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

Carolyn Bath\(^1\), Tania Cukalac, Kate Porter and Michael L. Dyall-Smith*

Department of Microbiology and Immunology, The University of Melbourne, Parkville, Victoria 3010, Australia

\(^1\)Now at Animal Genetics and Genomics, Primary Industries Research Victoria, 475 Mickleham Rd, Attwood, Victoria 3049, Australia

**Running Head:** Haloviruses His1 and His2

*Correspondence Footnote*

Dr M. L. Dyall-Smith, Department of Microbiology and Immunology

The University of Melbourne, Parkville

Victoria, 3010 Australia.

Telephone: +613 8344 5693

Facsimile: + 613 9347 1540

E-mail: mlds@unimelb.edu.au

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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 possessed 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.
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, Bratbak, and Heldal, 1997) but represent the majority of reported halovirus isolates (Dyall-Smith, Tang, and Bath, 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; Zillig et al., 1996). These crenarchaeal viruses were classified in a novel family of archaeal viruses, the *Fuselloviridae*, 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); 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, Whitman, and Konisky, 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 genome structure, 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 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°24'22.1" S, 141°57'55.6" 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 (see Fig. 1).

The host range of His2 was investigated by spotting a high titred virus lysate ($10^{10}$ plaque-forming units (PFU) per ml) onto plate overlays containing one of seventeen members of the family Halobacteriaceae (see Materials and Methods), including members of six genera and three additional species of *Haloarcula* (*Har. marismortui*, *Har. sinaiiensis* and *Har. vallismortis*). Plaques were only observed on lawns of *Har. hispanica*.

His2-infected liquid cultures of *Har. hispanica* produced high virus titres ($\geq 10^{10}$ PFU/ml) but generally remained turbid. Occasionally, complete lysis would occur in small volume cultures (10 ml), but this did not produce higher virus titres. The clear plaques and occasional lysis of liquid cultures indicated His2 was lytic in nature. Lysogens could not be isolated (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). Both viruses were relatively stable over a wide pH range (Fig. 2C) and were sensitive to chloroform (Fig. 2D). 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 two-fold in titre over 1 h (Fig. 2B).

Single-step growth

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. 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. For His1 (Fig. 3C), a slow initial rise in virus titre occurred, followed by a gradual increase over the next 10–24 h. For His2, experiments performed in parallel, using identical conditions and virus, produced different growth curves and titres at 24 h after infection (Fig. 3A, $4 \times 10^{11}$ PFU/ml; Fig. 3B, $5 \times 10^{10}$ PFU/ml). In the example in Fig. 3A, a steep rise in titre was observed between 2–3 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.

**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 (Table 1, supplementary material) gave a final virus titre of $7 \times 10^{12}$ PFU/ml ($6.6 \times 10^{10}$ 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, purified His2 particles were often rounded, distorted or completely disrupted (Fig. 4). Glutaraldehyde fixation did not improve the maintenance 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 (Fig. 4B). 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 (Fig. 5). 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. No difference was seen when proteins were analysed under non-reducing 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 (Figure 1, supplementary material; and see below). In addition, a de-novo sequence (QWEAKALAYDYAAR) from one of these peptides (1655.78 Da) matched that predicted for 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 (Fig. 9A), 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, 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*I, *Dra*I and *Hind*III. Examples of *Sac*II and *Tsp*RI digests are shown in Fig. 6. 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 shown) and His2 in terminal fragments below this size. Fig. 6 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 6) electrophorese more slowly if they come from DNA that was not protease-treated. These terminal proteins were essential for
replication, as protease treated His2 (or His1) DNA was unable to transfect host cells (manuscript in preparation).

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 Materials and Methods). The His1 genome was 14,464 bp and His2 was 16,067 bp (Genbank accessions AF191796 and AF191797, respectively). The palindrome CTAG was absent from both genomes, and the inverse 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. 7). 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 Materials and Methods) the putative ORFs and sequence features of the two genomes were examined, and the results are summarised in Fig. 7 and 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 haloarchaeal proteins (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. 7) 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. 7).

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).

