Characterization of New Viruses from Hypersaline Environments

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ACADEMIC DISSERTATION

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Take it with a grain of salt

Original publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I Porter K. [*], Kukkaro P. [*], Bamford J. K. H., Bath C., Kivelä H. M., Dyall-Smith M. L., Bamford D. H. SH1: A novel, spherical halovirus isolated from an Australian hypersaline lake. (2005) *Virology* **335:** 22-33.

II Kivelä H. M. [*], Roine E. [*], Kukkaro P., Laurinavicius S., Somerharju P., Bamford D. H. Quantitative dissociation of archaeal virus SH1 reveals distinct capsid proteins and a lipid core. (2006) *Virology* **356:** 4-11.

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[*] These authors contributed equally.

Also unpublished data will be presented.

Abbreviations

ABV	*Acidianus* bottle-shaped virus
Aeh1	*Aeromonas hydrophila* bacteriophage 1
AFV1	*Acidianus* filamentous virus 1
ARV1	*Acidianus* rod-shaped virus 1
ATV	*Acidianus* two-tailed virus
bp	base pairs
BSA	bovine serum albumin
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ds	double-stranded
EM	electron microscopy
HCTV-1	*Haloarcula californiae* tailed virus 1
HHPV-1	*Haloarcula hispanica* pleomorphic virus 1
HHTV-1	*Haloarcula hispanica* tailed virus 1
HIV-1	human immunodeficiency virus 1
HRPV-1	*Halorubrum* pleomorphic virus 1
HRTV-1	*Halorubrum* tailed virus 1
ICTV	International Committee on Taxonomy of Viruses
kb	kilobase pairs
kDa	kilodalton
LC	lipid core
MOI	multiplicity of infection
MPa	megapascal
mRNA	messenger RNA
OM	outer membrane
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
pfu	plaque forming units
PG	phosphatidylglycerol
PGP-Me	phosphatidylglycerophosphate methyl ester
PGS	phosphatidylglycerosulfate
p.i.	post infection
PM	plasma membrane
RNA	ribonucleic acid
rRNA	ribosomal RNA
SCTP-1	*Salicola* tailed virus 1
SCTP-2	*Salicola* tailed virus 2
SDS	sodium dodecyl sulphate
ss	single-stranded
STIV	*Sulfolobus* turreted icosahedral virus
TEM	transmission electron microscopy
TMV	tobacco mosaic virus
UV	ultraviolet
VLP	virus-like particle
VP	virion protein

Summary

Viruses of Archaea are the least studied group of viruses. Fewer than 50 archaeal viruses have been reported which constitutes less than one percent of all the isolated prokaryotic viruses. Only about one third of the isolated archaeal viruses infect halophiles. The diversity of haloviruses, virus ecology in highly saline environments and the interactions of haloviruses with their hosts have been little studied. The exiguous knowledge available on halophilic systems is not only due to inadequate sampling but also reflects the extra challenge highly saline systems set on biochemical studies.

In this study six new haloviruses were isolated and characterized. Viruses included four archaeal viruses and two bacteriophages. All of the other isolates exhibited head-tail morphology, except SH1 which was the first tailless icosahedral virus isolated from a high salt environment. Production and purification procedures were set up for all of these viruses and they were subjected to stability determinations.

Archaeal virus SH1 was studied in more detail. Biochemical studies revealed an internal membrane underneath the protein capsid and a linear dsDNA genome. The overall structure of SH1 resembles phages PRD1, PM2 and Bam35 as well as an archaeal virus STIV. SH1 possesses about 15 structural proteins that form complexes under non-reducing conditions. Quantitative dissociation provided information about the positions of these proteins in the virion. The life cycle of SH1 was also studied. This lytic virus infects *Haloarcula hispanica*. Adsorption to the host cells is fairly inefficient and the life cycle rather long.

Finally, virus responses in a variety of ionic conditions were studied. It was discovered that all of the studied viruses from low salt, marine and high salt environments tolerated larger range of salinities than their bacterial or archaeal hosts. The adsorption efficiency was not determined by the natural environment of a virus. Even though viruses with the slowest binding kinetics were among the haloviruses, fast binders were observed in viruses from all environments. When the salinity was altered, the virus adsorption responses were diverse. Four different behavioral patterns were observed: virus binding increased or decreased in increasing salinity, adsorption maximum was at a particular salt concentration or the salinity did not affect the binding. The way the virus binding was affected did not correlate with the environment, virus morphology or the organism the virus infects.

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A. INTRODUCTION

1. Virosphere

Viruses are fascinating, being something in between living organisms and dead material. Viruses are obligate parasites that can replicate only inside their host cells. Outside of a host, the purpose of a virus particle is to be an inert package that protects the genome from physical, chemical and enzymatic damage, until it delivers the vital nucleic acid into a susceptible host cell. When a virus has succeeded to infect a host and virion components have been produced within the host cell, the virus particles will be assembled into new progeny that are ready to be released from the host cell to initiate a new life cycle.

At its simplest, a virion contains nucleic acid and proteins to protect it. In addition, some viruses contain a lipid membrane. The genome of a virus can be either DNA or RNA, single-stranded (ss) or double-stranded (ds). Also the diversity of shapes and sizes makes viruses a variable group of biological entities. The classification of this diverse group has been assigned to the International Committee on Taxonomy of Viruses (ICTV) that has organized viruses into orders, families, subfamilies, genuses, and species (http://www.ncbi.nlm.nih.gov/ICTVdb/). To date ICTV has approved three orders, 73 families, 9 subfamilies, 287 genera, and over 5450 viruses that belong to more than 1950 species (http://www.ictvonline.org/). Virus classification is based mainly on virus morphology, nucleic acid type and host organism.

Viruses are best known for their pathogenic nature, the diseases they cause to humans, domestic animals and plants. However, they also influence our environment in many other ways. Viruses have an important role in the regulation of carbon, nitrogen and phosphorus cycling in the world's oceans and they are vehicles in ubiquitous genetic events in nature (Wilhelm and Suttle, 1999; Weinbauer, 2004). Nanotechnology and architecture have also been influenced by virus structures. Virus particles that have evolved to endure harsh environments still contain plasticity and metastability that technological and medical research is trying to utilize (Douglas and Young, 2006).

Viruses are everywhere. Most probably all organisms have viruses infecting them and viruses seem to outnumber their hosts (Bamford *et al.*, 2005a). In aquatic environments, no matter whether it is a sea (Wommack *et al.*, 1992; Wommack and Colwell, 2000), a fresh water environment (Wommack and Colwell, 2000), or an environment with high salinity like the Dead Sea (Oren *et al.*, 1997), the virus abundance has been observed to be higher than the host abundance. The total amount of virus particles in the biosphere has been estimated to be $10^{31} – 10^{32}$ (Bergh *et al.*, 1989; Comeau *et al.*, 2008) and sea waters have been predicted to contain over 10^{30} viruses (Suttle, 2005). Although viruses are plentiful in aquatic environments, the virus abundance and diversity has been estimated to be even greater in soil environments where the range of viral abundance varies less than in aquatic samples (Srinivasiah *et al.*, 2008). Viruses have not only been isolated from common environments such as oceans (Espejo and Canelo, 1968) and sewage (Olsen *et al.*, 1974) but also from extreme conditions like hot springs (Jaatinen *et al.*, 2008), fermented fish sauce (Pauling, 1982) and Arctic sea ice (Borriss *et al.*, 2003). However, viruses have still been sampled scarcely and new morphologies are constantly discovered (Prangishvili *et al.*, 2006a; Pietilä *et al.*, in preparation; Kukkaro *et al.*, in preparation). The diversity found among recently isolated

archaeal viruses is also amazing; genomes of such viruses show little sequence homology to anything else in the biosphere (Prangishvili *et al.*, 2006b). No wonder that Comeau *et al*. (2008) assume that the phages and archaeal viruses compose the largest reservoir of unexplored sequences on this planet.

2. Extreme ecological niches; environments with high salt

Extreme environments are surprisingly diverse and include both natural environments and those that have arisen due to human activities, both intentional and accidental. These environments can be classified into geochemical extremes such as hypersaline, alkaline and acidic environments and physical extremes which include for example extremely hot, cold and high pressure environments (van den Burg, 2003). Solar salterns are hypersaline environments set up for commercial production of salt and resemble natural high salt environments derived from sea water by evaporation. Man-made environments with such abnormal characteristics as high radioactivity and toxic chemicals have been thought to be unsuitable for life. However, microbes have been detected to degrade toxic compounds (Berne *et al.*, 2007) and research has been done on utilizing microbes to clean up the contaminated sites (Monti *et al.*, 2005; Germaine *et al.*, 2006).

It was long considered that extreme environments were devoid of life. However, we now know that organisms can exist in almost every extreme ecological niche. Some microbial communities are found employing a niche that embraces multiple extreme characteristics (Pikuta *et al.*, 2007). Growth characteristics of several extremophile types are listed in Table 1. Most of the identified extremophiles belong to the archaeal domain but also bacteria and eukaryotic organisms have been identified (van den Burg, 2003). I will now focus on environments with high salinity and halophilic organisms, the environment relevant to my studies.

Table 1. Classification of extremophiles.

Type	**Growth characteristics** [a]
Halophile	High salt, e.g. 2-5 M NaCl
Alkalophile	pH >9
Acidophile	pH <2-3
Thermophile	Temperature 60 – 80°C (thermophile)
	Temperature >80°C (hyperthermophile)
Psychrophile	Temperature <15°C
Barophile / Piezophile	Pressure up to 130 MPa

[a] Characteristics as in (van den Burg, 2003)

2.1. Hypersaline environments

Two of the largest and best studied salt lakes are the Great Salt Lake (USA), which is slightly alkaline, and the Dead Sea in Middle East which is slightly acidic (Satyanarayana *et al.*, 2005). Also thalassohaline environments (ionic composition similar to sea water) have been well studied around the world. These include natural salt lakes and evaporation ponds as well as solar salterns, which

consist of a series of shallow ponds connected in a sequence of increasingly saline waters. Salted food products are also considered high salt environments. Besides these environments, which are mainly extreme in respect of the salt concentration, there are environments around the world that encompass several extreme qualities. These include evaporation ponds in the Antarctica (Bowman *et al.*, 2000) and several hypersaline alkaline soda lakes such as Lake Magadi (Kenya) (Wood *et al.*, 1989) and Wadi An Natrun (Egypt) (Mesbah *et al.*, 2007). The soda brines lack divalent cations, magnesium and calcium, because of their low solubility in high pH of the lakes (Satyanarayana *et al.*, 2005). The Dead Sea differs from many other highly saline environments by its ion composition that has concentrations of divalent cations, magnesium and calcium, exceeding those of monovalent cations, sodium and potassium. The prevalent anions in the Dead Sea are chloride and bromide (Buchalo *et al.*, 1998) and the pH is relatively low (pH 6) (Oren, 2002a). This kind of environment where the ionic composition differs from the seawater is called athalassohaline. The Great Salt Lake has a similar chemical composition as typical ocean water. The major ions are sodium and chloride, followed by sulfate, magnesium, calcium and potassium (http://geology.utah.gov/online/). Table 2 shows a comparison of six major ions found in typical ocean water, the Great Salt Lake and the Dead Sea.

Table 2. Chemical compositions (dry weight percents) of ocean, Great Salt Lake and Dead Sea (modified after Utah Geological Survey, http://geology.utah.gov/online).

Source	Potassium	Sodium	Magnesium	Calcium	Chloride	Sulfate
Ocean (typical)	30.8	1.1	3.7	1.2	55.5	7.7
Great Salt Lake	32.8	2.0	3.3	0.2	54.5	7.2
Dead Sea	12.3	2.3	12.8	5.3	67.2	0.1

2.2. Halophilic organisms

How do we define a halophilic organism? Sharp boundaries are difficult to set because microorganisms preferring different salt concentrations, from fresh water to saturated salt, can be found. Growth optima depend also on the composition of the media and the growth temperature. By one classification extreme halophiles have growth optimum in a range of 2.5 – 5.2 M salt, borderline extreme halophiles in 1.5 – 4.0 M salt and moderate halophiles in 0.5 – 2.5 M salt. Halotolerant organisms do not require the high salt concentration but can tolerate it (Oren, 2008).

