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SH1: A NOVEL, SPHERICAL HALOVIRUS ISOLATED FROM AN AUSTRALIAN HYPERSALINE LAKE

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Running title
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Abstract

A novel halovirus, SH1, with a spherical morphology is described. Isolated from a hypersaline lake, SH1 is divalent, producing clear plaques on Haloarcula hispanica and a natural Halorubrum isolate. Single-step growth curves gave a latent period of 5 - 6 h and a burst size of around 200 PFU/cell. The host can differentiate to form tight clusters of thick cell-walled forms, and these were shown to be resistant to infection. Purified virions had no visible tail, were about 70 nm in diameter, and displayed a fragile outer capsid layer, possibly with an underlying membrane component. The structural proteins of the virion were analysed by SDS-PAGE and several were found to be cross-linked, forming protein complexes. The genome was linear, dsDNA, of approx. 30 kb in length. This morphology and linear genome are features not observed in any other euryarchaeal viruses, but have properties similar to the bacterial virus PRD1.

Introduction

More than 5000 viruses have been described but only about 39 of them infect archaeal hosts (http://www.ncbi.nlm.nih.gov/ICTV/; Dyall-Smith et al., 2003; Haring et al., 2004). The majority of these attack thermophilic archaia belonging to the kingdom Crenarchaeota (Prangishvili et al., 2001; Prangishvili, 2003; Rice et al., 2004). About 18 viruses of methanogenic and extremely halophilic archaia (kingdom Euryarchaeota) have been described, of which just a handful have been studied beyond a basic description (Bath and Dyall-Smith, 1998; Dyall-Smith et al., 2003; Klein et al., 2002; Luo et al., 2001). Two interesting themes emerging from studies of archaeal viruses are the discovery of novel viral morphologies and that the majority of the viral genes sequenced show no or little similarity to previously obtained sequences. These indicate that our sampling of the virosphere is still scarce and new structures and functions wait to be discovered.

The first virus infecting a halophilic archaean was discovered in 1974 (Torsvik and Dundas, 1974). Currently, about 15 haloarchaeal viruses (haloviruses) have been reported (Dyall-Smith et al., 2003), with the great majority resembling tailed dsDNA bacteriophages, such as lambda. Direct electron-microscopy of salt lake waters shows that they maintain high levels of virus-like particles (10 times higher than the cell population) with recognisable morphotypes including head-tail and lemon shaped particles (Guixa-Boixareu et al., 1996;
Oren et al., 1997). The search for viruses in Australian salt lakes has led to the isolation of a virus with yet another viral morphotype. SH1 is a spherical virion with layered shell appearance and no indication of any tail structure (Dyall-Smith et al., 2003). It plaques on *Haloarcula hispanica*.

Very recently the crenarchaeal dsDNA virus STIV structure was resolved using cryo-electron microscopy and image reconstruction (Rice et al., 2004). It was proposed that the capsid architecture resembles that of PRD1, a bacterial virus with an internal membrane (Abrescia et al., 2004; Cockburn et al., 2004). In this investigation we describe the details of the isolation, propagation, purification and initial characterization of halovirus SH1. We also propose that this dsDNA virus most probably has a lipid membrane and that several virion proteins are cross-linked. It is of interest whether SH1 resembles STIV and bacterial viruses such as PRD1, which have been suggested to be structurally related to several eukaryotic viruses (Benson et al., 2004).

**Results**

**Virus isolation and plaque morphology.** Hypersaline water samples from Serpentine Lake (32° 00’ S; 115° 31’ E) on Rottnest Island (Western Australia, Australia) were screened for haloviruses by plating directly on lawns of *Haloarcula hispanica*, *Halofex volcanii* and *Halorubrum coriense*. A novel halovirus was isolated from a single plaque on the lawn of *Har. hispanica* and was designated SH1, based on its isolation site (Serpentine Lake) and host (*hispanica*). Plaques were fully developed after three days at 30 °C using overlay plates of 18% MGM (see Materials and Methods), and were around 2 mm in diameter, clear, and with defined edges. At 25 °C incubation, plaque morphology was similar but plaques took 7 days to develop. We also isolated a spontaneous variant of *Har. hispanica* with attenuated virus production, designated as var.1, to support the propagation of SH1 (see below).

**Virus host range.** SH1 was unable to produce plaques on lawns of 12 characterised haloarchaeal strains from the genera *Haloarcula, Halobacterium, Halofex, Halorubrum* and *Natrialba* (Table 1). A selection of 13 natural isolates closely related to *Halorubrum* were also tested. These came from studies of microbes in an Australian saltern crystalliser pond, in
which it was shown that strains related to *Halorubrum* represented the dominant microbial group (Burns et al., 2004a; Burns et al., 2004b). SH1 was able to produce plaques on one of these isolates, CSW 2.09.4. The 16S rRNA gene of this isolate was identical (over 684 nt) to that of *Halorubrum sodomense* (Burns et al., 2004a). The efficiency of plating was examined by passaging on *Har. hispanica* and CSW 2.09.4 the two sensitive hosts, and then plaque titrating on both strains. There appeared to be no significant difference between the two strains for supporting SH1 growth.