The mol% G+C of both genomes was 39-40 while that of their 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. 8). Comparison to the sequence databases showed 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, Fornelos, and Mahillon, 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, Kx$_3$NSxYG, 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 Table 2, ORF 29) has similar sequences in four haloarchaea, three of which have been completely sequenced. *Har. marismortui, Hfx. volcanii* and *Natronomonas pharaonis* each encodes two VP1-like proteins, and the plasmid pHK2 of *Hfx. lucentense* encodes one. On closer inspection, the ORFs encoding VP1-like proteins in *Har. 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. 9). 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 common parasites of thermophilic and extremely halophilic archaea and, in the former organisms, are represented by numerous isolates (Prangishvili and Garrett, 2005). 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 almost certainly replicate via protein-priming, a mode that has not been reported previously in archaeal viruses (or indeed, in Archaea). 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). Round haloviruses, such as SH1, may also use this strategy. The SH1 genome is linear dsDNA, with ITR sequences (Bamford *et al.*, 2005) and terminal proteins attached (unpublished data). However, the identity and type of DNA polymerase used by SH1 is not currently known.

Spindle-shaped viruses appear to be widespread, having been observed in hypersaline waters in Europe (Diez *et al.*, 2000; Guixa-Boixareu *et al.*, 1996) and the Middle East (Oren, Bratbak, and Heldal, 1997), and isolated in Australia (Bath and Dyall-Smith, 1998)(this study). His1 was isolated from a coastal saltern 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 closely 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 halovirus 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, Stedman, and Zillig, 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, Tang, and Bath, 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 CAIs suggested that His1 and His2 are poorly adapted to grow in Haloarcula, and no virus-encoded tRNA 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, Bratbak, and Heldal, 1997).

Another interesting feature of the His1 and His2 genomes was the avoidance of certain tetrameric sequences, particularly CTAG and its inverse, GATC. These palindromes are absent in the genomes of haloviruses HF1, HF2 and SH1 (accessions NC_004927, NC_003345, NC_007217), but 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, both palindromes are methylated in Hfx. volcanii (Allers and Mevarech, 2005), and DNA entering Hfx. volcanii cells and that is not Dam-methylated at GATC sites, is restricted (Holmes, Nuttall, and Dyall-Smith, 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, Nuttall, and Dyall-Smith, 2004).

The major capsid protein of His2 is predicted to be exported and membrane-anchored, and the presence of homologues of VP1 in haloarchaeal genomes is intriguing, particularly as three of these species (Har. marismortui, Hfx. volcanii and Nmn. 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. 9B). These segments are not part of His2-like prophage genomes as the sequences flanking these ORFs: a) are not similar to His2 (or His1); b) vary considerably between genomes, and copies within the same genome; c) include cell metabolic genes; and d) include no recognisable viral DNA polymerase nearby or indeed anywhere in these genomes. Another intriguing relationship is that between His1 ORF 13 and related ORFs of Natronomonas, Haloarcula, and halovirus SH1. In the latter case, it is ORF 15 of SH1, which does not encode a virus structural protein but, like His1 ORF 13, is predicted to contain three transmembrane regions (Bamford et al., 2005). The cell homologues of His1 ORF 13 do not appear to be part of His1 or SH1 prophages, as cell metabolic genes closely flank them. Both of these examples (VP1 and ORF 13) suggest that frequent recombination events occur between these viruses and their hosts.

In the relatively few cases where the predicted ORFs of His1 and His2 showed significant matches to known sequences (excluding the DNA polymerase genes), the His1 predicted ORFs tended to show greatest similarity to Nmn. pharaonis ORFs while the His2 ORFs tended to be most closely related to ORFs of Har. marismortui. Even when a predicted
ORF of one virus showed a close match to an ORF of a haloarchaeon, the same relationship could not be found among the ORFs of the other virus. While the two viruses clearly derived from a common ancestor (based on similar DNA polymerase sequences, genome structure and virus morphology) they have diverged considerably over time, both in sequence, gene arrangement, and gene complement. The evidence suggests that recombination events between haloviruses, and between haloviruses and their hosts is a prominent factor driving their evolution, a model that is consistent with the widely accepted view that the genomes of prokaryotic viruses, while often organised in a modular fashion, evolve rapidly by recombination with hosts and related viruses (Hendrix et al., 2000). His1 and His2 represent divergent members of a novel, and probably widespread virus group that shows morphological similarity to fuselloviruses and other spindle-shaped archaeal viruses. However, at the molecular level, His1 and His2 are not related to the latter viruses, and both haloviruses have now been classified within the recently approved group, Salterprovirus (Bath and Dyall-Smith, 2004).
Materials and Methods