Halophiles can survive because of their ability to maintain osmotic balance by accumulating salts such as sodium and potassium chloride up to concentrations that are isotonic to their environment (van den Burg, 2003) or by organic compatible solute strategy where the intracellular salt concentration is kept low and the osmotic pressure is balanced with compatible solutes (Oren, 1999). In the first option, all the intracellular systems need to be adapted to work in high salt concentrations but it is energetically a better option than maintaining low intracellular salt concentration. Compatible solutes can either be produced by the cell or taken up from the environment (Oren, 1999). Halophiles comprise a great metabolic diversity. They include oxygenic and anoxygenic phototrophs, aerobic heterotrophs, fermenters, denitrifiers, sulfate reducers, and methanogens. However, the diversity of these metabolic types decreases with salinity

(Oren, 2002a) and not all the known metabolic types have been observed to function in high salt (Oren, 1999). Halophilic proteins are highly negatively charged to keep them soluble (van den Burg, 2003) and contain plenty of acidic amino acids and little hydrophobic amino acids (Oren, 1999). Because of the biotechnological interest in halophilic organisms and their enzymes, halophilic organisms are continuously isolated from different locations such as hypersaline lakes in Inner Mongolia (Pan *et al.*, 2006) and Algerian Sahara (Hacene *et al.*, 2004). Halophiles are also used in bioremediation of oil-contaminated high salt ecosystems (Pikuta *et al.*, 2007).

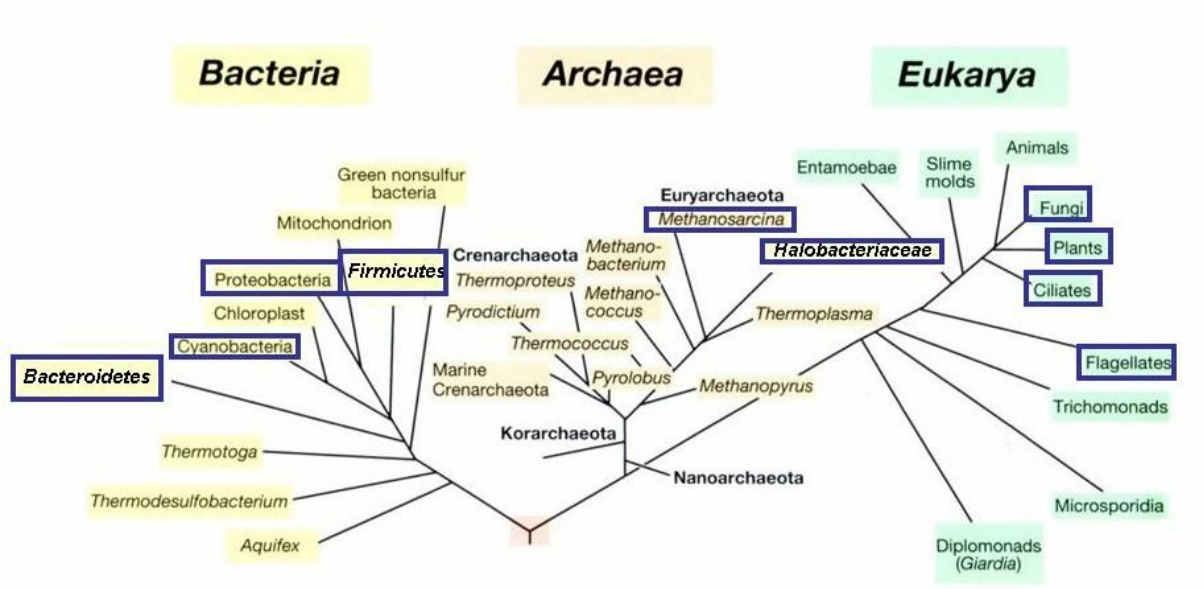


Figure 1. Halophilic microorganisms in the phylogenetic tree of life. The blue boxes mark the groups that contain halophilic organisms. Adapted from Oren (2008).

2.2.1. Eukarya in highly saline environments

A vast majority of identified organisms requiring high salt concentration for growth are archaea. However, they are not the sole organisms inhabiting environments with high salt, since both bacteria and eukaryotes have also been identified (Figure 1). Many of the eukaryotes inhabiting hypersaline environments are not halophilic but halotolerant. *Dunaliella*, a unicellular green alga, has been observed to be responsible for most primary production in hypersaline environments world wide. It is a well studied example that was first observed in 1838 in salt evaporation ponds in France (Oren, 2005). A variety of diatoms, eukaryotic algae, have been observed in salinities of ~2 M NaCl. These include species of *Amphora coffeaeformis*, *Nitzschia* and *Navicula* (Satyanarayana *et al.*, 2005). Protozoa (*Porodon utahensis* and *Fabrea salina*) (Satyanarayana *et al.*, 2005) and halotolerant yeast (*Debaryomyces hansenii*) (Sharma *et al.*, 2005) have also been described in saline environments. A variety of halophilic fungi has been reported in the Dead Sea (Buchalo *et al.*, 1998) and growing on salted fish (Wheeler and

Hocking, 1993). There is also a macroorganism that is found in a variety of high salt environments: the brine shrimp *Artemia* (Oren, 2002a). Eukaryotes seem to have adapted to high osmotic pressure by compatible solute strategy and no representatives have been found to have high intracellular salt concentration (Oren, 1999).

2.2.2. Halophilic bacteria

It was surprising when as high numbers as ~10^6 bacteria in a milliliter of water were found in a Spanish solar saltern, since these environments were thought to be inhabited solely by archaea (Anton *et al.*, 2000). Nevertheless, bacteria have been observed to contribute to the total biomass much less than archaea (Oren and Rodriguez-Valera, 2001). Many of the bacteria found in high salt environments are rather moderate than extreme halophiles (Oren, 2002a) but there are also bacteria, e.g. *Salinibacter* that have similar high salt requirement as the most halophilic archaea. *Salinibacter* have been found to be abundant in many high salt locations (Benlloch *et al.*, 2002) and constitute as much as 25% of the total prokaryotic community in several solar salterns in Spain (Anton *et al.*, 2000). Both Gram-negative and positive bacteria are present in highly saline environments and often have close relatives that are non-halophilic (Oren, 2002a). Many species of cyanobacteria have been reported in highly saline environments but their diversity has not been studied extensively (Brock, 1976; Satyanarayana *et al.*, 2005; Green *et al.*, 2008). The strategy that halophilic bacteria mostly use for surviving the osmotic stress is compatible solutes but halophilic anaerobic bacteria belonging to *Halanaerobiales* have been observed to have high intracellular salt concentrations (Oren, 1999).

2.2.3. Salt-loving archaea

Halophilic archaea can be distinguished from halophilic bacteria because of their archaeal characteristics, especially by the presence of ether-linked lipids (Pikuta *et al.*, 2007). The majority of the halophilic archaea requires 1.5 M NaCl for maintaining cellular integrity and have red pigmentation. The red color is derived mostly from carotenoids. Some haloarchaea also have a bacteriorhodopsin containing purple-membrane which is a light-dependent transmembrane proton pump that can support periods of phototrophic growth (Pikuta *et al.*, 2007). An archaeal group, *Halobacteriales*, has been observed to use the "salt in" strategy to cope with the osmotic stress (Oren, 1999). Other archaea seem to produce compatible solutes.

Archaea belonging to the family *Halobacteriaceae* have been observed in many locations to be the main component of the microbial biomass (Oren, 2002a) and members of this family are the most salt requiring organisms within Archaea (Oren, 2008). It was already 1980 when Walsby recognized square archaeal cells in hypersaline brine collected near the Red Sea (Walsby, 1980) but only recently such an organism was cultivated (Bolhuis *et al.*, 2004; Burns *et al.*, 2004a). This flat and square archaea has been observed to dominate hypersaline microbial communities (e.g. Anton *et al.*, 1999; Benlloch *et al.*, 2001; Benlloch *et al.*, 2002) and is now formally described as *Haloquadratum walsbyi*, a member of a novel genus within the family of *Halobacteriaceae* (Burns *et al.*, 2007). Halophilic archaea have also been reported within the class of *Methanothermea* (order

Methanosarcinales) but all the identified haloarchaea belong to the kingdom Euryarchaeota with no representatives within the other major kingdom of Archaea, Crenarchaeota (Oren, 2008). Crenarchaea consists mainly of thermophiles instead.

3. Haloviruses

Virus abundances observed in hypersaline environments are similar to what has been observed in other aquatic environments (Weinbauer, 2004; Suttle, 2005). Many studies have reported $10^7 – 10^9$ virus-like particles in a milliliter of water in high salt environments (Guixa-Boixareu *et al.*, 1996; Oren *et al.*, 1997; Bettarel *et al.*, 2006). In the study of virus abundance in the Dead Sea the amount of virus-like particles (VLP) was 0.9 – 9.5 times higher than the prokaryotic abundance, the value depending on the sampling time (Oren *et al.*, 1997). In two Spanish solar salterns the amount of VLPs was about one order of magnitude higher than the amount of prokaryotes (Guixa-Boixareu *et al.*, 1996). In these salterns the number of VLPs was observed to correlate with the prokaryotic abundance rather than with chlorophyll *a* suggesting that most of the viruses were prokaryotic ones. Also the virus abundance was observed to increase with increasing salt concentration and larger burst sizes were detected in the most saline ponds. However, the salinity effect seems to vary within the location studied, since in a Jamaican salt pond decreased viral abundance was observed with increasing salt concentrations (Wais and Daniels, 1985). Also the viral diversity has been observed to reduce with increasing salt concentration (Diez *et al.*, 2000). The effect that viruses have on prokaryotic mortality seems to depend on the location as well. In solar salterns (Spain) and in an alkaline hypersaline lake (USA) viruses did not seem to be a significant loss factor (Guixa-Boixareu *et al.*, 1996; Brum *et al.*, 2005) whereas they were observed to have a major role in the decline of prokaryotic communities in the Dead Sea (Oren *et al.*, 1997). The occurrence of different morphologies among VLPs in high salt environments has been studied using transmission electron microscopy (TEM). The most often observed morphologies have been spindle-shaped, tailed icosahedral and tailless icosahedral particles (Guixa-Boixareu *et al.*, 1996; Oren *et al.*, 1997; Diez *et al.*, 2000) but also other morphologies e.g. star-shaped particles have been observed (Oren *et al.*, 1997). In the study by Guixa-Boixareu *et al*. (1996) the abundance of spindle-shaped particles was observed to increase with increasing salinity.

3.1. Viruses of halophilic archaea

The diversity of morphologies discovered among isolated euryarchaeal viruses, which mostly consists of haloviruses, is much narrower than those found among crenarchaeal viruses (Figure 2). However, when more archaeal haloviruses are being isolated, new morphologies are found (see below) (Pietilä *et al.*, in preparation; Kukkaro *et al.*, in preparation). This is no surprise since only 44 archaeal viruses had been reported by 2007 and only about one third of these were viruses that infect halophilic archaea (Ackermann, 2007). Also the diversity found among genome types of isolated haloarchaeal viruses is minimal; all viruses exhibit linear dsDNA genomes (Prangishvili *et al.*, 2006b), except a newly isolated

pleomorphic virus HRPV-1 which has a circular ssDNA genome (Pietilä *et al.*, in preparation).

The ecology of archaeal haloviruses has not been much explored. This is probably due to the difficulty of differentiating viruses infecting archaea and bacteria in natural samples. However, in the study of two Spanish solar salterns the abundance of square archaea was observed to correlate with the abundance of spindle-shaped VLPs (Guixa-Boixareu *et al.*, 1996). When the samples were examined by TEM the square archaea were observed to be infected by viruses of other morphologies as well.

Most of the isolated haloarchaeal viruses have a tailed icosahedral morphology and they belong to families *Myoviridae* and *Siphoviridae* (Ackermann, 2007). Also the first archaeal virus discovered, *Halobacterium salinarium* virus Hs1, was a head-tail virus (Torsvik and Dundas, 1974). The best studied examples of tailed icosahedral haloviruses are *H. salinarium* virus ϕH, *Natrialba magadii* virus ϕCh1 as well as HF1 and HF2 which infect several haloarcheal species. Even though other archaeal virus genomes have little homology to sequences in the databases, the genomes of the archaeal head-tail viruses are different: they have several homologus matches to head-tail bacteriophages (Prangishvili *et al.*, 2006a).

ϕH belongs to the family *Myoviridae* and has an icosahedral head with a diameter of 64 nm and a contractile tail measuring 170 nm in length with tail fibers attached to it. ϕH was isolated after spontaneous lysis of its host, a laboratory strain of *H. salinarium* (Schnabel *et al.*, 1982). The virus is temperate and the genome is maintained as a circular plasmid during the lysogeny (Schnabel, 1984). The circular provirus genome of ϕH is subject to a lot of variation. This is due to duplications and inversion of an L segment of the genome that is flanked by insertion elements (Schnabel, 1984). The genome is proposed to be packaged by head full mechanisms since the ends of the genome are terminally redundant (Schnabel *et al.*, 1982).