**The life cycle of SH1.** In single-step growth experiments using a MOI of 50, a sharp increase in the number of viruses took place between 5 and 6 h after infection. An example is shown in Fig.1A, where the rise began at ~ 6 h post infection (p.i.), and the average burst size was ~ 200 PFU/cell. The latter result was supported by single burst experiments, that indicated an average burst size of 230 PFU/cell (data not shown). Cell lysis occurred either coincident with the titer rise or was delayed by several hours in some experiments. When *Har. hispanica* var.1 was used as a host it was observed that the adsorption of SH1 to these host cells was very slow. The maximal number of infected centers formed was reached in three hours (Fig. 1C) and, under the conditions of infection saturation, some 20% of the cells were able to form colonies (probably uninfected cells). The number of free viruses gradually increased starting at ~ 5 h p.i., as with the wild type *Har. hispanica* host. Compared to the non-infected control, culture turbidity began to decrease approx. 7 h p.i., reaching a stable level of approx. Klett 540 80 at 27 h p.i., indicating the presence of non-lysed cells (Fig. 1B). On average only 30 – 40 particles per infected cell were released.

To investigate the cause for the attenuated virus production in var.1, samples were collected during the infection cycle (MOI 15) and examined by thin section electron microscopy. In uninfected wild type and var. 1 cultures, single thin-walled cells and large clusters of thick-walled cells were detected. The appearance of large cell clusters was growth phase-dependent and much more abundant in var 1. compared to the wt strain (Fig. 2). Such cell clusters were also present early in the infection (Fig. 3A, C). The thick-walled cluster forms of wt *Har. hispanica* have been previously observed by Cline and Doolittle (Cline and Doolittle, 1992), but at lower frequency (0.1 - 0.01%). We observed viruses attached only to the single thin-walled cells (Fig. 3B, arrow). The newly assembled viral particles were also only detected inside the thin-walled cells (Fig. 3C, arrow), commencing at approximately 5 h p.i.. Both empty (arrowhead) and DNA containing virus particles (arrow) were observed in
the cell interior (Fig. 3D). A few non-lysed virus-containing cells were still detected at 19 h p.i., but at 93 h 45 min p.i. only thick-walled cells remained (Fig. 3E). The released viruses were often seen attached to cell debris as indicated in Figure 3F. The adherence of the thick-walled cells to each other but not to the thin-walled cells can be attributed to the properties of the external layer. The thick-walled cells were larger and their cytoplasm more electron dense compared to the thin-walled ones.

The remaining cells after SH1 caused cell lysis (liquid culture) were collected and subjected to thin section EM, and whole cell protein pattern (Hantula et al., 1990) and 16S rRNA sequence analyses (Fig. 3E and data not shown). The whole cell protein pattern and the 16S rRNA sequence (957 nt) did not deviate from those of the initial Har. hispanica culture (accession U6854) confirming that the remaining thick-walled cells were not contaminants. Spontaneous SH1-resistant variants of var.1 and wt Har. hispanica were also obtained from confluently lysed plates. The whole cell protein pattern, 16S rRNA sequence (932 nt) and the cell morphology (thin section EM) were determined for one such SH1-resistant strain. It showed no detectable deviation from the parental strain showing both thin-walled and thick-walled cell types.

**SH1 production and purification.** Virus could be obtained directly from the plate stock or from liquid cultures (see Material and Methods). The best yields obtained from a liquid culture were in the order of $\approx 2 \times 10^{11}$ PFU/ml. Virus in the lysate was concentrated by PEG$_{6000}$ precipitation and purified by linear rate zonal 5 – 20% (w/v) sucrose gradient centrifugation, resulting in “1 x” purified virus. The virus band from the sucrose gradient was loaded on top of CsCl (mean CsCl density, 1.30 g/ml, in SH1 buffer; see Materials and Methods) and centrifuged to equilibrium. This resulted in the formation of a sharp light scattering virus band at the density of 1.33 g/ml (Fig. 4B). The virus after CsCl centrifugation and concentration by differential centrifugation was designated as “2 x” purified. The recovery as well as specific infectivity (PFU/A$_{260}$) for each purification step of a typical virus batch is indicated in Figure 4A. The specific infectivity of the final pelleted 2 x purified virus was $2.1 \times 10^{12}$ PFU/mg protein (as determined by Bradford assay using BSA as a standard). Figure 5A shows the protein pattern along the purification procedure. Negative stain EM of SH1 particles in culture supernatant and after 2 x purification showed identical isometric particles, many with a clearly icosahedral profile (Fig. 5B, C). The particles had an average
diameter of about 70 nm and displayed an external proteinaceous shell with a compact core particle (particles devoid of the outer-shell are indicated with an arrow in Fig. 5C) of about 50 nm in diameter. Both uranyl acetate and potassium tungstate staining gave identical results (Fig. 5B, C) whereas molybdate staining failed, leading to disrupted particles.

