular weights, amino acid compositions, codon usage and other protein analyses were performed with DNA Strider 1.2 (CEA, France), and the proteomic programs available at the ExPASy mirror site (http://au.expasy.org) of the Swiss Institute of Bioinformatics. Predicted ORFs were based on a number of criteria. Most ORFs were included based on significant \( \geq 10^{-10} \) matches to entries in the GenBank database and/ or being \( \geq 40 \) aa long and predicted by Glimmer2. Finally, if manual inspection revealed ORFs with start and/or stop codons that overlapped the ends of already included ORFs and filled in gaps within long clusters of closely spaced ORFs, then these were also included.

Sequence similarity comparisons (BLASTN, BLASTP) were carried out against the NCBI nonredundant sequence databases (http://www.ncbi.nlm.nih.gov/). Sequence alignments were performed using ClustalW (http://www.ebi.ac.uk/clustalw) and T-Coffee (http://www.ch.embnet.org/software/TCoffee.html). DNA sequences were scanned for potential tRNA genes using the online base composition tools at http://molbiol-tools.ca/Jie_Zheng/. Preliminary sequence skew plots were produced using the online base composition tools at http://wustl.edu/eddy/tRNAscan-SE. Cumulative GC-skew and AT- tRNAscan-SE 1.21 (Lowe and Eddy, 1997)(http://www.genetics.wustl.edu/eddy/tRNAscan-SE). Protein p

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2006.02.005.

**References**