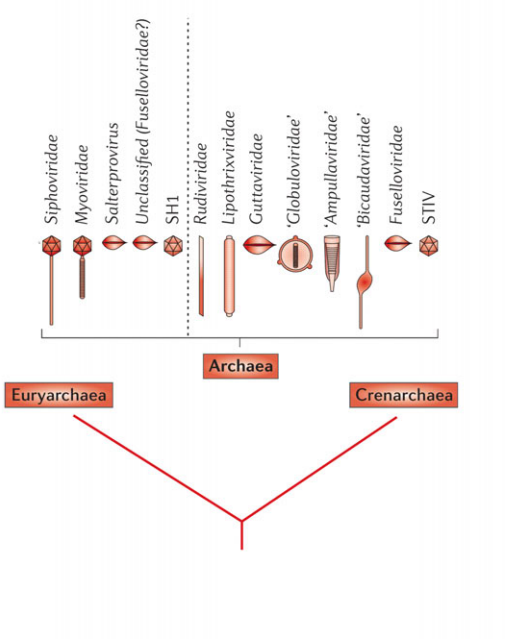


Figure 2. Morphologies found within archaeal viruses; comparison of euryarchaeal and crenarchaeal viruses. Euryarchaeal and crenarchaeal morphotypes are divided by the dashed line. The proposed virus families are shown in inverted commas and those approved by the International Committee on Taxonomy of Viruses with out. Viruses are not drawn to scale. Modified from Prangishvili *et al.* (2006a).

Haloalcalovirus ϕCh1 is also a member of the *Myoviridae* family and was discovered after spontaneous lysis of its host *Natrialba magadii*, the same way that ϕH was isolated. It is the only archaeal virus known to contain both DNA and RNA in its virion. It is a temperate virus that exists as a chromosomally integrated provirus. The virus head has a diameter of 70 nm and the tail is 130 nm long (Witte *et al.*, 1997). The genome of ϕCh1 has been sequenced. It is intriguing that a comparison of ϕCh1 genome to the partially sequenced genome of ϕH reveals a close relationship between the viruses although they inhabit considerably different environments in respect to pH (Klein *et al.*, 2002).

HF1 and HF2 belong to the family *Siphoviridae*. These lytic viruses were isolated from the same Australian solar saltern at the same time. They have identical morphologies: head diameter 58 nm and tail length 94 nm. HF1 has a very broad host range including *Halobacterium*, *Haloferax* and *Haloarcula* species whereas HF2 is know to infect only *Halorubrum saccharovorum* (previously know as *Halobacterium saccharovorum*) and a natural isolate Ch2 (Nuttall and Dyall-Smith, 1993a). The genomes of these viruses that are nearly 80 kb in size, have a 48 kb region that is identical except for a single base change. This suggests a recent recombination event has happened between these two viruses or yet another HF-like virus. The total genome identity is over 94% (Tang *et al.*, 2004).

Only two spindle-shaped haloviruses have been isolated, although this has been found to be one of the dominant morphotypes in the natural salt water samples (Guixa-Boixareu *et al.*, 1996; Oren *et al.*, 1997; Diez *et al.*, 2000). This is also one of the two morphotypes observed among both euryarchaeal and crenarchaeal viruses (Figure 2). The two isolates, His1 and His2 infecting *Haloarcula hispanica*, were isolated at different times. His1 was discovered from an Australian solar saltern whereas His2 was isolated later from a salt lake in Australia (Bath and Dyall-Smith, 1998; Bath *et al.*, 2006). His1 and His2 have similar particle morphology, with dimensions of about 44 × 77 nm and 44 × 67 nm, respectively, but differ in stability to raised temperature, low salt and chloroform. The viruses are lytic and proposed to exit the host without cell lysis. The genomes of His1 and His2 show little sequence similarity and they seem to be only distantly related. Both viruses have genomes with inverted terminal repeats and terminal proteins which suggest that the viruses replicate by protein-priming (Bath *et al.*, 2006).

Two newly isolated pleomorphic membrane containing viruses are dissimilar to morphologies observed this far among haloarchaeal viruses. HRPV-1, infecting *Halorubrum* sp., has a pleomorphic appearance with spike structures protruding from its external membrane (Pietilä *et al.*, in preparation), whereas HHPV-1, infecting *H. hispanica*, has a dsDNA genome, external membrane and a tadpole shape (Kukkaro *et al.*, in preparation). HRPV-1 and HHPV-1 were isolated from solar salterns in Italy, Trapani and Margherita di Savoia respectively. Neither one of these viruses lyses their host cells but the number of free viruses in the media increases in the course of time.

3.2. Bacteriophages in high salt

Halophages have not been studied extensively and the information available is scattered. Recently a bacteriophage ϕgspC, with an unusually large genome, was isolated from the Great Salt Plains National Wildlife Refuge (USA) (Seaman and Day, 2007). It is claimed to be the first phage with *Myoviridae* morphology infecting genus *Halomonas*. Also four additional phages infecting *Halomonas* were isolated from the same location but only one of them, ϕgspB, was studied in some detail. ϕgspC is a temperate phage with a wide host range and a genome of 340 kb. The genome is proposed to have genes increasing not only the fitness of the phage but also the fitness of the host. Both ϕgspB and C show high tolerance to a range of temperatures, salinities and pH (Seaman and Day, 2007).

The host of phage F9-11 is a moderately halophilic bacterium *Halomonas halophila* (previously know as *Deleya halophila*). The phage was isolated from a lysogenic host strain originating from a hypersaline soil sample (Spain). F9-11 exhibits head-tail morphology and virions

stay infectious in a wide range of salinities (0 – 30% w/v) for a long period of time (Calvo *et al.*, 1988). Phage Ps-G3 infects a moderately halophilic bacterium, *Pseudomonas* sp. G3, and is relatively stable without salt. This phage was isolated from a Canadian salt pond and has head-tail morphology (Kauri *et al.*, 1991).

4. Virus morphology

Only four morphologies are found among isolated haloviruses. Most of the viruses from high salt are of head-tail morphology. In addition, two spindle-shaped and two pleomorphic viruses have been reported. Year 2003 there also was a preliminary report on isolation of the icosahedral halovirus SH1 (Dyall-Smith *et al.*, 2003). Structures of the haloviruses have not been studied except for SH1 (Jäälinoja *et al.*, 2008) which structure will be discussed in detail in the section Results and discussion.

Virus morphology is usually based on either of the forms, helix or a sphere (with icosahedral symmetry) (Voyles, 2002). Crick and Watson suggested in 1956 that it is easier for the virus to force its host to make large amounts of identical small proteins than just a couple of copies of a large protein that could form a shell for the nucleic acid. The small proteins could then interact only in certain ways and produce symmetrical capsids, either helical or spherical (Crick and Watson, 1956). It has been calculated that a nucleic acid can contain a genetic code for a protein up to 15% of the nucleic acid's own weight (Cann, 2005). This means that it is impossible for a virus to code for proteins so large that only a few copies would be needed to encapsidate the genome. The interactions that hold a virus particle together include protein-protein, protein-nucleic acid and protein-lipid interactions. The forces behind the interactions are hydrophobic and electrostatic but only seldom covalent (Cann, 2005).

4.1. Helical structures and viruses

No helically arranged viruses have been isolated from high salt environments. However, the tails of the head-tail viruses are helical and structured as a tube with a hollow space inside. Similarly, helical viruses are built in this tube like format and the nucleic acid occupies the space inside. To form a cylinder, copies of a single protein are arranged in a ribbon like format where the proteins also interact with adjacent ones on both sides when the ribbon forms a tube (Figure 3A). The viruses built this way may comprise additional proteins to cap the ends of the cylinder. Tobacco mosaic virus (TMV) is the best studied representative of the helical viruses (Klug, 1999). Many plant viruses have this morphology but the reason why it is so common among them is unknown. Members of the family *Rudiviridae* infecting crenarchaea also exhibit naked helical morphology (Vestergaard *et al.*, 2005; Vestergaard *et al.*, 2008b). In contrast, no naked helical animal viruses are known (Cann, 2005). Some helical virus particles are rigid but many longer virions show some degree of flexibility which prevents an easy breakdown by forces it confronts. The particle length of a helical virus depends on the genome length. Virus capsids of different lengths can be observed among phages belonging to the family *Inoviridae*. The length depends on whether a virion contains less or more than a genome length

of virus DNA. This ability of the capsid extension has been utilized in making cloning vectors of a phage M13, for example (Hines and Ray, 1980).

4.2. Icosahedral capsids

An icosahedron contains 20 facets and 12 vertices (Figure 3B). The protein interactions are not quite as simple in this morphology as they are in helical capsids. Capsid proteins occupy only nearly same environments and the interactions they have with adjacent proteins are not identical. This quasi-equivalence theory permits self assembly of icosahedral capsids and was proposed for viruses by Caspar and Klug (1962). The minimum number of proteins needed to assemble an icosahedral virus capsid is 60 copies, three copies of a single protein forming each facet. In addition to the major capsid proteins, a virus can contain additional proteins serving many functions. Proteins involved in genome packaging and ejection are often included in the virion. Additional proteins can also be found in spikes that protrude from the vertices. Despite the icosahedral symmetry of a virus, all the proteins do not need to be icosahedrally assembled. For example, some viruses contain a unique vertex that is composed differentially than the other 11 and functions in the genome packaging (Gowen *et al.*, 2003; Karhu *et al.*, 2007). When an icosahedron is formed the help of scaffolding proteins is often needed for assembly. Scaffolding proteins are non-structural and are not found in the mature virion but only from the procapsids (Dokland, 1999).

Phages of the family *Microviridae*, ϕX174 for example, encompass icosahedral morphology (Ilag *et al.*, 1995). Icosahedral viruses (without membranes) are found among animal (e.g polio virus) and plant viruses (e.g. cowpea mosaic virus) but there are no archaeal virus isolates with this morphology (Prangishvili *et al.*, 2006a). Some viruses exhibit variations of a basic icosahedron. Interesting examples are geminiviruses which have two icosahedrons joined to form a virus capsid (e.g. Böttcher *et al.*, 2004).

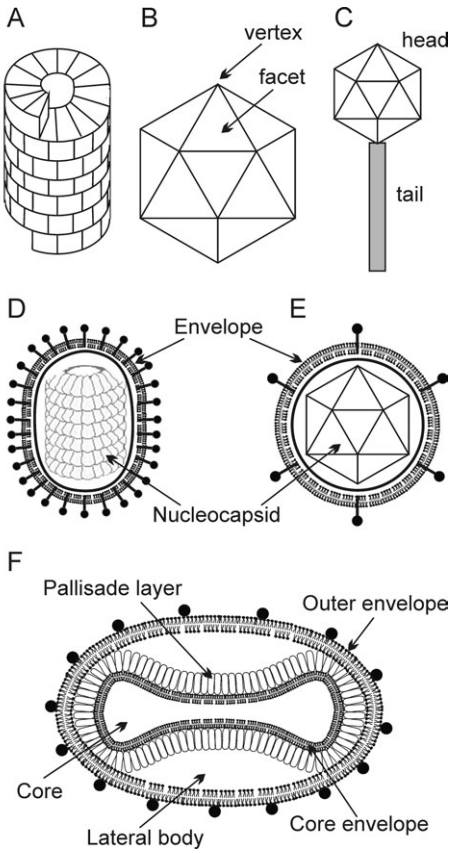


Figure 3. Virus morphologies. Viruses are built often with either helical (A) or icosahedral (B) symmetry. Head-tail viruses (C) exhibit binary symmetry, with both helical and icosahedral parts. Enveloped viruses can have helical (as in the case of influenza A virus) (D) or icosahedral nucleocapsid (ϕ6) (E) or the nucleocapsid appears amorphous as with poxviruses (F). Viruses are not drawn to scale.

4.3. Head-tail viruses

Head structures of the head-tail viruses are icosahedral. They can be either symmetrical, like the head of phage λ (Dokland and Murialdo, 1993), or elongated (prolate) as with phage T4 (Fokine *et al.*, 2004). In the prolate head, the elongation is due to extra copies of the capsid proteins added to the horizontal axis (in relation to the tail) of the head. A head-tail virus also encompasses a helical structure, the tail (e.g. Plisson *et al.*, 2007). That is why head-tail viruses are said to have a binary symmetry. The tail is attached to the head at one end and it can have additional structures, like tail fibers, at the other (Figure 3C). Head-tail viruses belong to the order *Caudovirales* which can be divided into three families based on the tail structure (Ackermann, 2007). Viruses of the family *Myoviridae* have long contractile tails whereas viruses of *Siphoviridae* have long but non-contractile tails. The described head-tail haloviruses belong to these two families. Members of the third family, *Podoviridae*, have short tails.