The low buoyant density of the virion, sensitivity to organic solvents and the appearance of membranous material upon virus disruption (see below) suggested the presence of lipids in the virion. For this reason, it was feasible to try to use sucrose gradients to equilibrate SH1. However, it appeared that the material that gave a single peak in CsCl centrifugation gave two adsorbing bands in a 30 – 70% (w/v) sucrose gradient: one with the viral proteins and some DNA (density, ~ 1.28 g/ml), and another, slower sedimenting one with almost only DNA (data not shown). It appeared that, like bacteriophage PM2 (Kivelä et al., 1999), SH1 was unstable in high sucrose concentrations, preventing the use of this purification method.

**SH1 stability.** The infectivity of SH1, when stored in the culture supernatant at 5 °C, stayed unaltered for several months. For obtaining more information on the virus stability and for defining a lower ionic strength minimal buffer to be used in biochemical analyses, SH1 stability was tested in a number of conditions.

The temperature dependence of the SH1 infectivity in high salt conditions is depicted in Figure 6A. Infectivity was stable up to 50 °C and dropped sharply beyond this temperature. No viability was detected if the incubation was continued overnight at 70 °C (data not shown). At pH values below 6, SH1 rapidly lost infectivity over a 30 min exposure time, but was stable to at least pH 9 (Fig. 6B). SH1 was very sensitive to chloroform, with three orders of magnitude loss of titer in 15 min when shaken with a 20% v/v of chloroform (Fig. 6E).

Dilution experiments indicated that SH1 is very sensitive to low ionic strength conditions (Fig. 6F, G). A series of experiments were carried out to examine the effects of different salts in varying concentrations to the stability of SH1. 1 × purified virus was collected by centrifugation and resuspended in an appropriate buffer at 4 °C, with HVD as a control. The titer was determined 1, 2 and 5 days after the resuspension. These experiments showed that the presence of KCl or MgSO₄ had little effect on the virus stability over the five-day incubation period. The critical ions for stability were NaCl and MgCl₂ (Fig. 6C, D). In
separate experiments, \( \text{Mg}^{2+} \) alone in 2 M concentration was shown to maintain virus infectivity (data not shown). These experiments indicate that the stability is ionic strength dependent and that divalent cations are an absolute requirement. Based on these experiments, an SH1 buffer, containing 40 mM Tris-HCl pH 7.2, 40 mM MgCl\(_2\), 2.5 mM CaCl\(_2\) and 1 M NaCl, was developed for use in future biochemical studies. Low salt and chloroform treated viruses were also examined using negative-stain EM. In both cases, disintegrated particles with detached outer layers and membraneous, pleomorphic material were seen (Fig. 6G for low salt conditions).

**SH1 structural proteins and protein complexes.** 2 × purified virus preparations were heated in sample buffer with or without β-mercaptoethanol (48 mM) and separated by tricine SDS-PAGE in gels with 8 or 17% (w/v) acrylamide. The different acrylamide concentrations allowed more accurate determinations of the relative molecular masses for large and small proteins. This analysis revealed 15 distinguishable protein bands after Coomassie blue staining, with apparent molecular masses ranging from 4 to 185 kDa (Fig. 7). Interestingly, if the protein samples were not reduced, five additional strong bands were observed (280, 250, 73, 62, 52 kDa) with concomitant reduction of the intensity of the bands of 24, 35 and 40 kDa.

**Genome of SH1.** Nucleic acid could be isolated from purified virus preparations but precautions were necessary to eliminate nuclease activity. To prevent nucleic acid degradation three phenol-chloroform extractions after SDS/proteinase K were necessary. Alternatively the proteinase K treatment could be replaced by 1 h incubation at 56 °C.

The SH1 genome was sensitive to DNase (RNase-free), exonuclease III, nuclease Bal-31, T7 exonuclease and type II restriction endonucleases, but not to Mung bean nuclease and RNase A. This indicated that the genome was linear, double-stranded DNA, with free (i.e. not covalently closed) termini. Of the 64 restriction endonucleases tested (Table 1, supplementary material), 24 were able to digest SH1 DNA (Fig. 1, supplementary material). The pattern of restriction site usage indicated that the DNA has a high G + C content and some enzymes produced digests with under-represented bands, indicating incomplete digestion of the genome.
To construct a physical map of the SH1 genome, exonuclease treatment with Bal-31, prior to digestion with the restriction enzymes \textit{Ase}I, \textit{Mse}I, \textit{SnaBI} and \textit{Ssp}I was utilized in conjunction with double-digests of the genome. The results of these experiments enabled the order in which the fragments were arranged in the genome to be determined (Fig. 8). The estimated size of the SH1 genome is 30.9 ± 1.0 kb.