Media

A concentrated salt stock solution (30% w/v) 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 1M Tris.Cl (pH 7.5). From this stock, halovirus diluent (HVD) and modified growth medium (MGM) containing 12, 18, or 23% (v/v) SW were prepared as previously described (Nuttall and Dyall-Smith, 1993; Porter 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. sinaiiensis” ATCC 33800 (Torreblanca et al., 1986), Har. vallismortis ATCC 29715$^T$ (Gonzalez, Gutierrez, and Ramirez, 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), Hfx. mediterranei ATCC 33500$^T$, Hfx. volcanii NCIMB 2012 (Torreblanca et al., 1986), Halorubrum coriense ACM 3911$^T$ (Kamekura and Dyall-Smith,
1995), *Halorubrum lacusprofundi* ATCC 49239\textsuperscript{T} (Franzman et al., 1988), *Hrr. saccharovorum* NCIMB 2081 (Ventosa et al., 1999), *Haloterrigena turkmenica* NCMB 784 (Ventosa et al., 1999) and *Natrialba asiatica* 172P1 JCM 9576\textsuperscript{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. sinaiiensis*’, 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. 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) 1 000-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 (5 000 × 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 OD$_{550}$ 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 > 10$^{10}$ 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% (w/v) sucrose (in HVD) (Beckman SW28; 20 000 rpm, 10 h, 10 °C). The pellet was resuspended in HVD, loaded onto a linear 30-70% (w/v) sucrose-HVD 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 on a 20 µl drop of 2% (w/v) uranyl acetate (May & Baker Ltd., England) for 30 seconds. 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 ten 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 T1E 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 were purchased from New England Biolabs Inc., Beverly, USA or Boehringer-Mannheim, Germany. Nuclease Bal-31 was purchased from New England Biolabs Inc., Beverly, 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**

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 manufacturers 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 Sequencher™(Gene Codes Corporation, USA). Analysis and annotation of the His1 and His2 genome sequences, including ORFs and ITR sequences, was 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 pI, 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 (E ≥ 10^{-10}) matches to entries in the GenBank database and/or being ≥ 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 tRNAscan-SE 1.21 (Lowe and Eddy, 1997) (http://www.genetics.wustl.edu/eddy/tRNAscan-SE). Cumulative GC-skew and AT-skew plots were produced using the online base composition tools at http://molbiol-tools.ca/Jie_Zheng/.
Acknowledgements

This research was supported by grants from The University of Melbourne (MRGS) and the Australian Research Council to MD-S. The support of the Department of Microbiology and Immunology is greatly appreciated. CB was supported by a Melbourne University postgraduate scholarship. KP was supported by an Australian Postgraduate Award. We thank Anna Friedhuber, Jocelyn Carpenter and Simon Crawford for assistance with transmission electron-microscopy, and Voula Kanellakis and Tiffany Cowie for help with DNA sequencing. We thank Brendan Russ for critical reading of the manuscript. Proteomic studies were facilitated by access to the Australian Proteome Analysis Facility established under the Australian Government’s Major National Research Facilities program.
REFERENCES


Figure Legends.

**Figure 1.** Plaques of His1 (A) and His2 (B) on *Har. hispanica*. Dilutions of virus were plated with cells in 18% MGM top layer agar, poured on 18% MGM base plates, allowed to set, and incubated at 30 °C (His1) or 37 °C (His2) for 48–72 h for visible plaques. Plates were 8.5 cm in diameter.

**Figure 2.** Stability curves for His1 and His2. Virus lysates were exposed to various conditions, and then diluted in HVD and the surviving virus concentrations determined by plaque assay (see Materials and Methods). Open squares (□) represent His1 titres and filled triangles (▲) represent His2 titres. A, temperature stability. Virus lysates were incubated at different temperatures for 1hr, then cooled to 4 °C. B, stability at lowered salt concentration. Virus was diluted 1:1000 in pure water and incubated at RT. C, stability to changes in pH. Virus was diluted 1:100 in buffers of different pH and incubated for 30 min at RT. D, sensitivity to chloroform. Virus was mixed with chloroform, in a ratio of 1:4 (chloroform to virus), and incubated at RT with constant mixing. Error bars represent one standard deviation of the data.

**Figure 3.** Halovirus single-step growth curves for His2 (A and B) and His1 (C). *Har. hispanica* cells were infected at a multiplicity of 30 for 1 h at 37 °C, after which the cells were washed thoroughly of unadsorbed virus. The cultures were incubated at 30 °C (His1) or 37 °C (His2) and the OD$_{550}$ and the titres of infectious centres followed at hourly intervals for 24 h. The cultures depicted in A and B were infected in parallel, under identical conditions. Error bars represent one standard deviation of the data.
Figure 4. Negative-stain electron microscopy of purified His2. A, typical preparation stained with uranyl acetate (2%) and showing particles with varying degrees of distortion (taken at 20,000 ×). B and C show examples of relatively well-preserved particles (taken at 52,000 ×). Scale bars represent 100 nm.