4.4. Viruses with membranes

4.4.1. Icosahedral viruses with an internal membrane

Bacteriophages belonging to the families *Tectiviridae* and *Corticoviridae*, for example, have a lipid bilayer inside the icosahedral protein capsid and the membrane surrounds the genome (Bamford, 2005; Abrescia *et al.*, 2008). It was first shown with PM2, the sole member of the *Corticoviridae* family, that a phage can have lipids as a structural component of a virion (Espejo and Canelo, 1968). A crenarchaeal virus *Sulfolobus* turreted icosahedral virus (STIV) has also similar structural arrangement as the internal membrane containing phages and is actually proposed to have common ancestry with members of the *Tectiviridae* family based on the coat protein structure (Maaty *et al.*, 2006). With PRD1, the type virus of the *Tectiviridae* family, the membrane of the virus has been observed to form a tail-tube structure upon infection and genome delivery (Grahn *et al.*, 2002).

4.4.2. Enveloped viruses

Viruses with protein capsids surrounded by a membrane are known to infect organisms from all three domains of life. Even though some phages, archaeal and plant viruses have this feature, most of the enveloped viruses infect animals. Pleomorphic haloviruses HHPV-1 and HRPV-1 have an external membrane but the nature of the nucleocapsid is unknown (Kukkaro *et al.*, in preparation; Pietilä *et al.*, in preparation). Nucleocapsid inside the membrane can be of a helical (influenza A virus; Nayak *et al.*, 2004) or icosahedral symmetry (ϕ6; Huiskonen *et al.*, 2006) (Figures 3D and E). There are also viruses that have no clearly structured nucleocapsid and appear amorphous (Cyrklaff *et al.*, 2005) (Figure 3F). The shapes of enveloped viruses vary enormously from a bottle shaped archaeal virus *Acidianus* bottle-shaped virus (ABV) (Häring *et al.*, 2005a) to rod-shaped (e.g. Arnold *et al.*, 2000) and just fairly round ones (e.g. Paredes *et al.*, 2004; Ganser-Pornillos *et al.*, 2008). Also the spindle-shaped viruses of the family *Fuselloviridae* infecting thermophilic archaea are enveloped (http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/i

ndex.htm) and the spindle-shaped haloviruses His1 and His2 may also contain a membrane but direct chemical assays are needed to prove it (Bath *et al.*, 2006). The virus membranes are host derived. The membrane has viral proteins inserted in to it but it may also contain small amounts of host derived proteins (Cann, 2005). The membrane proteins often function in host recognition and attachment or they form transport channels to the membrane. Enveloped viruses tend to use the host membrane as a place to direct assembly (Cann, 2005).

5. Early virus-host interactions

A crucial point for the outcome of virus infection is finding a susceptible host cell and the binding of a virion to the receptor molecule. At this stage an inert virus particle is activated to initiate the replication cycle of the virus. Often entry and virus uncoating are a programmed series of multiple reactions. This process starts at the cell surface with receptor binding and ends for example at the nucleus with genome decondensation (Bartlett *et al.*, 2000). The entry process affects the virus particle so that such things as penetration, capsid destabilization and genome uncoating will be enabled. These events can be triggered by receptor binding, low pH, re-entry to reducing environment and covalent modifications induced by enzymes (Greber et al., 1996; Ojala *et al.*, 2000; Simmons *et al.*, 2005; Nitschke *et al.*, 2008). Viruses infecting different organisms encounter some common problems upon the early stages of infection; a virus needs to bind specifically to the receptor of a vital cell and find a way to get the genome across a cell membrane. The genome can either enter the cell leaving the capsid outside the cell as in the case of most of the bacteriophages, or the whole virus particle can enter the cell. Viruses with dsRNA genome often deliver the genome to the cell in a protein capsid to avoid exposure of the RNA to the cell cytosol (Huismans *et al.*, 1987; Romantschuk *et al.*, 1988; Jiang and Coombs, 2005). Some viruses encounter additional barriers. For example phages, archaeal, fungal and algal viruses need to get through the cell wall. These viruses may carry enzymes in the virion that facilitate the process or the virus can be introduced to the cell by an invertebrate vector. Another option, used by fungal viruses, is to spread when cell to cell fusion occurs and no cell wall penetration is needed (Poranen *et al.*, 2002). Those eukaryotic viruses that replicate in the nucleus have the nuclear membrane as an additional barrier. Herpes virus has solved this problem by injecting the genome through a special vertex and a nuclear pore complex to the nucleus leaving the protein capsid in the cytosol (Sodeik *et al.*, 1997; Newcomb *et al.*, 2001). This is similar to the strategy many bacteriophages use to transfer their genome into a cell (see below).

5.1. Virus binding to a receptor

No receptor or receptor binding protein of an archaeal virus has been identified. Molecules used as virus receptors are variable and often difficult to identify. They include different proteins, lipids and carbohydrates which are normally exposed on the cell surface. Some viruses are known to use several receptors, either concomitantly or sequentially. Probably the best know example is human immunodeficiency virus 1 (HIV-1) which uses both CD4 and a chemokine receptor

during the attachment (Berger *et al.*, 1999). Similar behavior has been observed also with many bacteriophages. First a phage binds to a primary receptor, usually reversibly, and the binding to a secondary receptor makes the adsorption irreversible (Weinbauer, 2004). The binding to a primary receptor may cause conformational changes in the virus particle as has been observed with phage T4 (Leiman *et al.*, 2004). It is interesting that some fast mutating viruses e.g. Sindbis virus, can change the receptor it uses with only one or two dominant mutations in the gene that codes for the attachment protein (Klimstra *et al.*, 1998). Some viruses are also known to use an alternative receptor if their first choice is not present (Vlasak *et al.*, 2005). Receptors of animal viruses can be of many functions: e.g. ion transporters, signaling proteins and adhesion factors. These receptors can vary from ubiquitous to rare or be cell type specific. In addition to receptors, animal viruses also might bind to attachment factors which are relatively non-specific and help to concentrate viruses on the cell surface (Marsh and Helenius, 2006). Phage receptors are known to include, for example, lipopolysaccharide (LPS) (e.g. P22; Israel *et al.*, 1972), pili (e.g. ϕ6; Bamford *et al.*, 1976) and in the case of phage SPP1 both teichoic acids and a membrane protein YueB (Baptista *et al.*, 2008).

The receptor binding proteins of phages with head-tail morphology reside in the tail whereas icosahedral viruses often have the binding proteins at their vertex spikes as with phage PM2 (Huiskonen *et al.*, 2004). Naked animal viruses with no spikes, attach directly by their capsids to the receptor. Attachment proteins of enveloped viruses reside in the membrane. Archaeal virus *Acidianus* filamentous virus 1 (AFV1) has claw like structures at both ends of the filamentous body. These claws have been observed to mediate the attachment of the virion to pili of the host cells (Bettstetter *et al.*, 2003).

Even though adsorption of a virus to a receptor is highly specific, the interactions tend to be weak. However, multiple receptor binding sites on the virus enable an increase in affinity and result in nearly irreversible binding (Smith and Helenius, 2004). The interactions in adsorption, between a receptor binding protein and a receptor molecule, are probably electrostatic. Charged amino acids bind other charged amino acids or to carboxyl groups on the N-acetyl-sugars. For these interactions to take place, the molecules involved in the attachment need to have three-dimensional shapes that enable close enough contact (Adams, 1959; Voyles, 2002). The protein ionization and the three-dimensional shape of a protein are both affected by ionic strength and pH of the medium. Addition of divalent cations, for example, may help in virus adsorption by providing a bridge between two negatively charged groups. Divalent cations may also influence the three-dimensional shape of the proteins (Voyles, 2002). The rate of adsorption is affected by the absolute concentration of cells and viruses. Dilution of a virus host mixture 100-fold reduces the adsorption rate by a factor of 10^4. A large range of adsorption rate constants have been observed for different animal viruses: numbers have five orders of magnitude variation (Voyles, 2002).

5.2. Penetration

Genome delivery across the cell membrane either involves membrane fusion, pore formation or membrane permeabilization (Sieczkarski and Whittaker, 2005). Eukaryotic cells offer a variety of endocytic pathways, trafficking and sorting mechanisms that viruses have learned to utilize (Marsh and Helenius, 2006). Pathways that phages use to get across the bacterial cell membrane are

related to bacterial conjugation systems instead. These pathways enable the phage genome to pass through specific protein complexes that span through the membrane (Poranen *et al.*, 2002). The mechanisms of virus penetration to archaeal cells are unknown. Both prokaryotic and eukaryotic viruses are known to take advantage of cell filaments to be able to have directional movement (Jacobson, 1972; Romantschuk and Bamford, 1985; Sodeik *et al.*, 1997; Suomalainen *et al.*, 1999; Boyko *et al.*, 2000). This assists a virus to move to the place of replication.

The membrane fusion of enveloped viruses can either occur at the plasma membrane (PM) or if the virus enters via endocytic pathway, the virus membrane fuses with the vesicle membrane. Both pH-dependent (Lavillette *et al.*, 2006; Cote *et al.*, 2008; Rojek and Kunz, 2008) and pH-independent (Bamford *et al.*, 1987; Pedroso de Lima *et al.*, 1992; Marchant *et al.*, 2005) fusion proteins are known, the latter being able to fuse directly with PM or an outer membrane (OM) of gram negative bacteria. Penetration of non-enveloped viruses is less understood than the membrane fusion events of enveloped viruses. As with enveloped viruses, non-enveloped viruses are known to use both pH-dependent (Prchla *et al.*, 1994; Ashok and Atwood, 2003) and pH-independent (Perez and Carrasco, 1993; Ashok and Atwood, 2003) pathways for penetration.

Most of the bacteriophages deliver only the genome into the host cell. This might be because the prokaryotic cell envelope is a much more difficult barrier to penetrate than the single PM of animal cells (Poranen *et al.*, 2002). In the case of head-tail phages the virus genome is released from the phage head through a special vertex and the tail (Letellier *et al.*, 1999). The tail has structures that are specialized in the entry functions. When the genome is delivered across a cell membrane, the formed pore can be made of cellular proteins (phage λ; Roessner and Ihler, 1986; Berrier *et al.*, 2000) or the phage itself can carry pore forming proteins (phage T5; Feucht *et al.*, 1990). In the case of phage T4 the virus induces fusion of the OM and the PM resulting in a channel through which the genome can be delivered (Tarahovsky *et al.*, 1991; Tarahovsky *et al.*, 1995).

6. Virus life cycles

After a virus or its genome has entered the cell interior, the life cycle of a virus will continue. There are different paths the life cycle can take, depending on a virus.

In a lytic cycle the virus replication begins when the virus genome has entered a host cell. Viruses often need to control the gene expression so that certain genes will be expressed at a right time, e.g. proteins that are involved in host cell lysis should not be present before the virions are fully assembled and ready to confront the cell exterior. Viral proteins that are produced first usually aid in recruiting the host resources for a virus. Viral nucleic acids are replicated and structural protein synthesis often begins sometime after that (Voyles, 2002). The genome type of a virus as well as the type of host cell influence the protein synthesis and genome replication. In a prokaryotic cell the genome replication and transcription are not separated in space and can occur on the DNA concurrently. If a virus has a positive strand RNA genome, the genome can serve directly as mRNA for protein translation (Gamarnik and Andino, 1998). Viruses with RNA genomes encounter the problem of making new RNA molecules from an RNA template. Retroviruses which have positive strand RNA make a dsDNA copy of the genome and insert it to the host genome (e.g.

Bushman *et al.*, 1990) whereas other RNA viruses usually encode for an RNA dependent RNA polymerase (e.g. Van Etten *et al.*, 1973; Satija and Lal, 2007).

When both nucleic acids and viral proteins have accumulated in the cell the assembly process of new virions begins. There are two ways the assembly generally occurs. Either the capsids are preformed and the genome is packaged into it or the genome and the capsid proteins coassemble. However, in either of the cases the information needed for the proper assembly is in the amino acid sequence and the three-dimensional structure of the virion proteins (Caspar and Klug, 1962). The self assembly of a virus particle into an infectious virus from its constituents was demonstrated with TMV and is an example of coassembly (Fraenkel-Conrat and Williams, 1955). The place for virus assembly in a cell depends on the replication site as well as on the mechanisms of virus release. For example, enveloped viruses acquire the membrane often upon exit from the cell meaning that part of the assembly and exit occur concurrently.

The new progeny can exit the host cell in different ways. Budding from a host cell leaves the host intact and still viable. Spindle-shaped haloviruses His1 and His2 have been proposed to exit the host cell without causing cell lysis (Bath *et al.*, 2006). Usually enveloped viruses use this strategy, but exit without cell lysis has been proposed also for the naked archaeal virus *Acidianus* rod-shaped virus 1 (ARV1) (Vestergaard *et al.*, 2005). When host cells are not destroyed it enables a virus to establish persistent infection. When the host cell lyses the virus particles are released at once. This is a regulated process which needs to ensure that the virus particles are mature at the time of lysis (Rydman and Bamford, 2003). The head-tail virus Ja1 is an example of the lytic haloviruses (Wais *et al.*, 1975). Some viruses also have maturation steps that need to take place before the virion is infective, for example, retrovirus maturation occurs after release (Fu *et al.*, 2006). Archaeal virus *Acidianus* two-tailed virus (ATV) even has previously unseen major morphological development outside the host cell (Prangishvili *et al.*, 2006c). After a tailless lemon-shaped particle has been released from the host, two long tails will be developed, one to each end of the particle.