\textbf{Discussion.}

The characteristics of SH1 indicate that it is a novel virus infecting members of two genera within the family \textit{Halobacteriaceae} (Kingdom Euryarchaeota). It is only the second known halovirus (after HF1) able to infect \textit{Halorubrum} spp. (Dyall-Smith et al., 2003). Virus particles have a polyhedral morphology with a distinct proteinaceous outer layer and no discernable tail, and employ a lytic pattern of growth with a burst size of around 200. SH1 possesses a linear, dsDNA genome of ~ 30 kbp that was able to be digested by Bal-31, ExoIII and T7 nucleases, indicating the termini were accessible and not covalently closed.

The availability of a spontaneous host variant (var. 1) with increased number of thick-walled cell types allowed us to demonstrate that only cells lacking the external layer are susceptible to SH1. It will be of interest to obtain further information on the ecology of SH1 and its host in relation to the variation of the host cell types. Another intriguing observation was that, in single-step growth experiments, lysis of wild-type cultures by SH1 sometimes occurred after the period of major virus release. Our thin-section electron microscopic images suggest a release mechanism based on cell disruption (Fig. 3). However, many viruses of thermophilic archaea can exit their host cells without lysis, such as the \textit{Sulfolobus} virus SSV1 (Prangishvili et al., 2001). The cell wall of \textit{Haloarcula} (like \textit{Sulfolobus}) does not contain peptidoglycan, and is very thin and flexible, consisting of a single surface protein layer (S-layer) directly above the cell membrane (Grant et al., 2001).

In previous studies of virus-like particles (VLP) occurring in salt lakes, three major morphotypes were observed, lemon-shaped, polyhedral and head-tail. While polyhedral VLPs were observed in high numbers, it could not be determined whether they represented genuine viruses or the dislodged heads from head-tail phages (Guixa-Boixareu et al., 1996; Oren et al., 1997), an artefact that can easily occur during preparation of the head-tail haloviruses for
electron-microscopy (Nuttall and Dyall-Smith, 1993b). Given the sensitivity of many haloviruses to low salt concentrations, including SH1, the use of negative-stain electron-microscopy in studies of salt lake VLPs could have underestimated or entirely missed certain morphotypes. Now that an example of a polyhedral halovirus has been isolated, the significance and diversity of this morphotype in natural salt lakes can begin to be studied.

Our optimised concentration and purification procedure to obtain $2 \times$ purified virus reproducibly yielded about 15% of the original infectivity in the lysate. This is a comparable or even better yield compared to similar purification procedures for membrane containing dsDNA bacteriophages PRD1 or PM2 (Bamford and Bamford, 1991; Kivelä et al., 1999). The specific infectivity obtained for SH1 ($\sim 2 \times 10^{12}$ PFU / mg protein) is lower than what is obtained for PRD1 ($\sim 1 \times 10^{13}$ PFU / mg protein, Bamford and Bamford, 1991). Obviously the larger size (more protein per infective unit) of the SH1 virion accounts for some of this difference but $2 \times$ purified SH1 preparations seem to have more non-plaque forming particles compared to PRD1 although the morphology of virions in the lysate and after extensive purification was indistinguishable (Fig. 5). The stability of the virus (infectivity) in the original lysate (stored in the growth medium at $5 \, ^{\circ}C$) is good with very little decay in several months. The virion is sensitive to lowered salt conditions and depletion of divalent cations, in line with its adaptation to its native high salt environment.

There were only minor changes in the protein pattern when $1 \times$ and $2 \times$ purified viruses were compared (Fig. 5), supporting the idea that the structural protein species observed in $2 \times$ purified virus reflect the minimal number, described in more detail in Figure 7. The low buoyant density, appearance of membranous material in negatively stained specimens of disrupted particles, and chloroform sensitivity, suggests the presence of lipids in SH1 virion. Confirmation for this has been recently obtained in our ongoing biochemical studies on the virion composition (to be published).

Viruses use cross-linking to stabilize their capsid. The best studied example is the autocatalytic cross-linking taking place during capsid maturation in the lambdoid phage HK97 (Gan et al., 2004). Another examples include chilo iridescent virus, where the capsid is stabilized by extensive S-S bridging (Devauchelle et al., 1985), and in archaean viruses, halovirus $\Phi$Ch1 also uses cross-linking to stabilize its capsid (Klein et al., 2002). We
observed that in non-reduced preparations several new protein bands appeared in the gel
electrophoresis profile compared to the pattern obtained with mercaptoethanol-treated
preparations. Five of the new protein bands were abundant and marked with an asterisk in
Figure 7. The appearance of these bands correlated to the reduction of the intensity of three
major bands observed in the reduced samples (those designated 40, 35 and 24 kDa). We
assume that these proteins are SH1 capsid components.