Figure 5. Protein profile of purified His2. Virus was disrupted by heating in sample buffer (containing SDS and 2-mercaptoethanol) and proteins were separated by SDS-PAGE on a 4–12% NuPAGE Novex BisTris gel (Invitrogen, USA) and stained with Brilliant Blue G. The positions of molecular weight standards are indicated at the left (sizes indicated on the left in kDa). Virus proteins (VP1–VP4) are named, in order of MW, on the right.

Figure 6. Detection of terminal proteins of His2. DNA was extracted from purified virus particles either with protease (+) or without protease treatment (−), digested with SacII or TspRI, then separated by agarose gel electrophoresis. Terminal fragments of the expected size are indicated by white arrowheads. MW, DNA size standard (sizes in kB are given at the left).

Figure 7. Diagram of the His1 and His2 genomes. The major features are shown, including the predicted ORFs (light arrows), terminal inverted repeat sequences (dark arrowheads), and regions of nucleotide sequence repeats (shaded boxes). Scale bars, in kb, 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 and AT-skew are shown below each genome diagram, with inflection points indicated by circles.

**Figure 8.** 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.

**Figure 9.** Predicted features of the His2 VP1 protein are shown in A. The predicted signal peptide and predicted C-terminal membrane anchor regions are boxed, and the positions of potential asparagine-linked glycosylation sites are arrowed. A region of four consecutive serine residues near the anchor region (that may also provide potential glycosylation sites) is also indicated.

B. Diagram of the right end of the His2 genome, aligned with one of the two regions of the *Har. marismortui* genome that show strong 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 indicated. The ORF numbers for His2 are the same as in Table 2, 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 both His2 ORF 33 and *Har. marismortui* ORF rrn2294.
Table 1. Predicted ORFs of halovirus His1

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<tr>
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<td>Predicted coiled-coil, aa 77-98 (Coils).</td>
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<tr>
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<td></td>
<td>Putative type B family DNA polymerase. Similar to polymerases AAS15054 (mitochondrion <em>Daucus carota</em>; E=10⁻⁵); and AAR99740 (bacteriophage PR772, E=10⁻⁴). Overlaps next ORF.</td>
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<td>Similar to hypothetical proteins NP2552A (<em>Nmn. pharaonis</em>, E=10⁻¹⁵), rnaC3142 (<em>Har. marismortui</em>, E=10⁻⁸), and AAY24941.1 (halovirus SH1 ORF15, E=10⁻¹³). Predicted to contain 3 transmembrane regions (TMHMM). Overlaps next ORF.</td>
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<td>CxxC motif.</td>
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ORF, open reading frame; pI, isoelectric pH (predicted from the amino acid sequence); CxxC, amino acid sequence composed of cysteine-X-X-cysteine (where X is any amino acid); SignalP and TMHMM are online protein analysis programs (see Materials and Methods)

a. c, suffixed to the ORF number indicates the ORF is on complementary strand compared to non-suffixed ORFs.
b. RBS, ribosome binding site (Shine-Dalgarno sequence). Complementary to the 3’ end of the Har. hispanica 16S rRNA, 3’-TCCTCCACT...-5’.
Table 2. Predicted ORFs of the His2 genome