The virus infection is not always productive. Some bacteriophages and archaeal viruses can enter lysogenic life cycle at which time the viral genome exists as a prophage; the genome is either integrated into the host genome as with haloarchalovirus ϕCh (Witte *et al.*, 1997) or it exists as a plasmid as halovirus ϕH (Schnabel, 1984). These viruses are called temperate. Most of the viral genes are not expressed at the lysogenic stage, only repressor proteins, which inhibit the production of proteins that lead to lytic cycle, are synthesized. Viruses may be induced to lysogenic stage by signals from the environment. In the case of a thermophilic ATV a shift in temperature from 75°C to 85°C induces the virus to enter lysogeny (Prangishvili *et al.*, 2006c). A temperate virus can be induced again to enter the lytic cycle, which can take place because of an environmental trigger or it can be spontaneous. Archaeal viruses ϕH and ϕCh1 were both discovered after spontaneous induction of the virus (Schnabel *et al.*, 1982; Witte *et al.*, 1997). When a provirus is integrated into a host genome and the excision process occurs upon entrance to the lytic life cycle, the excised virus genome occasionally contains fragments of a host genome as well. These events lead to specialized transduction and this way contribute to the lateral gene transfer (Canchaya *et al.*, 2003). Sometimes integrated viruses stay in the genomes of the host cells as defective prophages or proviruses and can be found from genomes of many organisms (e.g. Fischetti, 2007; Krupovic and Bamford, 2008; Lee *et al.*, 2008). For example, human endogenous retroviruses are estimated to contribute about 8% of a human genome (Lee *et al.*, 2008).

7. Viruses used in this study

Haloviruses used in this study were isolated in the course of this research, except HHPV-1. Non-halophilic viruses were well studied virus-host systems.

Virus isolation takes advantage of the lytic properties of a virus. The top agar overlay technique is often used in prokaryotic virus isolations (Adams, 1959). In this method natural samples are plated directly with different indicator strains that can be either characterized organisms or isolates from natural samples. After incubation viruses are detected on the host lawn because of the plaques they produce. The plaques are then picked and further purified.

Viruses can be enriched from the natural samples before plaiting, either by physical means or by taking advantage of the virus capacity to replicate in a host. Tangential flow filtration (Alonso *et al.*, 1999) and ultrafiltration (Suttle *et al.*, 1991) are techniques where viruses are concentrated from samples with large volumes, like sea water. In a culture enrichment method viruses are propagated by replication in the host. This can be achieved by incubating either a prokaryote free sample with a single specific organism, or nutrients are added to an untreated sample. The latter allows the growth of any prokaryote present in the sample which leads to amplification of viruses associated with these organisms (Zemb *et al.*, 2008). Enrichment by cultivation enables the detection and isolation of viruses that may be present in minor amounts in the original sample.

The top agar overlay technique alone can be used in isolation of both lytic (Nuttall and Dyall-Smith, 1993a) and temperate viruses (Jiang *et al.*, 1998). In isolation of temperate viruses, an additional step, where viruses in the lysogenic life cycle are induced to enter the lytic cycle, can be used. Agents often used for the induction are mitomycin C or UV radiation (Mei *et al.*, 2007; Beilstein and Dreiseikelmann, 2008). When the virus has entered the lytic life cycle it can be detected producing plaques on a host lawn as described above.

The previously described viruses used in the study include well-studied type species (P22, PRD1, PM2, ϕ6) as well as a new isolate, HHPV-1. A preliminary report of the halovirus SH1 isolation informed about a new morphology found among viruses infecting haloarchaea (Dyall-Smith *et al.*, 2003). Archaeal halovirus HHPV-1 has been introduced in the section Haloviruses (see above).

PRD1, P22 and ϕ6 are all bacteriophages from low salt environments. PRD1 was isolated from sewage (Olsen *et al.*, 1974). It is a lytic internal membrane containing tailless icosahedral virus with a linear dsDNA genome that replicates via protein priming and it is the type species of the family *Tectiviridae* (Savilahti and Bamford, 1993; Abrescia *et al.*, 2004; Bamford, 2005). PRD1 infects several gram-negative bacteria including *Escherichia coli* and *Salmonella enterica* that contain a conjugative plasmid of the incompatibility group P, N or W (Olsen *et al.*, 1974). The virus adsorption is host growth phase dependent and is mediated by the protein P2 (Mindich *et al.*, 1982; Kotilainen *et al.*, 1993; Huiskonen *et al.*, 2007). It is known that the receptor or the receptor complex is coded by the conjugative plasmid and is functional only on metabolically active cells (Kotilainen *et al.*, 1993; Grahn *et al.*, 1997; Daugelavicius *et al.*, 1997).

There is no documentation of the origins of phage P22, but it is known that it was isolated around 1952 after induction from a *S. enterica* lysogen (http://www.asm.org/division/m/fax/p22fax.html). It infects only *S. enterica* that has an O-antigen polysaccharide on their surface, the so called "smooth" strains. This head-tail virus with a linear dsDNA genome, isometric icosahedral head, short tail and six tail fibers is the type species of the genus

"P22-like viruses" within the family *Podoviridae* (Vander Byl and Kropinski, 2000; Tang *et al.*, 2005; Chang *et al.*, 2006; Lander *et al.*, 2006). A P22 virion binds host lipopolysaccharide O-antigens on the host cell surface via its tail fibers and it possesses endoglycosidase activity that digests the O-antigens (Israel *et al.*, 1972; Steinbacher *et al.*, 1997). In an adsorption model by Israel (1978) it is proposed that only three of the six tail fibers function in binding.

The type organism of the *Cystoviridae* family, ϕ6, is a lytic enveloped dsRNA phage that infects a gram-negative pathogenic plant bacterium *Pseudomonas syringae* (Vidaver *et al.*, 1973). The phage encompasses two layers inside the envelope: an outer protein shell and a core particle into which the three genome segments are packaged (Semancik *et al.*, 1973; Van Etten *et al.*, 1974; Butcher *et al.*, 1997; Huiskonen *et al.*, 2006). The core particle contains RNA polymerase activity (Van Etten *et al.*, 1973; Olkkonen *et al.*, 1991). ϕ6 attaches with a spike protein P3 to host pilus that then facilitates virus access to the host cell surface by retraction (Vidaver *et al.*, 1973; Mindich *et al.*, 1976; Van Etten *et al.*, 1976; Romantschuk and Bamford, 1985).

PM2 is a marine bacteriophage that was isolated from the coastal waters of Chile (Espejo and Canelo, 1968). It is a lytic phage, known to infect two *Pseudoalteromonas* strains (Kivelä *et al.*, 1999). PM2 has an icosahedral protein capsid and an internal membrane which surrounds a circular supercoiled dsDNA genome (Espejo *et al.*, 1969; Abrescia *et al.*, 2008). It is the type species and the sole member of the *Corticoviridae* family. Pentameric vertex protein P1 binds the PM2 receptor which has not been identified but is known to be non-extractable and only functional on the host cell surface (Huiskonen *et al.*, 2004; Kivelä *et al.*, 2004). Adsorption of PM2 is aeration dependent.

B. AIMS OF THE PRESENT STUDY

Only a handful of archaeal viruses have been isolated and very few have been studied in some detail. Our knowledge is still inadequate to say much about the diversity of morphologies in different environments or among viruses infecting different groups of Archaea. Neither receptors nor receptor binding proteins of archaeal viruses have been characterized and both entry and exit mechanism are obscure. Also studies on life cycles of archaeal viruses are scarce. Several structures of viruses infecting archaea from the kingdom Crenarchaeota have been determined (Rice *et al.*, 2004; Häring *et al.*, 2005b; Vestergaard *et al.*, 2008a). However, before studies on haloarchaeal virus SH1, no work had been done on virus structures of euryarchaeal viruses. It would be interesting to know if viruses residing in highly saline environments have evolved structures that help them to cope with the harsh environment. Highly saline environments and their virus ecology have not been much explored. Only a few studies exist (Wais and Daniels, 1985; Guixa-Boixareu *et al.*, 1996; Oren *et al.*, 1997; Diez *et al.*, 2000; Brum *et al.*, 2005; Bettarel *et al.*, 2006) and they do not explore the ecology much beyond viral abundance, occurrence of different morphologies and effect on prokaryotic mortality.

The aim of this study was to shed light on different aspects of archaeal viruses and viruses of highly saline environments in particular. Specifically:

- To isolate new haloviruses and establish cultivation and purification methods for them.
- To study stability of newly isolated viruses.
- To explore the effects of ionic strength on virus adsorption and infectivity with viruses from different salinities.
- To study an archaeal halovirus SH1 in more detail:
    - identify the structural proteins and other components of the virus
    - study location of the structural proteins in the virion using biochemical methods
    - explore the life cycle of SH1

C. MATERIALS AND METHODS

Bacteria and archaea used in this study are listed in Table 3, whereas bacteriophages and archaeal viruses can be found in Table 4. Methods used are summarized in Table 5 and described in the original publications. The references to the methods can be found from the articles.

Table 3. Archaea and bacteria used in this study.

Archaea and bacteria	Relevant usage	Refence
Bacteria		
Pseudoalteromonas sp. strain ER72M2	III: Host for PM2	Kivelä *et al.*, 1999
Pseudomonas syringae pathovar phaseolicola HB10Y	III: Host for ϕ6	Vidaver *et al.*, 1973
Salicola sp. PV3	III: Host for SCTP-1; Tested for SCTP-2, HCTV-1, HHTV-1, HRTV-1 and SH1 susceptibility	III
Salicola sp. PV4	III: Host for SCTP-2; Tested for SCTP-1, HCTV-1, HHTV-1, HRTV-1 and SH1 susceptibility	III
Salmonella enterica serovar Typhimurium LT2 strain DS88	III: Host for PRD1 and P22	Bamford and Bamford, 1990
Archaea		
Haloarcula californiae ATCC 33799	III: Host for HCTV-1; Tested for HHTV-1 and HRTV-1 susceptibility	Javor *et al.*, 1982
Haloarcula hispanica ATCC 33960	I, II, III: Host for HHPV-1, HHTV-1 and SH1; All the studies on SH1 done using this host	Juez *et al.*, 1986
Haloarcula japonica TR1	III: Tested for HCTV-1, HHTV-1 and HRTV-1 susceptibility	Takashina *et al.*, 1990
Haloarcula marismortui ATCC 43049	I, III: Tested for HCTV-1, HHTV-1, HRTV-1 and SH1 susceptibility	Oren *et al.*, 1990
Haloarcula sinaiiensis ATCC 33800	I: Tested for SH1 susceptibility	Torreblanca *et al.*, 1986
Haloarcula vallismortis ATCC 29715	III: Tested for HCTV-1, HHTV-1 and HRTV-1 susceptibility	Torreblanca *et al.*, 1986
Halobacterium salinarum NCIMB 763	I: Tested for SH1 susceptibility	Ventosa and Oren, 1996
Haloferax gibbonsii ATCC 33959	I: Tested for SH1 susceptibility	Juez *et al.*, 1986
Haloferax lucentense NCIMB 13854	I: Tested for SH1 susceptibility	Gutierrez *et al.*, 2002
Haloferax volcanii ATCC 29605	I: Tested for SH1 susceptibility	Mullakhanbhai and Larsen, 1975
Halorubrum coriense ACAM 3911	I: Tested for SH1 susceptibility	Nuttall and Dyall-Smith, 1993b
Halorubrum lacusprofundi ACAM 34	I: Tested for SH1 susceptibility	Franzman *et al.*, 1988
Halorubrum saccharovorum NCIMB 2081	I: Tested for SH1 susceptibility	Tomlinson and Hochstein, 1976
Halorubrum sp. CSW 2.09.4	I: Host for SH1	Burns *et al.*, 2004a and Burns *et al.*, 2004b
Halorubrum sp. s1-1	III: Host for HRTV-1; Tested for HCTV-1, HHTV-1 and SH1 susceptibility	III
Haloterrigena turkmenica NCIMB 784	I: Tested for SH1 susceptibility	Ventosa *et al.*, 1999
Natrialba asiatica JCM 9576	I: Tested for SH1 susceptibility	Kamekura and Dyall-Smith, 1995

Table 4. Archaeal viruses and bacteriophages used in this study.