This work describes conditions where an extremely halophilic virus-host system yields
large amounts (typically 2 - 3 mg of protein per 1 liter batch) of highly purified virus material
and sets the basis for further studies on SH1. We also observe that the gross morphology of
the SH1 virion is similar to that of membrane-containing bacteriophages PRD1 and PM2, and
that its genome structure is similar to that of PRD1. We are currently collecting further data
on the virion composition and structure as well as determining the genome sequence. It will
be intriguing to see how well SH1 resembles STIV and the bacterial viruses with an internal
membrane. This will, hopefully, shed more light to the debated issue of evolution and origin
of viruses where studies on PRD1 and structurally related viruses have suggested a viral
lineage with long evolutionary roots and members infecting hosts in all three domains of life
(Bamford et al., 2002; Bamford, 2003; Benson et al., 1999; Benson et al., 2004).
Materials and methods

**Media and plates.** A 30% (w/v) stock of artificial salt water (SW) was prepared as described in the online handbook, The Halohandbook (http://www.microbiol.unimelb.edu.au/staff/mds/HaloHandbook/) and contained per litre: 240 g NaCl, 30 MgCl₂·6H₂O, 35 g MgSO₄·7H₂O, 7 g KCl, 0.5 g CaCl₂·2H₂O, 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, 20 or 23% (v/v) SW were prepared as previously described (Nuttall and Dyall-Smith, 1993b). Bacto-agar (Difco Laboratories, Detroit, USA) was added to give solid (14 - 15 g/L) or top-layer (4 - 6 g/L) media. These media were used also without added CaCl₂ (MGM-C).

**Haloarchaeal and halovirus strains.** The haloarchaeal strains used in this study are listed in Table 1. Additionally, from a collection of haloarchaea previously isolated from an Australian solar saltern (Burns et al., 2004a), 13 isolates were selected that were closely related to *Halorubrum*. The 16S rRNA genes of these isolates were partially sequenced to identify them (Burns et al., 2004a).

Haloarchaea were grown aerobically at 37 °C in either 18 or 23% (SW) MGM (or MGM-C), depending on the strain, with aeration (except for “*Haloarcula sinaiiensis*”, which was not aerated). Incubation periods varied from 1 to 4 days, depending on the strain.

**Isolation and culture of halovirus SH1.** Hypersaline water samples from Serpentine Lake (32° 00’ S; 115° 31’ E) on Rottnest Island (Western Australia, Australia), were centrifuged (5000 g, 10 min, room temperature) to remove cells and cellular debris. The samples were then screened for haloviruses by plating directly on lawns of *Har. hispanica*, *Halofex volcanii* and *Halorubrum coriense* using 12 or 18% MGM. After incubation for several days, a single plaque on the *Har. hispanica* plate was picked, re-plaque purified, and the isolated virus designated as SH1.

**SH1 host range.** 12 haloarchaeal strains from the genera *Haloarcula*, *Halobacterium*, *Halofex*, *Halorubrum* and *Natrialba*, and 13 natural *Halorubrum* isolates (see above) (Table 1) were screened for SH1 susceptibility. Lysates from SH1-infected *Har. hispanica* cultures containing $1 \times 10^{11}$ PFU/ml were spotted onto lawns of the selected haloarchaea on
12 or 18% MGM, and incubated for 3 - 5 days, depending upon the time taken for the host to form a visibly turbid lawn.

**SH1 single-step growth curve.** A 10 ml volume of an early exponential phase culture of *Har. hispanica* (3 x 10^7 CFU/ml in 18% MGM) was infected with SH1 (MOI, 50). Under these conditions the percentage of infected cells was close to 100%. After an adsorption period of 1 h at room temperature, the cells were washed twice with 50 ml of 18% MGM (at room temperature), resuspended in 50 ml 18% MGM and incubated at 37 °C, with shaking (100 rpm). Samples were removed at hourly intervals for measurements of absorbance (at 550 nm) and the number of infective centers. Plaque titrations (in duplicate) were performed immediately after sampling.

**Growth curve and thin-section electron-microscopy of SH1 infected *Har. hispanica* var.1.** Infection, incubation and sampling were similar to that described above for wt *Har. hispanica* but the virus inoculum was not removed and the culture medium was MGM-C. The level of free virus was determined by plaque assay from the supernatant after removing cells by centrifugation (microcentrifuge, 13,000 rpm, 1 min, room temperature). Cells were collected (13,000 rpm, 1 min, room temperature) and resuspended in 23% MGM-C for determining the level of infective centers and uninfected cells. The level of infective centers was determined by plaque assay and level of uninfected cells by viable count. Turbidity (cell density) was followed using a Klett-Summerson colorimeter (A_{540}). Samples for thin-section electron-microscopy were taken every hour (1 – 20 h p.i.) and 93 h 45 min p.i. from the infected culture (MOI, 15) and 17 h p.i. of the uninfected culture. In addition to this, another culture was prepared as above except an MOI 30 was used. Samples for thin-section electron-microscopy were taken from this culture 10 min, 20 min and 30 min p.i. Similarly, the frequencies of different cell morphologies of uninfected *Har. hispanica* wild-type and var 1. were examined by taking samples of the growing cells for thin section electron microscopy at different time points. The cells were propagated in 23% MGM-C at 37°C.

**Resistant forms of *Har. hispanica*.** Colonies of *Har. hispanica* arose on conflually lysed plates. Such colonies were picked, followed by six consecutive single colony purifications. The 16 S rRNA gene sequence of a representative of these variants was
determined in the DNA sequencing laboratory of The Institute of Biotechnology, University of Helsinki. These strains were also subjected to whole cell protein analysis (Hantula et al., 1990).