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<th>Start (bp)</th>
<th>Stop (bp)</th>
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<td>Predicted signal sequence (SignalP), and two N-glycosylation sites.</td>
</tr>
<tr>
<td>8c</td>
<td>2365</td>
<td>1994</td>
<td>123</td>
<td>4.8</td>
<td>aggagg</td>
<td>Predicted signal sequence (SignalP) and two predicted N-glycosylation sites. Overlaps next ORF.</td>
</tr>
<tr>
<td>9c</td>
<td>2559</td>
<td>2362</td>
<td>65</td>
<td>4.9</td>
<td>aggag</td>
<td>Two CxxC motifs.</td>
</tr>
<tr>
<td>10c</td>
<td>2845</td>
<td>2561</td>
<td>94</td>
<td>6.7</td>
<td></td>
<td>Two predicted transmembrane regions (TMHMM).</td>
</tr>
<tr>
<td>11c</td>
<td>2955</td>
<td>2875</td>
<td>26</td>
<td>9.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12c</td>
<td>3157</td>
<td>2978</td>
<td>59</td>
<td>9.4</td>
<td>gaggt</td>
<td>CxxC motif.</td>
</tr>
<tr>
<td>13c</td>
<td>3375</td>
<td>3160</td>
<td>71</td>
<td>6.6</td>
<td>gaggt</td>
<td></td>
</tr>
<tr>
<td>14c</td>
<td>5625</td>
<td>3463</td>
<td>720</td>
<td>4.8</td>
<td>gaggg</td>
<td>Putative type B family DNA polymerase. Similar [E=10^{3}] to linear plasmids of of maize mitochondria (P10582) and tectiviruses, e.g. Bacillus thuringiensis phage GIL16c (AAW33569).</td>
</tr>
<tr>
<td>15c</td>
<td>6106</td>
<td>5645</td>
<td>153</td>
<td>5.0</td>
<td>gaggt</td>
<td>Predicted coiled-coil, aa 119-147. (Coils)</td>
</tr>
<tr>
<td>16c</td>
<td>6480</td>
<td>6238</td>
<td>80</td>
<td>5.0</td>
<td></td>
<td>Overlaps next ORF.</td>
</tr>
<tr>
<td>17c</td>
<td>6587</td>
<td>6477</td>
<td>36</td>
<td>4.2</td>
<td>aggg</td>
<td>CxxC motif. Overlaps next ORF.</td>
</tr>
<tr>
<td>18c</td>
<td>6774</td>
<td>6577</td>
<td>65</td>
<td>8.2</td>
<td></td>
<td>CxxC motif. Overlaps next ORF.</td>
</tr>
<tr>
<td>19c</td>
<td>6980</td>
<td>6771</td>
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<td>4.5</td>
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<td>Overlaps next ORF.</td>
</tr>
<tr>
<td>20c</td>
<td>7069</td>
<td>6962</td>
<td>35</td>
<td>8.7</td>
<td></td>
<td>Two CxxC motifs.</td>
</tr>
<tr>
<td>21c</td>
<td>7293</td>
<td>7105</td>
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<td>Two CxxC motifs.</td>
</tr>
<tr>
<td>22c</td>
<td>7480</td>
<td>7298</td>
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<tr>
<td>23c</td>
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<td>7484</td>
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<td>Two CxxC motifs.</td>
</tr>
<tr>
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<td>8110</td>
<td>7814</td>
<td>98</td>
<td>4.0</td>
<td></td>
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<tr>
<td>25c</td>
<td>8425</td>
<td>8607</td>
<td>60</td>
<td>7.8</td>
<td></td>
<td>Hydrophobic carboxy-terminal region</td>
</tr>
</tbody>
</table>
Predicted to contain a signal sequence (SignalP), 7 transmembrane regions, and two N-glycosylation sites. Overlaps next ORF.

Two predicted transmembrane regions (TMHMM).

Similar to cell surface (glyco)proteins of *Methanosarcina* (E=10^{-8}) [AAM31623.1, AAM07567.1, AAZ70478.1,AAM30232.1]. Contains predicted signal sequence and membrane anchor, and 8 potential N-glycosylation sites, and internal repeats.

VP1, major capsid protein. Similar to CDS AAV47213 (rrnAC2399) [E=10^{-52}] and AAV47116 (rrnAC2291) of *Har. marismortui* ATCC 43049; to ORF4 of haloarchaeal plasmid pHK2 [E=10^{-15}]; two CDS of *Hfx. volcanii* (not yet annotated); and two CDS of *Nmn. pharaonis* (YP_326822).

Similar to rrn2292 of *Har. marismortui*. Contains predicted signal sequence (SignalP), a membrane anchor (THMM), and two predicted N-glycosylation sites. Overlaps next ORF.

Weak similarity to rrn2293 of *Har. marismortui*. Two predicted internal transmembrane regions (THMM) but no predicted signal sequence. Overlaps next ORF.

Two predicted transmembrane regions (TMHMM).

AAA ATPase motif (CDD 22776, smart00382). Similar to CDS AAV47119 (rrnAC2294) [E=10^{-45}] and AAV47217 (rrnAC2403) of *Har. marismortui* ATCC 43049; and to CDS in *Hfx. volcanii* (not yet annotated). Predicted helix-turn-helix motif, and P-loop NTP hydrolase motif. Overlaps next ORF.

Double methionine start. One potential membrane spanning region (THMM), possibly a signal sequence (SignalP). Overlaps next ORF.

Predicted transmembrane region (TMHMM). Two CxxC motifs.

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a. Legend the same as for Table 2.