Viruses and phages	Refence
Phages	
ϕ6	Vidaver *et al.*, 1973
PM2	Espejo and Canelo, 1968
PRD1	Olsen *et al.*, 1974
P22 c1¯7	Botstein and Levine, 1968
SCTP-1	III
SCTP-2	III
Viruses	
HCTV-1	III
HHPV-1	Kukkaro *et al.*, in preparation
HHTV-1	III
HRTV-1	III
SH1	I

Table 5. Methods used in this study.

Method	Used in		
Adsorption assays			III
Agarose gel electrophoresis	I		
Dissociation of virus particles		II	
Electron microscopy, negative stain	I		III
Electron microscopy, thin section	I	II	
Enzymatic treatments of SH1 DNA	I		
Gel filtration		II	
Growth and purification of SH1	I	II	
Growth and purification of HCTV-1, HHTV-1, HRTV-1, SCTP-1 and SCTP-2			III
Growth of ϕ6, HHPV-1, PM2, PRD1, P22 and SH1			III
Growth curve	I		
Isolation, DNA of SH1	I		
Isolation, hosts			III
Isolation, resistant forms of *Haloarcula hispanica*	I		
Isolation, viruses	I		III
Lipid isolation and analysis		II	
Mass spectrometry		II	
Plaque assay	I	II	III
Protein concentration measurement with the Bradford method	I	II	III
Protein quantification		II	
Sequencing, determination of 16S rRNA gene sequences	I		III
Sequencing, determination of N-terminal amino acid sequences		II	
Tricine-SDS-PAGE or SDS-PAGE and Coomassie Blue or EtBr staining	I	II	III
Virus host range study	I		III
Virus stability experiments	I		III

D. RESULTS AND DISCUSSION

1. Isolation of viruses and their hosts

1.1. Host isolation and identification

The collections of bacteria and archaea available in a laboratory are often not suitable for isolation of new viruses from natural samples. Often the halophilic prokaryotes that are available for cultivation in a laboratory do not reflect the abundances found in the environment (Oren, 2002b). For example, *Haloquadratum walsbyi* has long been known to contribute significantly to the prokaryotic communities in high salt environments, however only recently it was cultivated in laboratory conditions (Bolhuis *et al.*, 2004; Burns *et al.*, 2004a). The unavailability of *H. walsbyi* for cultivation for a long time may reflect the fact that no viruses have been isolated for this abundant archaea. This is regardless of the observation that cells of *H. walsbyi* have been often detected to be infected in nature by viruses of different morphologies (Guixa-Boixareu *et al.*, 1996).

For us to be able to isolate viruses infecting bacteria and archaea other than the ones available in the laboratory beforehand, we needed to isolate new prokaryotes. Water samples of high salinity were screened for archaea and bacteria and the acquired strains were used in virus isolation (III). Three prokaryotes that acted as virus hosts were analyzed for identification. Identification was done by partial 16S ribosomal RNA (rRNA) gene sequencing and comparing the obtained sequences to those available in the public data bases. The closest relative for one of the isolates was an archaea *Halorubrum aidingense*, whereas sequences of the other two isolates clustered among *Salicola* sp. sequences (III). Bacteria of the genus *Salicola* have only been recently isolated from highly saline environments around the world (Kharroub *et al.*, 2006; Maturrano *et al.*, 2006). They are rod-shaped heterotrophic gram-negative bacteria which grow in salinities of 10 – 30% (w/v) NaCl (Maturrano *et al.*, 2006). Genus *Halorubrum* was proposed in 1995 (McGenity and Grant, 1995) but the type species *Halorubrum saccharovorum* was already described in 1976 (Tomlinson and Hochstein, 1976). Many species have been described for this genus that has rod-shaped or pleomorphic archaea that grow in salinities of about 9 – 30% (w/v) NaCl (McGenity and Grant, 2001).

1.2. Virus isolation

New viruses were isolated from salt water samples acquired from three locations: Rottnest Island (Australia) (I), Trapani (Italy) (III) and Margherita di Savoia (Italy) (III). All together six new haloviruses were isolated (Table 4) either on prokaryotic strains available in the laboratory beforehand or on natural isolates from the water samples. All of the viruses were isolated from plaques they produced on a sensitive bacteria or archaea. Out of the six new isolates, two infected bacteria and four archaea. Two of the archaeal viruses, HHTV-1 and SH1, had the same host *H. hispanica* and HCTV-1 infected another *Haloarcula* species, *Haloacula californiae*. The fourth archaeal virus, HRTV-1, infected a *Halorubrum* sp. Both of the bacteriophages infected natural *Salicola* isolates. These phages, SCTP-1 and SCTP-

2, are the first phages reported infecting bacteria in the genus *Salicola*.

The isolations done in this study contribute considerably to the pool of reported haloviruses. By 2007 only 14 archaeal haloviruses, including SH1, had been reported (Ackermann, 2007). No information is available about the number of the isolated haloviruses of bacteria. Nevertheless we know that the number of isolated halophages is not high and even many of the putative hosts, the bacteria present in hypersaline environments, were only recently discovered (Anton *et al.*, 2000; Maturrano *et al.*, 2006). For example, there are no phages reported yet infecting species of the most abundant bacteria in many high salt environments, *Salinibacter*.

1.3. Host ranges of the viruses

All the isolated viruses were subjected to host range studies (I; III) but only SH1 was observed to infect a strain other than the one it was isolated on. Besides its isolation host *H. hispanica*, SH1 infected a natural isolate CSW 2.09.04 that is related to *Halorubrum*, based on partial 16S rRNA gene sequence (I; Burns *et al.*, 2004a; Burns *et al.*, 2004b). The host range of SH1 was largely studied with all together 28 strains representing both natural isolates and characterized haloarchaea (I; III). The host range of the other three archaeal viruses, HCTV-1, HHTV-1 and HRTV-1, was not studied with as large selection of strains as SH1; these viruses were tested on seven other strains besides the one they were isolated on (III). For the study of the halophages SCTP-1 and SCTP-2, no other halophilic bacteria besides their hosts, the two natural *Salicola* isolates, were available.

2. Production of virus particles

2.1. Propagation and purification of viruses

The six new virus isolates were all propagated by plate lysis method and purified by rate zonal centrifugation to gain 1 × purified virus material (I; III). For SH1, a liquid culture method was also used (I) and optimized to gain higher yields (II). Since SH1 was a subject of more detailed study, further purification by equilibrium centrifugation was performed to gain highly pure material which was designated as 2 × purified virus (I; II).

In the plate lysis method viruses were collected from top-layer agar plates incubated to show semi-confluent lysis by the virus. Viruses were precipitated with polyethylene glycol (PEG) 6000 or 8000 (average molecular weight) from the collected virus lysates and purified in a linear 5-20% (w/v) sucrose gradient with rate zonal centrifugation. The needed centrifugation time varied for each virus, depending on virus properties. For example, the largest virus SCTP-2, sedimented the fastest and had the shortest centrifugation time (III). After the centrifugation, all the viruses produced a light-scattering infective virus zone that was collected and viruses were pelleted by differential centrifugation if no further purification was performed.

Recovery of infectivity was calculated at several stages during the purification process. After the PEG concentration step, the recoveries varied from 45 to 70% when compared to the original lysate (100%) (I; III). The recoveries of 1 × viruses were not all calculated similarly; the value for SH1 was determined from the collected virus zone

after the rate zonal centrifugation since the purification of SH1 was continued. For the rest of the viruses the recovery was calculated after the final pelleting. The 1 × virus recoveries were in between 10 and 20% for HCTV-1, HHTV-1, HRTV-1, SCTP-1 and SCTP-2. For SH1 the recovery was 38%. The values obtained resembled those published for viruses purified with a similar method (Kivelä *et al.*, 1999; Ravantti *et al.*, 2003; Jaatinen *et al.*, 2008).

Specific infectivity of 1 × viruses was determined. It was either calculated as plaque forming units (pfu) /A_{260} or pfu/mg protein (determined by Bradford assay with BSA as a standard). SH1 had a value of 1.1 × 10^{11} pfu/A_{260} and the specific infectivity of the other viruses varied in between ~10^{10} – 10^{13} pfu/mg protein. HHTV-1 and HCTV-1 had the highest specific infectivities exceeding values published for many viruses, for example P23-77 (4.2 × 10^{12} pfu/mg protein; Jaatinen *et al.*, 2008) and Bam35 (1.6 × 10^{12} pfu/mg protein; Ravantti *et al.*, 2003). The lowest specific infectivity was observed with HRTV-1. The low value might be due to filamentous material, resembling flagella, that was observed to co-purify with the virus. This filamentous material was observed in electron micrographs taken of the 1 × purified material and the Coomassie stained tricine-SDS-PAGE also showed substantial amount of impurities (III).

Liquid culture methods used for SH1 propagation were adjusted for large scale purification (I). An early logarithmic *H. hispanica* culture was infected with SH1 using a multiplicity of infection (MOI) of 0.05. Cultures needed to be incubated for three days until the virus titer reached its maximum and turbidity reached the minimum. The yield with this method was ~2-5 × 10^{11} pfu/ml. When the method was further optimized the yield improved considerably (II). In the optimized method a mid-exponential *H. hispanica* culture was infected with MOI 40. Only ~5.5 h was needed until the lysis occurred. The lysate was then treated with DNase I and the 1 × purification was carried out as described above. Also the buffer used in the centrifugation gradients and virus resuspensions was changed to a more salt containing one. These changes enabled SH1 to infect the host cells more efficiently and to cause a faster lysis of the host culture. Also, the infectivity of SH1 might have been compromised with the buffer used in the original purification procedure since the salt concentration was set up to be minimal for sustaining the virus infectivity.

To obtain highly pure material, 1 × purified SH1 virus zone was collected after rate zonal centrifugation, layered on the top of CsCl (average density of 1.3 g/ml) and centrifuged to equilibrium. The infective virus produced a sharp light scattering zone at a density of 1.33 g/ml. This is slightly denser than what has been observed with viruses with similar morphology (Kivelä *et al.*, 1999; Ravantti *et al.*, 2003; Jaatinen *et al.*, 2008). The virus zone was collected and concentrated with differential centrifugation. Recovery of infectivity as well as specific infectivity was determined. Recovery of SH1 infectivity was ~16%. The specific infectivity improved when the liquid culture method was optimized from 1.6 × 10^{11} pfu/A_{260} and 2.1 × 10^{12} pfu/mg protein to 4-6 × 10^{11} pfu/ A_{260} and 4.5-6.5 × 10^{12} pfu/mg protein (I; II). The recovery obtained was slightly higher than often observed with other viruses purified with a similar method whereas specific infectivities seem to be no higher than the average values (Kivelä *et al.*, 1999; Ravantti *et al.*, 2003; Jaatinen *et al.*, 2008).

Equilibrium centrifugation of SH1 was also tested in a 30-70% (w/v) sucrose gradient (I). It appeared that this method could not be used with SH1 since the high sucrose concentration affected the virus infectivity. Bacteriophage PM2 has also been observed to be sensitive to high sucrose concentrations preventing usage of this method in purification (Kivelä *et al.*, 1999). When SH1 was centrifuged to equilibrium, the virus produced two light scattering zones, one consisting almost

solely of viral DNA and the other of viral proteins and some DNA. The latter zone produced at a density of ~1.28 g/ml and contained some infectivity.

2.2. Virus properties

Purified virus particles were subjected to negative-stain electron microscopy (EM) (I; III). It revealed five viruses of head-tail morphology and one tailless icosahedral virus. 96% of all the studied prokaryotic viruses are tailed (Ackermann, 2007), thus it was not surprising that the majority of the isolates also exhibited this morphology. Two of the head-tail viruses, HRTV-1 and SCTP-2 resembled viruses of *Myoviridae* whereas HCTV-1, HHTV-1 and SCTP-1 resembled those classified in *Siphoviridae*. SCTP-2 was notably larger than the other isolates of similar morphology. It had a head diameter of ~125 nm and ~145 nm long tail (III). The size of SCTP-2 is almost comparable to the largest bacteriophages ϕKZ (head diameter 130 nm, tail length ~185 nm; Krylov *et al.*, 2003), KVP40 (140 nm long and 70 nm wide head; Miller *et al.*, 2003) and *Aeromonas hydrophila* bacteriophage Aeh1 (134 nm long and 89 nm wide head, and 123 nm long tail; Chow and Rouf, 1983) and the virus is larger than bacteriophage T4 which is also among the largest viruses with 119.5 nm long and 86 nm wide head, and 100 nm long tail (Mesyanzhinov *et al.*, 2004). All the isolated giant head-tail bacteriophages belong to the family *Myoviridae* (Chang *et al.*, 2005).