**Large-scale growth and purification of SH1.** Two methods were used to propagate the virus, ‘plate lysate’ and ‘liquid lysate’ depending whether wt or var.1 were used respectively.

Liquid culture method. Liquid cultures of SH1 were grown by infection (MOI, 0.05) of an early logarithmic *Har. hispanica* culture in 18% MGM. Cultures were incubated at 30 °C, with aeration, for three days. At this time, although the cultures did not clear (i.e. complete cell lysis did not occur), SH1 titers reached a maximum and the absorbance at 550 nm ($A_{550}$) was at a minimum. Titers were determined by plaque assay at 30 °C on 18% MGM with an indicator lawn of *Har. hispanica*, using the method previously described (Nuttall and Dyall-Smith, 1993b).

Plate lysis method. SH1-seeded top layer agar plates containing *Har. hispanica* var. 1 cells were incubated until the plates showed semi-confluent lysis. The soft agar layer from each plate was collected into 3 ml of 23% MGM-C, pooled, and the culture incubated at 37 °C for a further 1.5 h, with aeration (190 rpm), after which cell debris was removed from the culture by low-speed centrifugation (Sorvall SLA3000; 10,000 rpm, 30 min, 4 °C) and titers determined by plaque assay.

Virus was precipitated from the cleared lysate by the addition of 10% (w/v) polyethylene glycol 6000 or 8000 MW, concentrated by centrifugation (Sorvall SLA3000; 10,000 rpm, 60 min, 4 °C), resuspended in SH1-buffer without calcium (40 mM Tris-HCl [pH 7.2], 40 mM MgCl$_2$ and 1 M NaCl) and titers determined by plaque assay. The virus preparation was purified in a linear 5 – 20% (w/v) sucrose (in the same buffer) gradient (Sorvall AH629; 22,000 rpm, 55 min, 20 °C) and the light-scattering virus zone collected. This product was designated “1 ×” purified virus. $A_{260}$ was measured, and titers determined by plaque assay. The 1 × purified virus was layered on the top of a CsCl solution (0.4 g/ml in SH1-buffer) and centrifuged to equilibrium (Sorvall AH629; 24,000 rpm, 22 h, 20 °C).
virus band was collected and diluted in 40 mM Tris (pH 7.2), 40 mM MgCl₂ in a volume ratio of 1:2 (band to solution). The virus was collected by centrifugation (Sorvall T647.5; 32,000 rpm, 3 h, 20 °C) and the pellet was resuspended in SH1-buffer. This material was designated as “2 x” purified virus. $A_{260}$ was measured, and titers were determined by plaque assay. The protein concentration was measured by the Coomassie blue method (Bradford, 1976) with BSA as a standard. After centrifugation to equilibrium in a CsCl gradient, some tubes were fractionated into 2 ml fractions, the $A_{260}$ and density of each fraction was measured, and samples taken for protein gel analysis.

Equilibrium centrifugation of SH1 in sucrose was done in 30 – 70% (w/v) sucrose (in SH1 buffer devoid of added calcium) gradient. The 1× purified virus was layered on the top of the gradient and centrifuged (Sorvall AH629; 24,000 rpm, 22 h 35 min, 20 °C). Tubes were fractionated to 2 ml fractions and the $A_{260}$ and $A_{280}$, virus titer (by plaque assay) and density of each fraction was measured. Samples from the gradient were also subjected to protein gel analysis.

**Electron Microscopy.** Negative-stain electron microscopy of virus preparations was performed by two methods. (1) Virus particles from SH1-infected *Har. hispanica* were allowed to adsorb to Formvar coated 200-mesh copper grids for 1.5 to 2 min. They were then negatively stained with 2% (w/v) uranyl acetate (May & Baker Ltd., Dagenham, UK) for 1 to 2 min. Excess liquid was adsorbed with filter paper and the grid was allowed to air dry. Grids were examined on a Philips CM 120 BioTwin transmission electron microscope, at an accelerating voltage of 120 kV. (2) Alternatively, using a similar method 2% (w/v) potassium phosphotungstate (pH 7.2) was used for staining and micrographs were taken with a JEOL 1200EX electron microscope operating at 60 kV.

Different buffering agents were tested in 23% MGM-C to replace Tris-HCl (pH 7.2), so that the medium could be used for both liquid culture and glutaraldehyde fixation, when preparing thin-section electron-micrographs. The use of 4-morpholine ethane sulfonic acid (MES; pH 6.7) to buffer the growth medium supported the cell growth as well as Tris-HCl (pH 7.2), so it was chosen. Thin-section electron-microscopy of uninfected and virus-infected *Har. hispanica* cultures was carried out as previously described (Bamford and Mindich, 1980), except that the cells were resuspended in 23% MGM-C, in which Tris-HCl (pH 7.2)
was replaced with MES (pH 7.2). Micrographs were taken with a JEOL 1200EX electron microscope operating at 60 kV.

**Halovirus SH1 stability.** After various treatments, virus titers were determined by plaque assay on *Har. hispanica*.

Thermal stability of SH1 was examined by two methods. (1) SH1-infected *Har. hispanica* lysates in 23% MGM-C were incubated for 15 min. (2) SH1-infected *Har. hispanica* lysates in 18% MGM were incubated overnight followed by plaque assay.

pH stability of SH1 was determined by dilution of SH1 lysates in 23% MGM-C in the appropriate pH buffer in a volume ratio of 1:100 (lysate to buffer). The buffer contained 61 mM Tris-HCl or potassium phosphate, 2.3 M NaCl, 82 mM MgCl$_2$, 79 mM MgSO$_4$ and 52 mM KCl. Incubation was at 22 °C for 30 min.

Chloroform stability to SH1 was examined by exposure of SH1 lysates in 18% MGM to chloroform in a volume ratio of 1:4 (chloroform to lysate). Incubation was at room temperature, with constant agitation. At various times, samples were removed and diluted in HVD.

SH1 stability in a depleted ionic environment was examined by diluting SH1 lysates in 18% MGM 1,000-fold with distilled water. Incubation was at room temperature, with constant agitation. At various times, samples were removed and diluted in HVD. SH1 stability in different compositions of salt medium was examined using cleared virus lysates or 1 x purified virus as follows. (1) Virus particles after rate zonal sucrose gradient centrifugation were collected (Beckman Ti50; 32,000 rpm, 3 h, 20 °C). The pellet was rinsed once with the buffer to be tested and resuspended on ice over night. The presence/absence of precipitate was verified by centrifugation (microcentrifuge 5,000 rpm, 4 min, room temperature). If large aggregates were observed, the sample was not used. HVD was used as a control medium and the results were normalized to this. (2) Virus particles were pelleted from SH1 lysates in 18% MGM (Sorvall AH 629; 23,000 rpm, 13 h, 10 °C). The pellet was resuspended in the buffer to be tested. Incubation was at room temperature, with constant agitation, for 24 h. After incubation, samples were diluted in HVD.
**SDS-polyacrylamide gel electrophoresis.** The proteins in purified virus preparations were resolved using a modified tricine-SDS-PAGE system (devoid of spacer gel) using separation gels with acrylamide concentrations of 8, 14 or 17% (w/v) (Schägger and von Jagow, 1987). Alternatively, SDS-PAGE was performed as described previously (Olkkonen and Bamford, 1989). The concentration of β-mercaptoethanol in sample buffer was 48 mM in reduced samples and in non-reduced samples β-mercaptoethanol was replaced with water.

**Preparation and analysis of SH1 DNA.** The isolation of protease treated DNA from purified SH1 preparations was performed according to the method of Ausubel *et al.* (Ausubel *et al.*, 1994), with two additional phenol-chloroform-isooamyl alcohol (25:24:1 v:v:v; Sigma-Aldrich Pty. Ltd, St. Louis, USA) extractions. Non-protease treated DNA was isolated by the same method, except that proteinase K was omitted from the protocol and virus preparations were incubated for 1 h at 56 °C prior to extraction. Genomic DNA and restriction fragments were separated on 1% (w/v) agarose gels in Tris-acetate-EDTA electrophoresis buffer (Ausubel *et al.*, 1994).

Exonuclease III, mung bean nuclease, nuclease Bal-31, T7 exonuclease and type II restriction endonucleases were purchased from New England Biolabs Inc., Beverly, USA. DNase (RNase-free) was purchased from Promega Co., Madison, USA. RNase A was purchased from Sigma-Aldrich Pty. Ltd, St. Louis, USA. All reagents were used according to the manufacturer’s specifications (New England Biolabs Inc., Beverly, USA; Promega Co., Madison, USA; Sigma-Aldrich Pty. Ltd, St. Louis, USA).
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**Figure 1.** Growth of SH1 in *Har. hispanica*. (A) A representative single-step growth curve. An early logarithmic culture of *Har. hispanica* was infected with SH1 (MOI, 50), washed of free virus, and the samples removed at various time for plaque assay (of infective centers) and free viruses. Error bars represent two standard deviations of the average titer. (B) Growth curve of SH1 on *Har. hispanica* var. 1 (in 23% MGM-C). Non-infected (triangles) and infected (circles) cultures of *Har. hispanica* were grown at 37 °C. An arrow indicates the time of infection with SH1 (MOI, 15). Turbidity was followed using a Klett-Summerson colorimeter (A$_{540}$). Bars show the amount of free virus (PFU/ml) in the culture medium. (C) The kinetics of formation of infective centers and reduction of the number of viable cells after infection of *Har. hispanica* var. 1 with SH1 (MOI, 15).

**Figure 2.** Cell morphologies of *Har. hispanica* wild-type and var.1. The growth curves of wild-type (continuous line) and var.1 (dashed line) cells grown in 23% MGM-C at 37°C. The circles indicate the time points for samples analyzed by thin section electron microscopy (a, b, and c). The inset shows the proportion of clustered thick-walled cells observed in the electron micrographs.