The sizes of the isolates HHTV-1, HRTV-1 and SCTP-1 were similar: they all had a head with ~55 nm diameter and the tail lengths were ~110 nm, ~85 nm and ~95 nm, respectively (III). HCTV-1 was slightly larger than these three with ~70 nm diameter head and ~80 nm long tail (III).

The tailless icosahedral virus SH1 had a particle diameter of about 70 nm. The micrographs also showed particles devoid of a proteinaceous shell, revealing a compact core which were ~50 nm in diameter (I). The more accurate size of SH1 has been later determined in a study that resolved the virion structure to 9.6-Å resolution (Jäälinoja *et al.*, 2008). The size of SH1 was 79.5 nm from vertex to vertex. In addition, the structural study revealed large horn-like spikes at the vertices and the whole particle measured ~115 nm from spike to spike. SH1 was the first euryarchaeal virus isolated with the tailless icosahedral morphology and only one other archaeal virus with similar morphology, STIV infecting a thermophile, has been reported (Rice *et al.*, 2004).

3. Virus stability

Sensitivity to chloroform of all the newly isolated viruses was tested (I; III). Sensitivity suggests the presence of a membrane, but disruption of a non-membrane containing virus particle is also possible as in the case of SNJ1, a head-tail haloarchaeal virus (Mei *et al.*, 2007). Among the studied viruses, SH1 was the most sensitive to chloroform: the infectivity dropped three orders of magnitude in 15 min when shaken with 20% (v/v) chloroform (I). The presence of a membrane in SH1 was later confirmed (Bamford *et al.*, 2005b). The two isolated phages, SCTP-1 and SCTP-2, were also somewhat chloroform-sensitive, but the effect was not quite as drastic as with SH1. After incubation in 5% chloroform (v/v) over night SCTP-1 infectivity dropped two orders of magnitude and SCTP-2 infectivity one order (III). No membrane containing head-tail viruses have been discovered, which suggests SCTP-1

and SCTP-2 to be devoid of a lipid component. However, lipid extraction should be performed to ensure the absence of a membrane. The other viruses were unaffected by chloroform.

Virus infectivity in different NaCl concentrations was tested with each virus used in study III. This included all the six new haloviruses isolated as well as a halovirus HHPV-1. Three mesophilic phages (PRD1, P22 and ϕ6) and a marine phage PM2 were also included. When a time that was adequate for virus adsorption was used in the incubation, all the head-tail viruses (P22, SCTP-1, SCTP-2, HCTV-1, HHTV-1 and HRTV-2) retained the infectivity above 50% in the studied salinity range (0 to 4.5 M NaCl). Adjusting salinity in this range affected other viruses, which contained either an inner (PRD1, PM2 and SH1) or an outer membrane (ϕ6 and HHPV-1). Low salt and marine membrane containing viruses had an infectivity drop below 50% in high NaCl concentrations whereas membrane-containing haloviruses were affected by low NaCl concentrations. Two of the most sensitive viruses were the enveloped viruses ϕ6 and HHPV-1. Overall, all the studied viruses were less sensitive to change in NaCl concentration than their host organisms (III) as has been observed earlier with viruses of halophilic archaea (Wais *et al.*, 1975; Pauling, 1982). Better tolerance of different salinities would give an advantage to the virus over the host in an environment with fluctuating salinity. It has been detected that haloviruses can either be sensitive (Nuttall and Dyall-Smith, 1993a; Mei *et al.*, 2007) or resistant (Vogelsang-Wenke and Oesterhelt, 1988; Daniels and Wais, 1998) to change in ionic conditions. This observation does not seem to be limited to only haloviruses, but is true for viruses from low salt as well, as observed in this study. Out of the three low salt viruses, infectivity of P22 was not significantly altered even in high NaCl concentrations whereas PRD1 and ϕ6 were affected by high salt.

Tolerance of SH1 to change in NaCl concentration was also tested with a longer incubation period than above (I). It was observed that SH1 tolerated decrease to 1 M NaCl over a five day period but 0.5 M NaCl had an effect on the infectivity already after one day incubation. In another similar experiment on SH1 the $MgCl_2$ concentration of the buffer was changed, but the NaCl concentration was unaltered. The concentration of $MgCl_2$ could be set to 40 mM without an effect on SH1 infectivity over five days, but 10 mM $MgCl_2$ concentration caused a decrease in the virus titer after two days (I). If Mg^{2+} was used alone with out NaCl, 2 M concentration was needed to sustain the infectivity of the virus. When the virus was diluted 1000-fold in pure water, one order of magnitude decrease was observed after a day. This shows that high ionic strength is needed to maintain the SH1 infectivity but is not alone sufficient: divalent cations are also compulsory. The same is true with an archaeal halovirus Hh-3: it is stabilized by Mg^{2+} and it needs high ionic strength for survival (Pauling, 1982). Halovirus Hh-1, on the other hand, has also been observed to need Mg^{2+} for stabilization but it is not specifically dependant on NaCl and can survive long periods of time in solutions of different ionic strengths (Pauling, 1982). The stability of an archaeal halovirus Ja1 is dependent on high ionic strength if divalent cations are not present; however, 20 mM $MgSO_4$ provides similar stability as 2 M NaCl or KCl (Wais *et al.*, 1975). Haloviruses HF1 and HF2 behave similarly as Ja1: they can maintain infectivity in all NaCl concentrations if 100 mM Mg^{2+} is provided (Nuttall and Dyall-Smith, 1993a).

Thermostability of SH1 was tested with 15 min incubations in various temperatures. SH1 was stable up to 50 °C after which the virus titer dropped rapidly (I). Halovirus SNJ1 seems to be slightly more sensitive to high temperatures than SH1; the SNJ1 titer drops after 20 min incubation at temperatures higher than 40 °C (Mei *et al.*, 2007). Archaeal halovirus His2

loses infectivity at temperatures higher than 50 °C (1 h incubation) similarly as SH1, whereas halovirus His1 retains infectivity until 60 °C (Bath *et al.*, 2006). The effect of pH on SH1 infectivity was studied in a range of pH 5 to 9. High pH values did not affect SH1 infectivity in 30 minutes, whereas a sharp decrease was observed below pH 6 (I). SNJ1 has been observed to behave similarly sustaining the infectivity in alkaline solutions but showing sensitivity to acidic solutions (Mei *et al.*, 2007), while infectivity of both His1 and His2 is relatively stable over a pH range of 3 to 9 (Bath *et al.*, 2006). Stability of SH1 was also followed in a lysate stock in which the infectivity remained unaltered for several months when stored at 5 °C (I).

4. SH1

4.1. The life cycle of SH1

Adsorption of SH1 to the host cells was slow when the life cycle was studied with a growth curve experiment (I). Maximal amount of infective centers (infected cells) was not reached until 3 h post infection (p.i.). The slow adsorption was confirmed with an adsorption test (III). Viruses could not infect all *H. hispanica* cells in the culture and this was especially predominant with a variant strain of the host. When the cause for this was studied, it was observed that the variant strain contained a higher proportion of cluster type cells having thick cell walls than what was normally detected in the host culture. Cline and Doolittle (1992) had observed the presence of clustered cells in a *H. hispanica* culture at low frequency (0.1 to 0.01%) whereas cells in the clusters constituted sometimes over 80% of the culture in the variant strain. SH1 was never observed to be attached on the surface of these cluster type cells and intracellular viruses were not detected either. After the virus lysis had occurred, only cells with thick cell wall remained in the culture indicating that this cell type could not be infected by SH1. It was confirmed that the culture had no contaminants and the 16S rRNA gene sequence of these cells matched the *H. hispanica* sequence.

In a single-step experiment the virus amount increased considerably between 5 and 6 h p.i. (I). Also when the virus titer of the growth curve was followed, the amount of extracellular viruses started to rise ~5 h p.i. and both empty and full intracellular virus particles were observed by TEM. The detection of empty particles and identification of an open reading frame putatively coding for an ATPase, suggest SH1 to package its genome into preformed capsids (Bamford *et al.*, 2005b). The culture turbidity started to decrease around 7 h p.i. reaching a stable level ~27 h p.i.. However, because of the presence of the cluster type cells that remained uninfected, some re-growth of the culture occurred soon after stabilization. After the cell lysis, the viruses were often observed attached to the cell debris. An average burst size of ~200 pfu/cell was calculated.

SH1 is a lytic virus. The host cell culture lysis occurs concurrently with the extracellular virus increase and the only cells remaining after the lysis are the ones that SH1 seems to be unable to infect. These observations are not in line with Bath *et al.* (2006) and Porter *et al.* (2007) claiming that SH1 exits the cell without host cell lysis. In a single-step growth curve that Porter *et al.* (2007) present to support the proposition, a rise in virus titer occurs 5 to 6 h p.i. as detected in our growth curve experiment. The turbidity of the host culture does not start to decrease yet at that stage but the growth is retarded. The decrease in the culture turbidity begins around 15 h p.i.. A

reason for that observation could be, that due to the inefficient adsorption of SH1 only a small portion of the cells have been infected before washing away the unbound virus. When this small portion of the cells lyse at the time of the first increase in the titer, most of the cells continue dividing and the growth is only retarded. After the first burst of the viruses, the virus amount is sufficient to infect most of the cells and viruses have an adequate adsorption period since the excess is not washed away. Consequently a host culture lysis is observed. Our optimized infection cycle however, is able to produce a decrease in host culture turbidity upon the initial virus titer increase.

4.2. Structural proteins and protein complexes

Structural proteins and their apparent masses were analyzed in tricine-SDS-PAGE from 2 × purified SH1 (I). The gel revealed ~15 protein bands out of which four were major. The apparent masses for the bands ranged from 4 to 185 kDa. When non-reducing conditions were used for the virus sample, five additional bands were observed in a gel (I). Concomitant reduction of intensity in three of the major bands (VP3, VP4 and VP7) indicated that these proteins could constitute the complexes.

In later studies the 15 protein bands were subjected to protein chemical studies for identification of the genes from the virus genome (Bamford *et al.*, 2005b). This resulted in identification of 11 of the bands. Four of the minor bands could not be identified either by N-terminal amino acid sequence analysis or by mass spectrometry. Three of the most abundant proteins VP3, VP4 and VP7 were suggested to be coat-associated and a major protein VP12 was proposed to be membrane-associated. In sedimentation and gel filtrations analyses VP2 behaved as an asymmetric protein (II) and the sequence predicted a fiber-like structure that is suitable for forming viral spikes (Bamford *et al.*, 2005b). Indeed, VP2 has been observed to be involved in forming the spikes, although not the distal part of them (Figure 4) (Jäälinoja *et al.*, 2008).

The protein complexes formed in non-reducing conditions were analyzed by peptide mass fingerprinting (Bamford *et al.*, 2005b). The study revealed complexes C1 and C2 to be composed solely of VP1 and C3 of only VP4. Complexes C4 and C5 both contained two different proteins: VP4 and VP7. However, when the capsid associated proteins were studied, after solubilization in dissociation experiments (see below), heteromultimers composed of VP4 and VP7 could not be detected. (II). VP4 and VP7 were detected in the virion in estimated ratio of one VP4 per two VP7 proteins (II). Later, in the structural study on SH1, it was suggested that VP7 forms the hexameric base of the capsomers and VP4 is present in two or three copies in a capsomer, depending on the location, as a decoration protein (Jäälinoja *et al.*, 2008). Even though reduction of intensity in the VP3 band was observed in non-reducing conditions, this viral protein was not detected in any of the complexes. VP3 has been later discovered to be involved in the spike formation with VP6 (Figure 4) (Jäälinoja *et al.*, 2008).

The complex structures formed by the proteins of SH1 could provide extra stabilization of the protein coat of the virus. Many protein complexes of halophiles are known to fall apart in low salt concentrations (Klein *et al.*, 2002) and cross-linking of proteins would give protection against that. A haloalcalovirus ϕCh1 (Klein *et al.*, 2002) and phage HK97 (Popa *et al.*, 1991) for example, are known to have cross-linked protein coats. However, usually protein-protein interactions in the virus do not involve covalent bonding (Cann, 2005).

For the nature of the SH1 protein multimerization in non-reducing conditions,

the most apparent explanation would be disulphide bonding in the way of chilo iridescent virus that employs S-S bridging in its capsid formation (Cerutti and Devauchelle, 1985; Devauchelle *et al.*, 1985). However, when the sequences of the SH1 proteins involved in the complex formation (VP1, VP4 and VP7) were studied, only one cysteine residue per protein was discovered (Bamford *et al.*, 2005b).