**Figure 3.** Thin section electron micrographs of the SH1 infection cycle. (A) Two different morphologies of *Har. hispanica* var.1, single celled (thin-walled) and clustered cells (thick-walled) were observed at 20 min p.i. (B) Viruses (arrow) were observed only attached to the thin-walled cells at 2 h p.i. (C) At 8 h p.i., progeny SH1 particles (arrow) only appeared inside the thin-walled cells and not in the thick-walled cell clusters, seen on the right. (D) DNA filled (arrow) and empty (arrowhead) SH1 virions were observed in the cells at 12 h p.i. (E) Only thick-walled cells remained in the culture at 93 h 45 min p.i. (F) After lysis at 5 h
p.i., viruses (arrow) were attached to cell debris. The scale bars represent 1 μm in A and E, and 200 nm in B, C, D and F.

**Figure 4.** Purification of halovirus SH1 obtained from solid culture. (A) Recovery of infectivity from different steps of purification. PEG<sub>8000</sub> precipitate represents virus precipitated from the *Har. hispanica* cell lysate with PEG<sub>8000</sub>. “1 ×” virus represents virus obtained after purification using 5 to 20% (w/v) sucrose gradient rate zonal centrifugation. “2 ×” virus represents virus obtained after further purification using CsCl isopycnic centrifugation and differential centrifugation. (B) SH1 purification profile after equilibration in a CsCl gradient.

**Figure 5.** Purification of halovirus SH1. (A) Protein profiles from different purification stages (Coomassie blue stained tricine-SDS-PAGE, 14% (w/v) acrylamide). Lane 1, PEG<sub>8000</sub> concentrated virus from the lysate. Lane 2, virus zone after rate zonal sucrose gradient centrifugation (“1 ×” purified virus). Lane 3, purified virus after centrifugation to equilibrium in CsCl (“2 ×” purified virus). Molecular masses of standard proteins (in kDa) are indicated on the left. (B) Particles observed in a lysate of SH1-infected *Har. hispanica* cells. (negative stain EM, 2% uranyl acetate stain). The scale bar in represents 100 nm. (C) Particles observed in “2 ×” purified virus preparation (negative stain EM, 1% phosphotungstate). Long arrow points to a particle with an intact outer shell and icosahedral profile, while the arrowhead points to a virus core. The scale bar represents 100 nm.

**Figure 6.** Stability of halovirus SH1. (A) SH1 temperature tolerance was measured in 23% MGM-C, with an incubation time of 15 min. (B) pH stability of SH1 was performed in Tris-HCl buffer (61 mM Tris-HCl, 2.3 M NaCl, 82 mM MgCl<sub>2</sub>, 79 mM MgSO<sub>4</sub> and 52 mM KCl)
(closed circles) and potassium phosphate buffer (61 mM potassium phosphate, 2.3 M NaCl, 82 mM MgCl₂, 79 mM MgSO₄ and 52 mM KCl) (open circles). Virus was incubated for 30 min at room temperature. The effect of ionic environment on SH1 stability was investigated by changing the concentration of (C) NaCl and (D) MgCl₂. HVD was used as a control.

Buffers comprised NaCl and MgCl₂ in the indicated concentrations, and either 20 mM Tris-HCl (pH 7.2) and 50 mM MgCl₂, when changing NaCl concentration or 20 mM Tris-HCl (pH 7.2) and 1 M NaCl, when changing MgCl₂ concentration. Incubation was at 4°C. (E) SH1 sensitivity to chloroform was measured by exposure of SH1 lysates in 18% MGM to chloroform in a volume ratio of 1:4 (chloroform to lysate). (F) SH1 sensitivity to lowered salt. SH1 lysates in 18% MGM was diluted 1000-fold in pure water, samples taken at various times, and virus titers determined by plaque assay. (G) Negative stained (1% phosphotungstate) electron micrographs of purified SH1 disrupted as in Fig. F. Bar represents 100 nm.

**FIGURE 7.** Protein patterns of purified SH1 particles. (A) 2 × purified virus in 17% tricine SDS-PAGE and (B) 8% SDS-PAGE under reducing (lanes 1) and non-reducing (lanes 2) conditions. Protein complexes (indicated with an asterisk) appear when virus proteins are not reduced with 2-mercaptoethanol. Molecular masses of proteins or protein complexes (in kDa) of SH1 are indicated on the right. Molecular masses of standard proteins (in kDa) used for determination of SH1 protein masses are indicated on the left.

**FIGURE 8.** Physical map of the SH1 genome. A physical map of the recognition sites of restriction endonucleases *Ase*I, *Mse*I, *SnaBI* and *Ssp*I was developed using nuclease Bal-31 and restriction endonuclease double digests. Recognition sites of restriction endonuclease are indicated by horizontal lines. Sizes (in kilobase pairs) of DNA fragments generated by
restriction endonuclease digestion are indicated on each fragment. The unlabelled $MseI$ fragments are, in order from left to right, 0.2, 0.3 and 0.2 kb.
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