4.3. Internal membrane

In the negative stain electron micrographs of SH1, particles devoid of proteinaceous shell were occasionally observed, revealing an inner core with membranous material (I). The additional observations that the virus was sensitive to chloroform and had a low buoyant density, suggested the presence of lipids (I). Bamford *et al.* (2005b) confirmed the existence of lipids and analyzed the lipid composition of the virus showing that it differs from the host lipid composition qualitatively and quantitatively. SH1 has three major phospholipids (phosphatidylglycerol (PG), phosphatidylglycerophosphate methyl ester (PGP-Me) and phosphatidylglycerosulfate (PGS)) whereas *H. hispanica* seems to have eight different lipid species. In SH1 the proportion of PGP-Me was higher and PGS lower than in the host. Neutral lipids were present in both SH1 and *H. hispanica* but in different proportions. This kind of selectiveness in acquiring the viral membrane from the host is known among several viruses (Laurinavicius *et al.*, 2004a; Laurinavicius *et al.*, 2004b).

The presence of an internal membrane was confirmed by dissociation studies (II). Conditions were determined, in which SH1 virion could be quantitatively dissociated. The resulted components were analyzed by rate zonal centrifugation and the presence of lipids was determined from different dissociation products. Two different conditions were discovered (3 M urea treatment and low ionic strength) where the coat associated proteins were solubilized and a faster sedimenting lipid core (LC) was observed. Lipids were associated with the LC. In the structural study of SH1 the lipid bilayer was distinct under the protein coat and it followed the shape of the capsid (Jäälinoja *et al.*, 2008). The presence of the LC confirmed the overall structural similarity to icosahedral internal membrane containing viruses such as PRD1, PM2, Bam35 and STIV (Abrescia *et al.*, 2004; Khayat *et al.*, 2005; Laurinmäki *et al.*, 2005; Abrescia *et al.*, 2008).

4.4. Components of the lipid core

Dissociation studies of SH1 were performed to determine the arrangement of structural proteins and the lipid component in the virion (II). Two conditions (3 M urea treatment and low ionic strength) were determined where SH1 could be quantitatively dissociated. In both conditions proteins associated with the protein shell were solubilized releasing a LC that contained a distinct set of membrane associated proteins. The viral DNA was either associated with the LC (3 M urea treatment) or released (low ionic strength). Proteins VP2, VP3, VP4, VP6, VP7 and VP9 were clearly protein coat associated and were dissociated in both conditions studied. VP5 was distributed about equally between the soluble fraction and the LC,

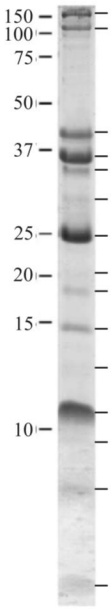
indicating close proximity to the membrane. VP1 was detected in the same fractions as the DNA: in low ionic strength it was mostly released whereas with 3 M urea treatment it stayed in the LC fraction. This observation inspires to speculate about VP1 being involved in capping of the DNA to the LC. The proteins that were LC associated in both treatments were VP10 and VP12, as well as VP13 (molecular mass 8.8 kDa) which was surprisingly also detected in a position around 37 kDa in the tricine-SDS-PAGE even though previously detected only at a position around 8 kDa (Figure 4A; I). VP12 was by far the most abundant protein of the LC constituting about 70%. The small amount of membrane associated proteins in SH1 indicates a different genome delivery system than in PRD1 (Bamford *et al.*, 2005b). Bacteriophage PRD1 has at least nine integral membrane proteins which are mostly involved in the DNA delivery (Grahn *et al.*, 2002). Jäälinoja *et al*. (2008) propose that the large ordered transmembrane complexes seen underneath the spikes and the peripheral proteins around it are involved in the genome translocation. The positions of the unidentified proteins VP8, VP11, VP14 and VP15 could not be determined because of their minor nature in the virion.

With the knowledge gained in the studies (I; II; Bamford *et al.*, 2005b; Jäälinoja *et al.*, 2008) we can now propose positions and functions of some structural proteins and create a schematic presentation of SH1 (Figure 4). However, it should be noted that clear evidence regarding the exact positions of most of the proteins is still lacking and the functions of the proteins are only speculations.

4.5. Genome

Preliminary analysis of the nucleic acid isolated from SH1 indicated a linear dsDNA molecule (I). The size estimation was ~31 kb (I). The genome of SH1 was later sequenced and it confirmed the linear dsDNA nature of the genome (Bamford *et al.*, 2005b). The exact length of the genome is 30 898 bp and the ends contain 309 bp long inverted terminal repeats. In the database search, the genome of SH1 revealed few significant matches to sequences in the databases. However, one of the 56 predicted open reading frames gave a hit to ATPases. The genome is organized in the bacterial manner to at least four operons that produce polycistronic transcripts. Genes for all identified structural components of the SH1 virion, except one, are located in the middle of the genome and are probably transcribed from a single operon (Bamford *et al.*, 2005b).

A



Protein	Molecular mass (kDa)	Position	Proposed function	Nature of the protein
VP1	152	peripheral membrane protein	capping of DNA to the LC	asymmetric, homomultimers
VP2	81	capsid associated	spike stem / base structure	asymmetric
VP3	38	capsid associated	spike	globular monomer
VP4	26	capsid associated	coat	homo and hetero (VP4 & VP7) multimers
VP5	32	peripheral membrane protein		
VP6	25	capsid associated	spike	
VP7	20	capsid associated	coat	homo and hetero (VP4 & VP7) multimers
VP8		not identified	ATPase	
VP9	17	capsid associated	pentamer at the vertexes	
VP10	17	membrane associated		
VP11		not identified		
VP12	10	membrane associated	major integral membrane protein	
VP13	9	membrane associated		
VP14		not identified		
VP15		not identified		

B

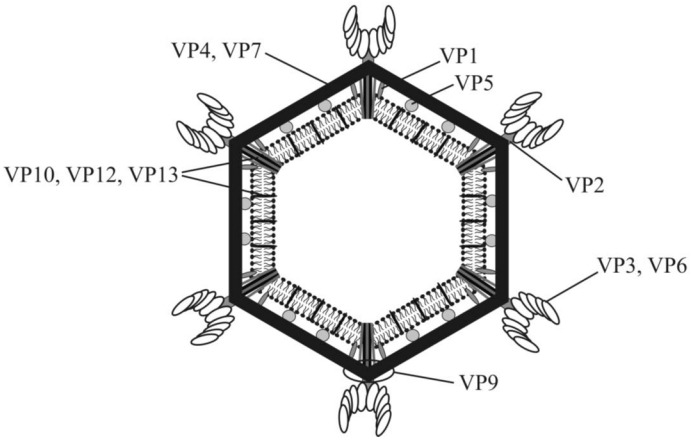


Figure 4. The predicted positions, functions and the nature of the structural proteins of SH1. Protein pattern of 2 × purified SH1 reveals 15 proteins (A). Molecular masses of the identified proteins have been calculated based on the sequence information. Molecular masses of the standard proteins are shown on the left. Position of the protein in a virion and the nature of the protein have been determined by quantitative dissociation experiments and protein analysis. (B) Schematic presentation of SH1 showing the putative positions of the identified structural proteins.

5. Virus-host interactions in environments with different ionic strengths

Viruses with efficient adsorption to their host cells are not limited to environments with low salt concentrations. However, when adsorption rate constants of 11 viruses (Table 4) from different environments were studied the slowest binders were found among viruses from high salt conditions (III). It was interesting,

that the two bacteriophages of the high salt viruses (SCTP-1 and SCTP-2) had high adsorption rate constants, similar to the values of low salt (P22, PRD1 and ϕ6) and marine (PM2) phages. Only one of the archaeal viruses (HHPV-1) had an adsorption rate constant similar to phages; the four other archaeal viruses (HCTV-1, HHTV-1, HRTV-1 and SH1) were slower binders. All together, phages were faster binders than archaeal viruses.

The study of the adsorption rate constants included viruses with different morphologies: head-tail, tailless icosahedral and enveloped. Even though two of the viruses with highest adsorption rate constants were of the head-tail morphology, also the slowest binder was a head-tail virus. Among the tailless icosahedral viruses a large variation in the adsorption rate constants was observed as well and as expected, no correlation between the virus morphology and the adsorption rate constant could be detected. The adsorption rate constants varied over four orders of magnitude between the fastest (P22) and the slowest (HHTV-1) binder which indicates different systems in the adsorption mechanism.

All the 11 viruses included in the adsorption rate constant comparison were subjected to adsorption efficiency determination in different ionic conditions (III). The used NaCl concentration range depended on the natural environment of each virus. The range was set to be as large as possible, close to the limits of host viability. Since the viruses always tolerated altered ionic conditions better than their hosts (see Virus stability) no restrictions were brought about by the viruses.

The observed responses on virus adsorption were diverse. The behavioral patterns included increasing and decreasing adsorption when the NaCl concentration was raised, maximal binding at a certain NaCl concentration and nearly unaffected binding in altered conditions. Responses varied among viruses from different environments and different morphologies and no typical pattern was discovered within any virus group. Also no difference could be observed between archaeal viruses and bacteriophages. The only pattern not discovered within haloviruses in this study was decreasing adsorption efficiency in increasing NaCl concentration as detected with the previously studied halovirus Hs1 (Torsvik and Dundas, 1980). Often the haloviruses were observed to have an adsorption maximum at a certain NaCl concentration, adsorption being less efficient in higher and lower salinity, similarly with the marine phage PM2. This illustrates how these viruses are adapted to the salt concentration they reside in, e.g. marine phage PM2 to the sea water and HHPV-1, HHTV-1 and SH1 to the salt concentration which is optimal for the growth of their host *H. hispanica* (Ventosa, 2001). However, not always did the viruses have an optimal adsorption at conditions optimal for the host. Two of the studied viruses, ϕ6 and SCTP-1, had more efficient binding at concentrations that were progressively worse for the host cells. It was predominant in all cases but one, that the salt concentration had an explicit impact on the adsorption.

This study increased our knowledge on the variability of responses seen on virus adsorption in altered ionic strength. The increased knowledge also raises more questions about the adsorption mechanisms. Adsorption of the viruses is probably mediated by electrostatic forces which are affected by the ionic conditions (Adams, 1959; Voyles, 2002). Some haloviruses however, bind with the same efficiency over a large range of ionic strengths (HRTV-1; III, S5100; Daniels and Wais, 1990). Do the adsorption mechanisms of haloviruses obey the rules observed with mesophilic viruses or might they have evolved alternative binding strategies?

E. CONCLUSIONS

These studies on halovirus SH1 and on the virus responses in altered ionic conditions have advanced our knowledge considerably.

Isolation and characterization of six new haloviruses in the course of this study made a significant increase to the number of isolated haloviruses of archaea and halophages. Most of the viruses were of head-tail morphology which has been prevalent among isolated haloviruses. SH1 however, exhibited a morphology isolated for the first time from an environment with high salinity. It seems that the viruses isolated from high salt environments do not reflect the proportions of different morphologies found in nature (Guixa-Boixareu *et al.*, 1996; Oren *et al.*, 1997; Dyall-Smith *et al.*, 2003). There should be further isolations to show whether such diversity is found among haloviruses that has been observed within the viruses of thermophilic archaea.

Halovirus SH1 was well-characterized in this and in the other parallel studies. It is a lytic virus with tailless icosahedral morphology. Under the protein shell it possesses an internal membrane and a linear dsDNA genome. The overall structure of SH1 resembles many of the viruses in the proposed PRD1-adenovirus lineage (Bamford *et al.*, 2002; Bamford, 2003; Benson *et al.*, 2004; Bamford *et al.*, 2005a). Viruses in this lineage are suggested to have a common ancestor based on the structure; a double β-barrel fold of the coat protein is the common feature among these viruses. The recently solved structure of SH1 revealed a capsid that is most likely composed of single β-barrels rather than double β-barrels (Jäälinoja *et al.*, 2008). It is possible that the double β-barrels of the viral capsid proteins are formed through gene duplication or gene fusion from the single β-barrels and the capsid architecture found in SH1 is a molecular fossil (Jäälinoja *et al.*, 2008). To ensure the β-barrel nature of the major capsid protein of SH1 a high resolution structure of the protein should be acquired.

Salt concentration has a predominant impact on virus adsorption. Generally when the salinity of the surrounding environment is altered, it influences virus adsorption dramatically. However, viruses that have an unaltered adsorption in a large range of salt concentrations are also known. The environment a virus normally resides in does not seem to define whether a virus is a fast or a slow binder. Neither does the environment seem to impact how the adsorption efficiency is altered when the salt concentration is changed. Viruses of low and high salt can react in multiple ways to change in salinity. The mechanisms of binding in high salt concentrations are unclear and shoud be investigated. A lot of research needs to be done to understand the early events on halovirus binding to the host cell. No receptors or receptor binding proteins have been identified from viruses infecting halophilic archaea so far. This might be partially due to insufficient knowledge on archaeal surface structures, the putative receptors for viruses.

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Petra

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